High in-vivo Stability and First-in-human Experience with [18F]AlF-RESCA-MIRC213: A 18F-Labeled Nanobody as PET Radiotracer for Diagnosis of HER2-Positive Cancers

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Research Article

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Abstract

Purpose [^{18}F]AlF-RESCA was introduced as a core particularly useful for ^{18}F-labeling of heat-sensitive biomolecules. However, no translational studies have been reported up to now. Herein, we reported the first-in-human evaluation of an ^{18}F-labeled anti-HER2 nanobody MIRC213 as a PET radiotracer for imaging HER2-positive cancers.

Methods In this study, [^{18}F]AlF-RESCA-MIRC213 was prepared at room temperature. Small-animal PET/CT and bio-distribution were performed on HER2-positive subcutaneous xenografts. Six breast cancer patients (3 HER2-positive and 3 HER2-negative patients) were included, and PET/CT images were acquired at 2 h and 4 h after injection of 222 ± 18.5 MBq of [^{18}F]AlF-RESCA-MIRC213. All patients underwent [^{18}F]-FDG PET/CT within a week for comparison purpose. Bio-distribution and dosimetry were calculated. Standardized uptake values (SUV) were measured in tumors and normal organs.

Results [^{18}F]AlF-RESCA-MIRC213 was prepared within 20 min at room temperature with the radiochemical yield of 50.48 ± 7.6% and radiochemical purity of > 98% (n > 10). The 2 h cellular uptake of [^{18}F]AlF-RESCA-MIRC213 in NCI-N87 cells was 11.22 ± 0.60 %IA/10^5 cells. Its binding affinity K_d value was determined to be 1.23 ± 0.58 nM using SK-OV-3 cells. After 2 h injection, the xenografted SK-OV-3 tumors could be readily detected by [^{18}F]AlF-RESCA-MIRC213 PET with SUVmax of 4.73 ± 1.18 ID%/g and 1.70 ± 0.13 ID%/g for the blocking group ($p < 0.05$). No significant radioactivity was seen in the bone in tumor-bearing animals. After approved by ethics committee (No.2021KT108). First-in-human PET translational study was conducted. In six all patients, there was no adverse reactions during study. The uptake of [^{18}F]AlF-RESCA-MIRC213 was mainly in lacrimal gland, parotid gland, submandibular gland, thyroid gland, gallbladder, kidneys, liver, and intestine. There was no significant radioactivity accumulation in the bone of cancer patients. [^{18}F]AlF-RESCA-MIRC213 had significantly higher tumor uptake in HER2-positive lesions than that in HER2-negative lesions (SUVmax of 3.62 ± 1.56 vs. 1.41 ± 0.41, $p = 0.0012$) at 2 h post-injection. Kidneys received the highest radiation dose of 2.17×10^{-2} mGy/MBq, and the effective dose was 1.76×10^{-2} mSv/MBq.

Conclusions [^{18}F]AlF-RESCA-MIRC213 could be prepared with high radiolabeling yield under mild conditions. [^{18}F]AlF-RESCA-MIRC213 has relatively high stability both in vitro and in vivo. The results from clinical transformation suggest that [^{18}F]AlF-RESCA-MIRC213 PET/CT is a safe procedure with favorable pharmacokinetics and dosimetry profile, and it is a promising new PET radiotracer for noninvasive diagnosis of HER2-positive cancers.

Introduction

Human epidermal growth factor receptor type 2 (HER2) is a prominent molecular target for cancer therapy. HER2 is overexpressed in many types of cancers, such as breast cancer (20% − 30%), gastric cancer (20% − 24%), and colorectal cancers (3% − 5%)[1–4]. There are two primary techniques used to

assay the HER2 levels: immunohistochemical staining (IHC) and fluorescence in situ hybridization (FISH) [5]. However, both IHC and FISH are not informative about the HER2 expression heterogeneity because of limitations of these assays. In addition, these methods are invasive and need a biopsy to evaluate the target status[6–9]. Therefore, it would be advantageous to have precise information on the HER2 status in all tumor lesions of cancer patients.

Positron emission tomography (PET) could definitely overcome some limitations of IHC and FISH and accurately quantitatively evaluate the expression of HER2 in tumors in a noninvasive manner. PET could be used to identify patients who would benefit from targeted anti-HER2 treatment and to monitor the efficacy of such treatments[10–12]. ^{18}F is the radionuclide of choice due to its easy availability and almost ideal nuclear decay characteristics for PET[13, 14]. Among various ^{18}F-labeling techniques, [^{18}F]AlF-chelation is a promising strategy that allows one-pot fluorination of biomolecules in aqueous solution[15]. William et al. first described ^{18}F-labeling of peptide using the [^{18}F]AlF core[16]. The results from clinical study clearly showed that [^{18}F]AlF-NOTA-PRGD2 had favorable imaging characteristics in lung cancer patients[17]. However, this strategy is mostly restricted to ^{18}F-labeling at high temperatures, which poses significant challenges for heat-sensitive biomolecules. Recently, a new bifunctional chelating agent (±)-H_3RESCA was developed for ^{18}F-labelling of antibody, nanobody and affibody[18, 19]. The [^{18}F]AlF-RESCA core is of great interest due to its highly ^{18}F-labelling efficiency at room temperature (RT).

Nanobodies are smallest antigen binding fragments with high antigen-binding affinity and fast blood clearance [20, 21]. Their short biological half-lives match well with the half-life of ^{18}F ($t_{1/2}$ = 109.8 min). In this study, we report synthesis and biological evaluation (potential to target the HER2-positive tumors, imaging characteristics, biodistribution, radiation dosimetry, and safety) of a [^{18}F]AlF-RESCA-labelled HER2 nanobody MIRC213, [^{18}F]AlF-RESCA-MIRC213. To our surprise, [^{18}F]AlF-RESCA-MIRC213 shows relatively high stability in vivo in small animals and breast cancer patients. This first-in-human experience of [^{18}F]AlF-RESCA-labelled biomolecules would be helpful for non-invasive detection of HER2 receptors expression in cancers.

Materials And Methods

Chemicals and Biochemicals

Chemicals, reagents, and solvents were purchased commercially and without further purification. (±)-H_3RESCA-Mal was purchased form Confluore Biological Technology Company (Xi'an, China). GGGGC oligopeptide was custom synthesized by GL Biochem (Shanghai, China). Na^{18}F was supplied by the Department of Nuclear Medicine, Peking University Cancer Hospital & Institute in Beijing. Disposable PD-10 Desalting Columns (PD-10 columns) were purchased from GE Healthcare (Piscataway, NJ, USA). The sterile filter (0.22 μm) was purchased from PALL (New York, USA). All cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). The SK-OV-3 cells grew in MCCOY'S 5A medium

supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The NCI-N87 and MCF-7 cells grew in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin and streptomycin. The BALB/c nude mice were obtained from Beijing Vital River Experiment Animal Technical Co., Ltd. (Beijing, China).

Production of Nanobody.

The HER2-targeting nanobody (MIRC213) was modified with LPETG-His6 at its C-terminus, which could be recognized by sortase A. Then the radiolabeling precursor of RESCA-MIRC213 was prepared by sortaseA-mediated transacylation. Briefly, MIRC213 (0.4 mM) was mixed with sortase A (8 μM) and G_4C oligopeptide (10 mM) in reaction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$) for 6 h at 4 ℃ to get MIRC213-G_4C. After purification by size-exclusion chromatography in 0.1 M PBS solution, MIRC213-G_4C was mixed with five molar excess RESCA to incubate for 2 h at 37°C under basic conditions (pH = 8.0). RESCA-MIRC213 was isolated from the reaction mixture by size-exclusion chromatography in 0.1 M PBS solution.

Radio-HPLC Method.

Radio-HPLC method for analysis of [^{18}F]AlF-RESCA-MIRC213 used an Agilent Technologies 1200 series of HPLC systems (Agilent Technologies, California, USA) and Superdex™ 75 Increase 10/300 GL columns (Cytiva, Washington, USA). The flow rate was 0.8 mL/min. The mobile phase was 100% phosphate-buffered saline buffer (PBS). The radiochemical purity (RCP) was reported as the percentage of area for the expected radiometric peak on each radio-HPLC chromatogram of [^{18}F]AlF-RESCA-MIRC213. The HPLC retention time for [^{18}F]AlF-RESCA-MIRC213 was at 16.8 min, while that of the {[^{18}F]AlF}$^{2+}$ was at 22.95 min, with less than 5%. The radio-TLC method used GE Whatman Grade 1 qualitative filter paper and PBS as the mobile phase. [^{18}F]AlF-RESCA-MIRC213 stayed at the origin while {[^{18}F]AlF}$^{2+}$ and $^{18}F^-$ migrated to solvent front.

Radiosynthesis of [^{18}F]AlF-RESCA-MIRC213

For ^{18}F-labeling, the freshly eluted 100 μL Na^{18}F solution in saline was mixed with 4 μL of 20 mM $AlCl_3$ in sodium acetate buffer solution (0.1 M, pH 4.5) at RT for 5 min. RESCA-MIRC213 (50 μL, 4 mg/mL) was added to the mixture above. After incubation at RT for 12 min, the reaction mixture was purified using a PD-10 column with PBS buffer (0.01 M, pH 7.4) as the eluent. All collected product was passed through a sterile filter (0.22 μm). The RCP of [^{18}F]AlF-RESCA-MIRC213 were analyzed by the radio-HPLC.

In Vitro Stability

[^{18}F]AlF-RESCA-MIRC213 (3.7 MBq, 200 μL) was mixed with 200 μL of 5% human serum albumins (HSA) or 200 μL PBS buffer (0.01 M, pH 7.4) and incubated at RT for 6 h. The incubated mixtures were analyzed by radio-HPLC.

Cell Uptake and Cell-Binding Affinity

NCI-N87 gastric cancer cell line and SK-OV-3 ovarian cancer cell line were HER2-positive cells. MCF-7 breast cancer cell line was HER2-negative cells. The HER2 expression was measured by flow cytometry, and the details are described in the Supporting Information. NCI-N87 cells and MCF-7 cells were plated on 24-well plates 24 h in advance (approx. 1×10^5 cells per well, 4 wells per group). Then, the 0.5 mL of [^{18}F]AlF-RESCA-MIRC213 stock solution (148 kBq/mL in fresh medium) added in every well. The medium was removed after 5, 30, 60, and 120 min incubation. Each well was washed twice with cold PBS buffer, then lysed with 200 μL, 1 M NaOH for 10 min. The radioactivity was measured with a Perkin Elmer Wizard-2470 γ counter (Waltham, MA, USA). In the blocking group, cells were co-incubated with 0.5 mL of [^{18}F]AlF-RESCA-MIRC213 stock solution (148 kBq/mL) and 100 μg cold MIRC213. The result was expressed as the percentage injected activity (%IA/10^5 cells). SK-OV-3 cells were plated on 48-well plates 24 h in advance (approx. 1×10^5 cells per well, 4 wells per group). Different concentrations of [^{18}F]AlF-RESCA-MIRC213 (0.0925 kBq, 0.37 kBq, 0.925 kBq, 1.85 kBq, 3.7 kBq, 9.25,18.5 kBq, 37 kBq, 92.5,185 kBq, and 370 kBq) in 500 μL of fresh medium were added into each well. After 120 min incubation, the medium was removed. Each well was washed the twice with cold PBS, lysed with 300 μL of 1 M NaOH for 10 min. The radioactivity was measured with γ counter. The total binding was derived and plotted against the concentration of [^{18}F]AlF-RESCA-MIRC213 to calculate the dissociation constant (K_d) [22].

Small-Animal PET/CT Protocol

All animal experiments were conducted according to protocols approved by the Peking University Biodistribution Studies Cancer Hospital Animal Care and Use Committee (EAEC 2022-01). Mice were raised under specific disease-free conditions and were handled and maintained according to the Institutional Animal Care and Use Committee guidelines.

The xenografted SK-OV-3, NCI-N87 and MCF-7 tumor models were established in BALB/c nude mice for biodistribution and PET studies. Imaging was performed with a micro PET/CT (Super Nova PET/CT, PINGSENG, Shanghai, China). [^{18}F]AlF-RESCA-MIRC213 (7.4 MBq in 200 μL solution) was injected intravenously to each animal via tail vein for PET studies. In the block group, each animal was co-injected with 7.4 MBq of [^{18}F]AlF-RESCA-MIRC213 and 1 mg of MIRC213 (n = 5). PET/CT images were acquired at 30, 60, and 120 min post-injection of [^{18}F]AlF-RESCA-MIRC213. The regions of interest (ROIs) were used to estimate the uptake in each organ.

Biodistribution Study

Biodistribution studies were performed in the xenografted SK-OV-3 and MCF-7 tumor models. Each animal was injected with [^{18}F]AlF-RESCA-MIRC213 (0.74 MBq in 200 μL solution) via tail vein. Animals were sacrificed at 2 h post-injection, and the organs of interest were harvested, washed with saline, dried with absorbent tissue, weighed, and counted on a γ-counter. The organ uptake was calculated as the percentage of injected dose (ID%) or the percentage of injected dose per gram of wet tissue (ID%/g). The blocking experiment was performed using MIRC213 (1 mg per animal) as the blocking agent. The tumor-to-background (T/B) ratios were expressed as the average plus standard deviation. Statistical analysis was performed by one-way analysis of variance (ANOVA). The level of significance was set at $p < 0.05$.

Clinical PET/CT of [^{18}F]AlF-RESCA-MIRC213

The clinical protocol was approved by the Ethics Committee of Beijing Cancer Hospital (No. 2021KT108). The informed written consent was obtained from each patient before the study. Six patients (age 18 to 75 years, ECOG score 0 or 1) who were highly suspected with breast cancer or with biopsy-confirmed HER2-expression were included in this study (Table 1). The patients excluded were those with abnormal liver and kidney function or women pregnant and lactating. Patients were injected intravenously with 222 ± 18.5 MBq of [^{18}F]AlF-RESCA-MIRC213, randomized to an injected protein mass of 0.5 mg, 1 mg and 2 mg to determine the optimal protein dose required to obtain high-contrast images. All patients underwent PET/CT at 2 and 4 h after injection. Imaging was performed from head to mid-thigh using a Philips Medical Systems Gemini TF scanner. CT was performed using 120 kev voltage, 100 mas current, pitch 0.8 mm, single-tube rotation time 0.5 s, and scan layer thickness 3 mm. CT reconstruction was performed using standard methods with a 512 × 512 matrix and layer thickness 3–5 mm. A 9–10 bed 3-dimensional models (90 s per bed) were used to acquire PET images, which were reconstructed using the ordered subset expectation maximization method. [^{18}F]-FDG PET/CT were acquired within 7 days for comparison purpose. The images were evaluated and quantified by two experienced nuclear medicine physicians. The standard uptake values (SUV) were measured using a standardized method[23]. Lesion analyses were restricted to diameter ≥ 1 cm or SUVmax value > 2.5 on [^{18}F]-FDG PET/CT. Based on traditional imaging examination and [^{18}F]-FDG results, 20 lesions were identified and were analyzed correspondingly by [^{18}F]AlF-RESCA-MIRC213 PET, including 10 HER2 lesions from HER2-positive patients (patient 2, 3, 5), and 10 HER2 lesions from HER2-negative patients (patient 1, 4, 6).

Table 1

Patient Characteristics

Patient no.	Age (y)	Injected dose (MBq)	Co-injected of MIRC213 (mg)	HER2		SUVmax		
				IHC FISH (HER2/CEP17)		Primary tumor	Contralateral breast	Liver (2 h pi.)
1	62	218.94	0.5	2+	< 2.0	1.1	0.5	11.8
2	46	215.71	1	3+	-	3.8	1.2	13.1
3	60	253.45	1	3+	-	7.5	0.5	5.1
4	58	220.52	1	1+	-	2.1	0.5	12.1
5	37	197.58	1	3+	-	4.4	1.1	6.3
6	66	249.38	2	2+	< 2.0	2.0	0.9	8.6

Statistical Analysis

All statistical analysis was completed using the SPSS software (version 22.0; IBM Corp.) and GraphPad Prism 6.0 software (San Diego, USA). *P* values less than 0.05 were considered statistically significant. Throughout the article, values are presented as mean ± SD.

Results

Synthesis of RESCA-MIRC213

MIRC213 was produced in high chemical purity over 95%. RESCA was site-specifically conjugated to MIR213 via the maleimide-thiol reaction (Fig. 1a). RESCA-MIRC213 was obtained with the average RESCA/nanobody ratio being ~ 1:1, as evidenced by the matrix-assisted laser desorption/ionization (MALDI) analysis (**Figure S1**). The purity of RESCA-MIRC213 was > 95% by HPLC analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**Figure S2**).

Radiochemistry

[^{18}F]AlF-RESCA-MIRC213 was prepared at RT within 12 min with the radiochemical yield of 50.48 ± 7.6% (n > 10) (Fig. 1a). Its RCP was > 95% (n > 10) after column purification (**Figure S3)**. The specific activity was 39.3 ± 4.3 GBq/μmol. [^{18}F]AlF-RESCA-MIRC213 was stable in PBS buffer and 5% HSA for 6 h (Fig. 1b), suggesting that [^{18}F]AlF-RESCA-MIRC213 has relatively high stability. Its lipid/water partition coefficient (Log P) value was − 2.45 ± 0.09.

Cell Uptake and Cell-Binding Affinity

Flow cytometry analysis was performed to verify the HER2-expression level in SK-OV-3, NCI-N87 and MCF-7 human tumor cells. MCF-7 cells showed a weak binding of HER2 antibody whereas SK-OV-3 and NCI-N87 cells stained intensely with the HER2 antibody (**Figure S4**). As expected, the cellular uptake of [^{18}F]AlF-RESCA-MIRC213 in NCI-N87 cells (11.22 ± 0.60%IA/10^5 cells) was significantly higher than that in MCF-7 cells (0.67 ± 0.09%IA/10^5 cells, $p < 0.001$) at 2 h, and the uptake in NCI-N87 cells was efficiently blocked with excessive cold MIRC213 to 1.02 ± 0.07%IA/10^5 cells ($p < 0.001$) of incubation (Fig. 1c). These results suggested that [^{18}F]AlF-RESCA-MIRC213 was able to target the HER2-positive tumor cells with high specificity. The K_d value of [^{18}F]AlF-RESCA-MIRC213 was calculated to be 1.23 ± 0.58 nM in binding to SK-OV-3 cells (Fig. 1d).

Small-Animal PET/CT

PET/CT images of the mice bearing subcutaneous SK-OV-3, NCI-N87 and MCF-7 xenografts were obtained at 30, 60 and 120 min after injection of [^{18}F]AlF-RESCA-MIRC213 (Fig. 2a). In general, [^{18}F]AlF-RESCA-MIRC213 underwent rapid clearance mainly through the renal route. There was a moderate clearance via the gallbladder and intestinal tract. The xenografted SK-OV-3 and NCI-N87 tumors were clearly visible at all time points, whereas its uptake in the xenografted MCF-7 tumors was barely seen.

The tumor/muscle ratios of SK-OV-3, NCI-N87 and MCF-7 at 2 h after injection were 22.71 ± 0.65, 14.95 ± 0.58 and 3.91 ± 2.00, respectively. The T/NT SUVmax ratios were shown in **Figure S5**. Co-injection of excess MIRC213 resulted in a significant reduction in tumor uptake of [^{18}F]AlF-RESCA-MIRC213 with the mean SUVs of 6.12 ± 1.73 vs 0.36 ± 0.01 in mice bearing xenografted SK-OV-3 tumors. No significant radioactivity was seen in the bone of tumor-bearing animals.

Bio-distribution and Western Blot analysis of HER2 expression

The bio-distribution data was shown in **Figure. 2b**. [^{18}F]AlF-RESCA-MIRC213 had the uptake significantly higher in SK-OV-3 tumors (4.73 ± 1.18 ID%/g) than that in MCF-7 tumors (1.36 ± 0.81 ID%/g, $p < 0.05$) at 2 h after injection. Co-injection of excess MIRC213 resulted in the decreased uptake in SK-OV-3 tumors (4.73 ± 1.18 ID%/g vs. 1.70 ± 0.13 ID%/g, $p < 0.05$), indicating the high in vivo specificity of [^{18}F]AlF-RESCA-MIRC213. These results were consistent with those from micro-PET. The tumor/muscle ratios at 2 h after injection were 28.66 ± 4.26 for SK-OV-3 tumors, 4.12 ± 2.55 for MCF-7 tumors and 4.96 ± 0.92 for the blocking group (Fig. 2c). Western blot was performed to verify the HER2-expression level in tumor tissues. As shown in Fig. 2d, the HER2-expression in the SK-OV-3 and NCI-N87 tumors was significantly higher than that in MCF-7 tumors. The bone uptake was 3.14 ± 0.45 ID%/g in the animals bearing SK-OV-3 xenografts and 2.95 ± 0.75 ID%/g in the animals bearing MCF-7 xenografts, which is similar to that reported for [^{18}F]AlF-RESCA-IL2 in BALB/c mice (3.9 ± 1.2 ID%/g) and in SCID mice inoculated with human activated PBMCs (4.3 ± 2.6 ID%/g) [24].

PET/CT in Cancer Patients

Pathological examination of primary tumor lesions was performed in all 6 breast cancer patients (from 2021/11- 2022/02). Among them, three cases were IHC HER2 3+ (high HER2 expression), and two cases were HER2 2+/FISH - and one HER2 1+ (low HER2 expression). According to screening criteria, 10 HER2-positive lesions and 10 HER2-negative lesions were compared and analyzed. In the 10 HER2-positive lesions, [^{18}F]-FDG showed obvious uptake with SUVmax of 10.72 ± 7.41 at 2 h post-injection. [^{18}F]AlF-RESCA-MIRC213 also showed high uptake (SUVmax 3.62 ± 1.56) in the same locations at the same time point. The tumor uptake remained relatively high with SUVmax of 3.22 ± 1.98 at 4 h post-injection of [^{18}F]AlF-RESCA-MIRC213. In the 10 HER2-negative lesions, [^{18}F]-FDG (SUVmax 8.99 ± 5.00) showed the uptake significantly higher than [^{18}F]AlF-RESCA-MIRC213 (2 h SUVmax 1.41 ± 0.41, 4 h SUVmax 1.41 ± 0.40) in the same locations. Obviously, [^{18}F]AlF-RESCA-MIRC213 had higher uptake in HER2-positive lesions than those in HER2-negative lesions, with SUVmax of 3.62 ± 1.56 vs. 1.41 ± 0.41 ($p = 0.0012$) at 2 h. The tumor-to-contralateral breast ratio of HER2-positive primary lesions was significantly higher (12.07 ± 5.22 vs. 4.70 ± 1.36, $p = 0.0015$) than that of HER2-negative primary lesions. With [^{18}F]-FDG, the SUVmax values of HER2-negative lesions overlaps with that of the HER2-positive lesions (SUVmax 10.72 ± 7.41 vs. 8.99 ± 5.00, $p = 0.5497$, SUVmax range of 1.1–24.2 and 2.3–14.9). The tumor uptake of [^{18}F]AlF-RESCA-MIRC213 was consistent with the HER2 expression of IHC tests, while the tumor uptake of

[^{18}F]-FDG was not related to HER2 status. These result further verified the HER2-targeting specificity of [^{18}F]AlF-RESCA-MIRC213.

Figure 3 showed representative PET/CT images and pathological data in patient 3, a 60-y female patient with HER2-positive primary breast cancer and axillary lymph node metastasis. The 2 h SUVmax of primary lesion of [^{18}F]AlF-RESCA-MIRC213 was 7.5, and [^{18}F]-FDG was 9.1. Besides, we observed a 0.5 cm axillary lymph node metastasis, which was not obvious on CT. There were more uptake in [^{18}F]AlF-RESCA-MIRC213 than [^{18}F]-FDG, with SUVmax of 3.3 vs. 1.1, respectively. The HE staining and IHC results of primary cancer were shown in Fig. 3c.

Figure 4 showed selected PET/CT images and pathological data in patient 4, a 58-y female patient with HER2-negative primary breast cancer and axillary lymph node metastases. The SUVmax of primary lesion and axillary lymph node metastases of [^{18}F]-FDG were significantly higher than that of [^{18}F]AlF-RESCA-MIRC213 (primary lesion 6.7 vs. 2.1, lymph node metastases 2.3 vs. 1.3, 3.9 vs. 1.5, 3.7 vs. 1.4). The HE staining and IHC results of primary cancer were shown in Fig. 4c.

Biodistribution

The demographics of 6 patients were presented in Table 1. There was no clinically detectable adverse pharmacological effects or significant changes in vital signs during the study. Figure 5 shows representative PET images of breast cancer patients co-injected with 0.5 mg, 1 mg and 2 mg cold MIRC213. Co-injection of cold biomolecule (antibodies, nanobodies and affibodies) is a common practice to minimize the liver radioactivity accumulation. Different amount of MIRC213 was used optimize the tumor/background ratio. Overall, the highest uptake was observed in the lacrimal gland, parotid gland, submandibular gland, thyroid gland, gallbladder, kidneys, liver, and intestines. Liver uptake was variable between different subgroups with the SUVmax values of 11.8, 13.1, 5.1, 12.1, 6.3, 8.6, respectively, at 2 h post-injection. The liver uptake of [^{18}F]AlF-RESCA-MIRC213 decreased at 4 h post-injection. Co-injection of 1 mg of MIRC213 could significantly reduce nonspecific uptake in the liver. Therefore, we chose this dose for all subsequent case studies. The uptake of [^{18}F]AlF-RESCA-MIRC213 in individual organs is presented in Fig. 6. The bone uptake in all breast cancer patients was low, which was consistent with the biodistribution and PET/CT studies in small animals (Fig. 2). Moreover, it was very similar to that reported for [^{18}F]AlF-NOTA-PSMA-BCH in prostate cancer patients (0.63 ± 0.16 vs. 0.91 ± 0.53, $p > 0.05$, 2 h post-injection) [25], and a [^{18}F]AlF-labeled somatostatin receptor (SSTR) antagonist [^{18}F]AlF-NOTA-JR11 in SSTR2-positive cancer patients [26].

Dosimetry of [^{18}F]AlF-RESCA-MIRC213

The organ radiation dosimetry and effective dose were shown in Table 2. The kidneys were the most critical organ with the highest absorbed dose of 2.17×10^{-2} mGy/MBq, followed by the urinary bladder wall (8.44×10^{-3} mGy/MBq), and thyroid (4.13×10^{-3} mGy/MBq). The average effective dose was 1.76×10^{-2} mSv/MBq.

Table 2

Human organ radiation dosimetry estimates for [^{18}F]AlF-RESCA-MIRC213.

Organ	Radiation Dosimetry (mGy/MBq)
Adrenals	2.80×10^{-3}
Brain	6.19×10^{-5}
Esophagus	7.49×10^{-4}
Eyes	1.06×10^{-4}
Gallbladder Wall	1.98×10^{-3}
Left colon	6.84×10^{-4}
Small Intestine	5.24×10^{-4}
Stomach Wall	1.52×10^{-3}
Right colon	5.29×10^{-4}
Rectum	5.96×10^{-4}
Heart Wall	1.22×10^{-3}
Kidneys	2.17×10^{-2}
Liver	1.01×10^{-3}
Lungs	3.66×10^{-3}
Pancreas	1.17×10^{-3}
Prostate	8.07×10^{-4}
Salivary Glands	1.46×10^{-4}
Red Marrow	4.59×10^{-4}
Osteogenic Cells	3.21×10^{-4}
Spleen	1.96×10^{-3}
Testes	2.46×10^{-4}
Thymus	5.82×10^{-4}

Organ	Radiation Dosimetry (mGy/MBq)
Thyroid	4.13×10^{-3}
Urinary Bladder Wall	8.44×10^{-3}
Total Body	4.51×10^{-4}
Data are in mGy/MBq (n = 6).	
The average effective dose was estimated to be 1.76×10^{-2} mSv/MBq.	

Dicussion

Nanobody has become a promising alternative to the traditional full-sized antibody in molecular imaging due to its smaller size (15 kDa) and high antigen-binding affinity[27]. Nanobody-based molecular imaging probes have been successfully translated to the bedside practice[28, 29]. In this study, we utilize MIRC213 as the HER2-targeting biomolecule, and the [^{18}F]AlF-RESCA core for ^{18}F-labelling at room temperature. [^{18}F]AlF-RESCA-MIRC213 showed a favorable biodistribution properties and high uptake in HER2-positive tumors. The uptake of [^{18}F]AlF-RESCA-MIRC213 correlates well with the tumor HER2 expression level. The uptake of [^{18}F]AlF-RESCA-MIRC213 in SK-OV-3 and NCI-N87 cells (high HER2 expression) was significantly was higher than that in MCF-7 cells (low-HER2 expression). The HER2 specificity of [^{18}F]AlF-RESCA-MIRC213 was demonstrated by the blocking with excess MIRC213 (Fig. 1c). [^{18}F]AlF-RESCA-MIRC213 shows a high uptake in the kidneys, which is consistent with the results from our previous study[30]. It also has a high uptake in the gallbladder and intestines. Thus, [^{18}F]AlF-RESCA-MIRC213 is excreted through the renal, gallbladder and intestinal tract excretion pathways.

It is interesting to note that the bone was nearly invisible on small-animal PET/CT images (Fig. 2a) even though [^{18}F]AlF-RESCA-MIRC213 shows some bone uptake in biodistribution (Fig. 2b), which is consistent with that reported for [^{18}F]AlF-RESCA-conjugated affibody, nanobody and protein[18, 24]. Al(EDTA)$^-$ (Al = aluminum, EDTA = ethylenediaminetetraacetate) and related complexes are thermodynamically stable but kinetically labile. It is not surprising that concerns are raised when RESCA is proposed as the bifunctional chelators (BFC) for ^{18}F-labeling of biomolecules. If the [^{18}F]AlF-RESCA core were to undergo demetalation and/or defluorination in vivo, one would have seen more extensive bone uptake. In addition, the bone uptake of [^{18}F]AlF-RESCA-MIRC213 is dependent on the tumor-bearing models (NCI-N87 vs. SK-OV-3 xenografts) (Fig. 2b).

Therefore, it is reasonable to believe that its bone uptake is not solely attributed to demetalation and/or defluorination. It has also been reported the bone uptake is dependent of on the animal species[32]. More importantly, [^{18}F]AlF-RESCA-MIRC213 displays undetectable bone uptake in all six breast cancer patients,

which is very similar to the results reported for [^{18}F]AlF-NOTA-PSMA-BCH in prostate cancer patients[25], and [^{18}F]AlF-NOTA-JR11 in the SSTR2-positive cancer patients[26]. These results strongly suggest that [^{18}F]AlF-RESCA is almost as good as [^{18}F]AlF-NOTA with respect to their in vivo stability. [^{18}F]AlF-RESCA is useful for highly efficient ^{18}F-labeling of heat-sensitive biomolecules. It must be noted that the in vivo stability of a new radiotracer is also dependent on its biological half-life, route of excretion, and metabolic stability. Therefore, caution must be taken in interpreting the elevated bone uptake of new radiotracers in small animals.

The high kidney uptake of [^{18}F]AlF-RESCA-MIRC213 follows a pattern similar to that of other radiolabeled polypeptides and small proteins[33–35], as expected from the uptake pattern of nanobodies in rodents[36]. The high uptake in the liver and intestine is likely nonspecific because the HER2 expression in these organs is relatively low[37]. To prove this hypothesis and assess the impact of MIRC213 on non-specific bindings, the patients were injected with [^{18}F]AlF-RESCA-MIRC213 and different amount of MIRC213. Patient 1 was co-injected with 0.5 mg MIRC213 and showed relatively high non-specific uptake in the liver (Fig. 3). Subsequently, Patient 2 was injected with [^{18}F]AlF-RESCA-MIRC213 and 1 mg MIRC213, and exhibited the best imaging contrast (Fig. 3). When 2 mg of MIRC213 was co-injected into Patient 6, the image quality was not as good as that with 1 mg of MIRC213 because of the relatively high uptake in normal organs (Fig. 3). On the basis of these findings, we thus chose 1 mg of MIRC213 as the dose for subsequent studies.

Accurate differentiation of HER2 spatiotemporal heterogeneity is of paramount importance in evaluation of HER2 status and noninvasive monitoring of the response in cancer patients treated with HER2-targeted therapy. In this study, the HER2-positive tumor lesions have significantly higher uptake than those HER2-negative lesions, with SUVmax of 3.62 ± 1.56 vs. 1.41 ± 0.41 (p = 0.0012) at 2 h post-injection. With [^{18}F]-FDG, the SUVmax of HER2-negative lesions overlaps with that of the HER2-positive lesions (SUVmax range of 1.1–24.2 and 2.3–14.9). The uptake of [^{18}F]AlF-RESCA-MIRC213 in tumor was consistent with the HER2 status from the IHC tests.

Rapid blood clearance of [^{18}F]AlF-RESCA-MIRC213 allows imaging as early as 2 h post-injection without the risk of false-positive signals due to the blood pool activity. Among all the normal organs, [^{18}F]AlF-RESCA-MIRC213 has the highest absorbed dose in kidneys (2.17×10^{-2} mGy/MBq) with the effective dose of 1.76×10^{-2} mSv/MBq, which was within a reasonable range[34]. The high uptake in the gallbladder, kidneys, and intestines remains a significant challenge although this may not interfere the interpretation of tumor lessons in chest regions. Therefore, future research should be directed towards minimization of its high uptake in kidneys, gallbladder, and intestines.

Conclusion

In summary, [^{18}F]AlF-RESCA-MIRC213 is readily prepared with excellent yield and high specific activity at RT in 20 min. The results from preclinical and clinical studies clearly show that [^{18}F]AlF-RESCA-MIRC213

is able to detect the HER2-positive tumors with reasonable radiation exposure. [^{18}F]AlF-RESCA-MIRC213 is also a promising PET radiotracer with the potential for noninvasive monitoring of anti-HER2 therapy.

Abbreviations

HER2: human epidermal receptor type 2; IHC: immunohistochemical staining; FISH: fluorescence in situ hybridization; SUV: Standardized uptake values; PET: Positron emission tomography; RT: room temperature; FBS: fetal bovine serum; HAS: human serum albumins; PBS: phosphate-buffered saline buffer; RCP: radiochemical purity; BFC: bifunctional chelators

Declarations

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Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by Ethics Committee of Beijing Cancer Hospital and Institute (No.2021KT108). Informed consent was obtained from all individual participants included in the study.

Conflict of Interest

The authors declare no potential conflicts of interest.

Informed consent

Informed consent was obtained from the 6 individual participants included in this study.

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Figures

Figure 1

(**a**) Production of [^{18}F]AlF-RESCA-MIRC213; (**b**) In vitro stability of [^{18}F]AlF-RESCA-MIRC213. (**c**) Uptake of [^{18}F]AlF-RESCA-MIRC213 in NCI-N87 and MCF-7 cells (n=4)Block=co-incubation with 100 µg of MIRC213. NS=not statistically significant. (**d**) Binding affinity determination of Al^{18}F-RESCA-MIRC213 in

the SK-OV-3 cells. Results are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, student's paired *t test.*

Figure 2

(**a**) [^{18}F]AlF-RESCA-MIRC213 PET images of mice bearing SK-OV-3, NCI-N87 and MCF-7 tumors (white arrow)Block=co-incubation with 1 mg MIRC213 in mice bearing SK-OV-3 xenografted. (**b**) Biodistribution in mice bearing SK-OV-3 and MCF-7 xenografted tumors. Block=co-injection with 1mg MIRC213 in mice bearing SK-OV-3 xenografted. (**c**) Tumor-to-muscle target ratios of [^{18}F]AlF-RESCA-MIRC213 in mice bearing SK-OV-3 and MCF-7 tumors at 2 h after injection. (**d**) Representative immunoblot image showing total HER2 expression in tissue lysates. Results are expressed as mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, student's paired *t test.*

Figure 3

PET and PET/CT images in breast cancer patients with HER2 3+ after injection of [^{18}F]-FDG and [^{18}F]AlF-RESCA-MIRC213 with 1 mg of MIRC213. (**a**) Representative maximum-intensity-projection (MIP) images of [^{18}F]-FDG (left) and [^{18}F]AlF-RESCA-MIRC213 (right) in patient 3. (**b**) PET/CT images (left), PET images (middle) and CT images (right) show primary lesion (red arrow) and lymph node metastasis (green arrow) of patient 3. (**c**) The HE staining (left) and HER2 IHC test (right) of primary lesion of patient 3.

Figure 4

4 PET and PET/CT images in breast cancer patients with HER2 1+ after injection of [^{18}F]-FDG and [^{18}F]AlF-RESCA-MIRC213 with 1 mg MIRC213. (**a**) Representative maximum-intensity-projection (MIP) images of [^{18}F]-FDG (left) and [^{18}F]AlF-RESCA-MIRC213 (right) in patient 4. (**b**) PET/CT images (left), PET images (middle) and CT images (right) show primary lesion (red arrow) of patient 4. (**c**) The HE staining (left) and HER2 IHC test (right) of primary lesion of patient 4.

Figure 5

Representative PET MIP images at 2 h and 4 h after injection of [^{18}F]AlF-RESCA-MIRC213. (**a**) Patient 1, injected with 0.5 mg of MIRC213. (**b**) Patient 3, injected with 1 mg of MIRC213. **(c)** Patient 6, injected with 2 mg of MIRC213.

Figure 6

Biodistribution of [^{18}F]AlF-RESCA-MIRC213 in patients with HER2-positive tumors (n=6).

Supplementary Files

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- SupplementarydataMIRC213EJNMMI.docx