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Original Article

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Comparison and evaluation of ^{89}Zr-labeled trastuzumab and Thio-trastuzumab : a potential immuno-PET probe for HER2-positive carcinomas

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ABSTRACT

^{89}Zr is a positron-emitting radioisotope, which has known as well-suited radioisotope for use in a monoclonal antibody-based imaging agent for immuno-PET. The purpose of this study was to quantitatively evaluate the diagnostic ability of general trastuzumab and thio-trastuzumab as HER2 positive receptors based on Df hexadentate iron chelator. Desferrioxamine-p-SCN (Df-Bz-NCS) and desferroxamine-maleimide (Df-Mal) were purchased from Macrocyclics (Dallas, TX, USA). The trastuzumab was purchased from Roche (Schweiz), and thio-trastuzumab was obtained from professor Hyo-Jeong Hong group (Kangwon National University). The radioisotope ^{89}Zr was produced by domestic purification system and KIRAMS using medical cyclotron (50 MeV, Scantronix). The conjugates of Df-trastuzumab and Df-thio-trastuzumab were prepared with Df-Bz-NCS and Df-Mal under basic aqueous solution (pH 8-9) at room temperature, respectively. The conjugates purified by PD-10 column were mixed with dried ^{89}Zr chloride. ^{89}Zr-labeled conjugates were purified and concentrated by Amicon ultra centrifugal filter. The preparation step and time of ^{89}Zr-labeled conjugates was shorted as 4 steps within 2 hours. ^{89}Zr-labeled conjugates showed the highly radiochemical purity of over 98%, and were very stable until 7 days by the analysis of radio-ITLC method. Each radio-labeled conjugates were also exhibited the highly stability in both PBS buffer and mouse serum. Immuno-PET imaging of ^{89}Zr-labeled conjugates in mice bearing gastric cancer xenograft tumors with HER2 expression showed high tumor uptake in the NCI-N87 HER2-expressing. However, ^{89}Zr-Df-Mal-thio-trastuzumab showed a relatively lower tumor-to-background ratio than ^{89}Zr-Df-Bz-trastuzumab, as well as whole-body distribution. In the results, ^{89}Zr-Df-Bz-trastuzumab was evaluated to have a relatively higher HER2 diagnostic ability than ^{89}Zr-Df-Mal-thio-trastuzumab.

Key Word: HER2, ^{89}Zr-Df-Bz-NCS-trastuzumab, ^{89}Zr-Df-thio-trastuzumab, PET/CT, NCI-N8

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Introduction

HER2 (Human Epidermal growth factor Receptor 2) is one of the representative ligand-orphan receptors overexpressed on the surface of malignant tumor cells (1,2). This amplification of HER2 is well known to induces the poor prognosis of tumors, and enhances tumor metastasis (3). Because of these unique properties, HER2 has been actively researched as a target marker for tumors over the past 30 years (4). The monoclonal antibody trastuzumab for HER2 targeting was approved by the Food and Drug Administration (FDA) to treat HER-2-positive breast cancer in 1998. Trastuzumab, as a humanized monoclonal antibody, has the characteristic of specific binding to the transmembrane domain of the tyrosine kinase receptor HER2. This selectively binding affinity of trastuzumab has been utilized for diagnosis and therapy of various cancer such as gastric and breast cancers (5). In particular, trastuzumab has been widely studied as a quantitative molecular diagnostic imaging agent for HER2-positive region in tumors by radiolabeling to positron-emitting nuclides such as ^{89}Zr or ^{64}Cu in the field of nuclear medicine (6,7). Combination of antibodies and positron-emitting radionuclides for PET applications requires a proper match between the biologic half-life of the protein and the physical half-life of the radioisotope. Therefore, the long half-life PET radioisotope ^{89}Zr ($T_{1/2}$ = 78.41 h, Eγ = 511, 908 KeV) is very appropriate radioisotope to develop immune-PET agents with antibodies for in vivo imaging of cancer (8 - 10). Based on these findings, ^{89}Zr-trastuzumab has been particularly applied in various research as a immuno-PET imaging agent of HER2-overexpressing tumors (11,12). In this process, the trastuzumab has been generally synthesized using desferrioxamine (Df) as bifunctional chelator for radiolabeling with ^{89}Zr (13,14). Recently, various Df coupling strategies have been reported using thioether, iron-N-succinyldesferrioxamine-tetrafluorophenol ester (Fe-N-suc-Df-TFP ester), p-isothiocyanatobenzyl-desferrioxamine(Df-Bz-NCS), and functionalized carbonylacrylic reagents as thio- mAbs to improve the diagnostic ability of ^{89}Zr-trastuzumab (15-17). Based on this, several studies have been applied to the evaluation of HER2/neu status or HER2 downregulation by the HSP90 inhibitor NVP-AUY922 and PU-H71 (18-20). However, among these various approaches, the efficacy of thio-trastuzumab as a HER2 overexpression tracer still have unclear limitations. Accordingly, the purpose of this study was to quantitatively evaluate the diagnostic ability of general trastuzumab and thio-trastuzumab as HER2 positive receptors based on Df hexadentate iron chelator.

Materials and Methods

1. General

Df-Bz-NCS and Df-Mal were purchased from Macrocyclics, Inc. (Dallas, TX, USA). The trastuzumab was purchased from Roche (Schweiz), and thio-trastuzumab was obtained from Professor Hyo-Jeong Hong group (Kangwon National University, Korea). The radioisotope ^{89}Zr was produced at the KIRAMS (Korea Institute of Radiological & Medical Sciences) using medical cyclotron (50 MeV, Scantronix). NCI-N87 (HER2 positive gastric cancer) cell line was purchased from ATCC (American Type Culture Collection, USA). The cell line was cultured in RPMI 1640 (Hyclone, Thermo SCIENTIFIC, Utah, USA) supplemented with 10% fetal bovine serum (J R Scientific (JRS) CA, USA) and 1% penicillin-streptomycin (Gibco, Life Technologies). ^{89}Zr radiolabeling reaction was assessed using instan thin-layer chromatography (ITLC-SG) paper (Pall Corp., Port Washington, NY) and analyzed on the gamma counter (1480 Wizard3, Perkin Elmer, MA, USA). All

activity measurements were performed in a dose calibrator (CRC-15R Capintec, U.S.A).

2. Synthesis of Df-trastuzumab and Df-thio-trastuzumab conjugates

We used the Df-Bz-NCS and Df-Mal as a bifunctional chelator for ^{89}Zr labeling to trastuzumab and thio-trastuzumab, respectively. First of all, Df-trastuzumab conjugate was mixed with Df-Bz-NCS (in 100 μL of DMSO) with 1.5 mg of trastuzumab in 300 μL distilled water for 2 hr at RT. Next, Df Df-thio-trastuzumab conjugate was mixed with Df-Mal (in 50 μL of DMSO) with 1.5 mg of thio-trastuzumab in 200 μL distilled water for 30 min at RT. pH of both derivatives were adjusted to 8 with 1 M $NaHCO_3$. The Df-trastuzumab and Df-thio-trastuzumab conjugates were purified by using size exclusion chromatography (PD-10 column, GE Health care).

3. ^{89}Zr labeling of Df-trastuzumab and Df-thio-trastuzumab

The pH was adjusted with 1mM NaOAc (pH 5 ~ 6) when purified by PD-10 column prior to ^{89}Zr labeling reaction. Then, the purified Df-trastuzumab and Df-thio-trastuzumab conjugates were labeled with dried $^{89}ZrCl_2$ at room temperature for 10 min, respectively. Radiochemical purity (>95 ± 2 %) was evaluated by radio-ITLC, using 0.1M citric acid mobile phase. Finally, ^{89}Zr-labeled Df-trastuzumab and Df-thio-trastuzumab conjugates were purified by using Amicon Ultra (centrifugal filters, 15mL 100K, MILLIPORE, IRELAND) to eliminate Free-^{89}Zr and Df.

4. Stability test

The stability of ^{89}Zr-labeled complexes was incubated by mixing ^{89}Zr-Df-Bz-trastuzumab (100 μCi) and ^{89}Zr- Df-Mal-thio-trastuzumab (100 μCi) with human serum (500 μL) and PBS (500 μL) at 37 ℃ for 24, 48, 72, 96, 138 and 168 h respectively.

5. Cell uptake studies (NCI-N87)

NCI-N87 (gastric carcinoma cell, HER2-expressing) were cultured in RPMI-1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin and grown at 37 ℃, respectively. NCI-N87 cells (2×10^6/2 ml) in culture media were seeded in each well of 6-well plate and were incubated for 1, 4, 24, 48, and 72 h in atmosphere containing 5% CO_2 at 37℃, respectively. (n = 3 each).

6. Small animal PET/CT imaging studies

Animal studies were performed according to approved protocol by the animal research committee of Korea Institute of Radiological and Medical Sciences (KIRAMS). Small animal PET/CT scans were conducted using a micro-PET/CT scanner (Inveon™, Siemens) at 24, 48, 72, and 96 h post injection. Micro-PET/CT scans were performed on xenograft gastric carcinoma bearing female BALB/C nude mice (n = 4). Nude mice were administered ^{89}Zr-Df-Bz-trastuzumab (n = 3) and ^{89}Zr-Df-Mal-thio-trastuzumab (n = 3) via tail vein injection (100 ± 1.0 μCi) under 1.5% isoflurane anesthesia. Acquired micro-PET/CT images were reconstructed by 2-dimensional order-subset expectation maximization (OSEM 2D). For each micro-PET/CT scan, regions of interest (ROI) were evaluated the tumor, normal tissue, and major organs on the whole-body images. The radioactivity concentration within the tumor, muscle, liver, and the other major organs was obtained from the mean value within the regions of interest and then converted to percentage of the injected dose/gram tissue (%ID/g).

7. Biodistribution studies

Tumor bearing nude mice were anesthetized with isoflurane (Abbott Lab. LTD, USA) mixed 35% O_2 in N_2. 10 ± 0.5 μCi of ^{89}Zr-Df-Bz-trastuzumab and ^{89}Zr-Df-Mal-thio-trastuzumab were administered via tail vein injection to each mouse (n = 4). Mice were sacrificed at

different time points (24, 48, 72, and 96 h) post injection (i.p). Biodistribution was obtained tumors, interest organs (muscle, liver, kidneys, bone, lung, spleen, heart, intestine, stomach, tail), blood and then were collected, weighted. The radioactivity concentrations were analyzed with automatic gamma counter (1480 Wizard3, Perkin Elmer) at each time point. The tumor and organ uptake were calculated % ID/g.

Result and Discussion

1. Preparation and analysis of 6 and Df-thio-trastuzumab

Desferrioxamine (Df) is one of chelators that can form a very stable complex with ^{89}Zr (21). In our studies, the trastuzumab was prepared by coupling Df-Bz-NCS to the lysine groups of trastuzumab mAb. Also, the thio-trastuzumab was prepared by coupling Df-Mal to the cysteine groups of thio-trastuzumab mAb (Figure 1). The conjugation reaction of monoclonal antibody (mAb) and desferrioxamine (Df) is very sensitive on pH. Accordingly, conditions of immuneconjugation for 2 chelates are listed in the table 1. Df-Bz-NCS-trastuzumab and Df-Mal-thio-trastuzumab analyzed a chelate-to-mAb ratio of 1.9, 1.3 by using MALDI-TOF, respectively (Table.1). In the same synthetic environment, Df-Bz-NCS-trastuzumab showed a relatively higher mAb conjugate rate (about 46%) than Df-Mal-thio-trastuzumab.

Table 1. Reaction condition of Df-Bz-NCS and Df-Mal Conjugation to Prepare ^{89}Zr-Df-mAb.

Df-Bz-NCS Chelate Conjugation to mAb					Df-Chx-Mal Chelate Conjugation to mAb				
pH	time (h)	Chelates (excess nm fold)	mAb (nmol)	Chelates/mAb (c/a)	pH	time (min)	Chelates (excess nm fold)	mAb (nmol)	Chelates/mAb (c/a)
8	2	1	20	1.9	8	30	1	20	1.3

2. Radiolabeling of Df-trastuzumab and Df-thio-trastuzumab

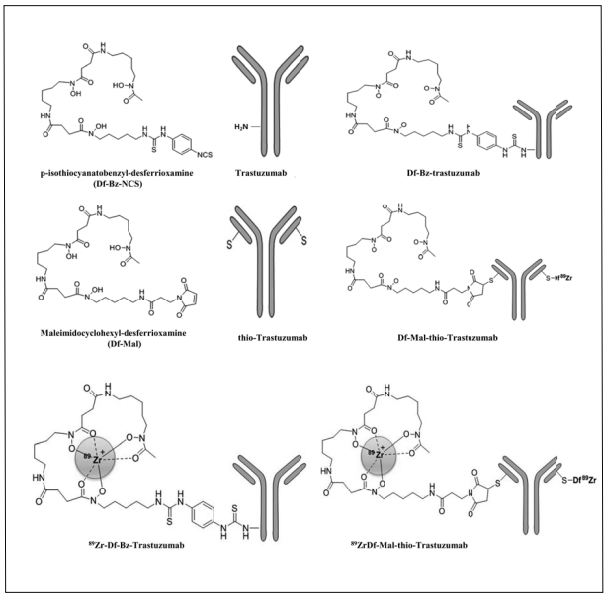

Figure 1. Scheme of mAb modification with the bifunctional chelate Df-Bz-NCS and Df-Mal.

Radiolabeling of Df-trastuzumab and Df-thio-trastuzumab with $^{89}ZrCl_2$ was labeled with high radiochemical purity (>95%, respectively) at room temperature for 10 minutes. ^{89}Zr-Df-trastuzumab and ^{89}Zr-Df-thio-trastuzumab were purified by using Amicon Ultra (centrifugal filters, 15mL 100K, MILLIPORE, IRELAND) in order to remove impurities. Final radiochemical purity after Amicon Ultra purification was >98% in all ^{89}Zr-labeled analogues.

3. *In vitro* evaluations

The stability of ^{89}Zr-Df-Bz-trastuzumab and ^{89}Zr-Df-Mal-thio-trastuzumab was determined in PBS and mouse serum. We observed a very high stability (>95%) for up to 7 days at 37 °C. As shown in figure 2, cell uptake of Df-trastuzumab and Df-thio-trastuzumab increased slowly for up to 48 h. The uptake of both Df-Bz-trastuzumab and Df-Mal-thio-trastuzumab labeled with ^{89}Zr was indicated the highest results at 48 hours. However, these results showed that Df-Bz-trastuzumab is approximately 2 times higher than Df-Mal-thio-trastuzumab.

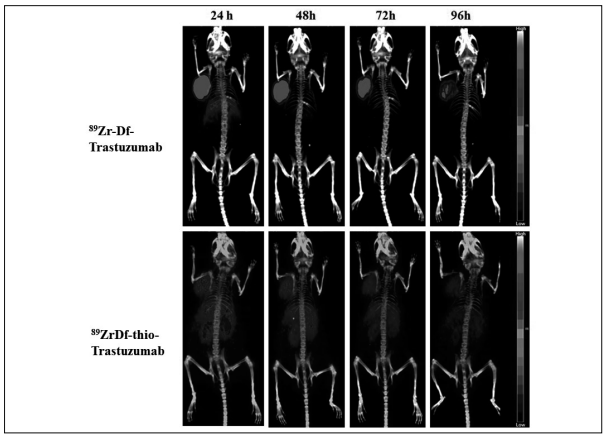

Figure 3. Tumor uptake of ^{89}Zr-Df-trastuzumab and ^{89}Zr-Df- thio-Trastuzumab on HER2 (NCI-N87) model. The immune-PET images were acquired at 24-96 h after tail vein injection (respectively 100 ± 1.0 μCi) with maximum intensity projections (MIP).

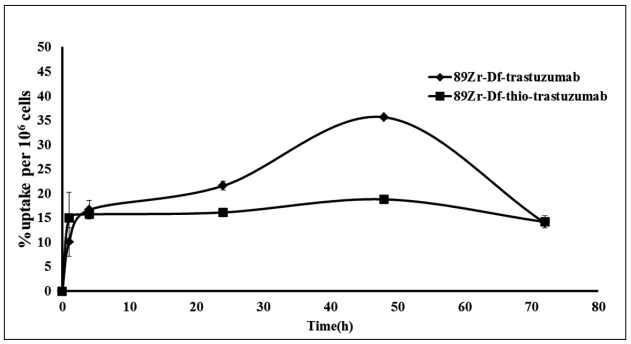

Figure 2. Cell uptake of Df-trastuzumab and Df-thio-Trastuzumab labeled with ^{89}Zr in accordance with over time. The 3μCi of each tracer was added to each well and incubated at 37 °C for 1, 4, 24, 48 and 72 h, respectively (n = 3 each).

4. *In vivo* imaging (PET/CT) studies

Immuno-PET/CT imaging of ^{89}Zr-Df-Bz-trastuzumab and ^{89}Zr-Df-Mal-thio-trastuzumab was evaluated by xenografted mouse models of NCI-N87 (HER2 positive). Each Immuno-PET/CT image of small mouse model was acquired at various time points (24, 48, 72, and 96 h) after injection of 100 ± 1.0 μCi of radiolabeled mAb. Representative PET images remarkably showed the tumor on each left shoulder (Figure. 3). Both ^{89}Zr-Df-Bz-trastuzumab and ^{89}Zr-Df-Mal-thio-trastuzumab were showed the relatively highest high tumor uptake rate at 48 h post injection as in the cellular uptake studies, respectively. However, ^{89}Zr-Df-Mal-thio-trastuzumab showed a relatively lower tumor-to-background ratio than ^{89}Zr-Df-Bz-trastuzumab, as well as whole-body distribution. In the results, ^{89}Zr-Df-Bz-trastuzumab was evaluated to have a relatively highe HER2 diagnostic ability than ^{89}Zr-Df-Mal-thio-trastuzumab.

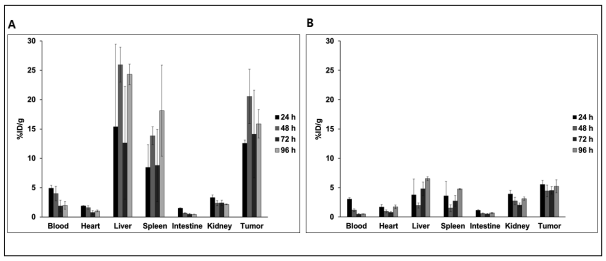

Figure 4. Biodistribution of ^{89}Zr-Df-Bz-trastuzumab and ^{89}Zr-Df-Mal-thio-Trastuzumab in NCI-N87 (HER2 expression) bearing female athymic nude mice (respectively, n = 4). Approximately 10 ± 0.5μCi of each tracer was administered via the tail vein.

5. Biodistribution studies

At 24, 48, 72, and 96 h post injection, the biodistribution results are presented in figure 4. ^{89}Zr-Df-Bz-trastuzumab and ^{89}Zr-Df-Mal-thio-trastuzumab showed the high uptake rates in tumor, liver and spleen at all time points. The tumor uptakes of ^{89}Zr-Df-Bz-trastuzumab (A) at 24, 48, 72 and 96 h were 12.58 ± 0.53, 20.55 ± 4.62, 14.14 ± 7.45, and 15.88 ± 2.42 %ID/g, respectively. It was showed the highest value similar to cell uptake study at 48 h p.i, whereas the ^{89}Zr-Df-Mal-thio-trastuzumab (B) at 24, 48, 72, and 96 h showed little different with 5.54 ± 0.66, 4.43 ± 0.98, 4.54 ± 0.63, and 5.21 ± 1.10 %ID/g, respectively. In particular, these were showed difference of approximately 5 times at 48 h p.i. The blood levels of ^{89}Zr-Df-Bz-trastuzumab declined from 4.91 ± 0.47%ID/g at 24 h to 1.99 ± 0.66%ID/g at 96 h, and ^{89}Zr-Df-Mal-thio-trastuzumab declined from 3.05 ± 0.23%ID/g at 24 h to 0.49 ± 0.04%ID/g at 96 h. The results of these biodistribution studies, demonstrated the organ distribution characteristics and tumor uptake rate of each mAb in the body, similar to the PET imaging results.

Conclusion

We synthesized ^{89}Zr-Df-Bz-trastuzumab and ^{89}Zr-Df-Mal-thio-trastuzumab in this study, and quantitatively compared the distribution characteristics and potential as a PET tracer for molecular diagnosis of HER2 overexpression site. This study showed that df-based pure ^{89}Zr-Df-Bz-trastuzumab could more effectively and stably track the HER2 than thiol-substituted ^{89}Zr-Df-Mal-thio-trastuzumab. These research results are expected to contribute to research on the development of an ^{89}Zr- trastuzumab-based HER2 tracer.

Acknowledgments

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