SPECT/CT Imaging of the Novel HER2-targeted Peptide Probe $^{99m}$Tc-HYNIC-H6F in Breast Cancer Mouse Models

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ABSTRACT

Objectives: Overexpression of human epidermal growth factor receptor 2 (HER2) plays important roles in tumorigenesis and tumor progression in breast cancer. Nuclear imaging of HER2 expression in tumors might detect all HER2-positive tumors throughout the body and guide HER2-targeted therapies for patients. We therefore aimed to develop a HER2-targeted peptide probe for breast cancer imaging. A novel single-photon emission computed tomography (SPECT) imaging probe, \(^{99m}\text{Tc-HYNIC-H6F}\), was prepared and then evaluated in breast cancer animal models.

Methods: The HER2-targeted peptide H6F (YLFFVFER) was conjugated with the bifunctional chelator hydrazinonicotinamide (HYNIC). \(^{99m}\text{Tc-HYNIC-H6F}\) was prepared, and the \textit{in vivo} characteristics of \(^{99m}\text{Tc-HYNIC-H6F}\) were investigated in MDA-MB-453 (HER2-positive) and MDA-MB-231 (HER2-negative) models using small-animal SPECT/computed tomography (CT). Moreover, to investigate the specificity of the H6F peptide toward HER2 and the potential applications in monitoring therapies involving trastuzumab, unlabeled H6F and trastuzumab were employed as blocking agents in cell competition studies and SPECT imaging.

Results: A standard tricine/trisodium triphenylphosphine-3,3’,3”-trisulfonate (TPPTS) labeling procedure demonstrated that the radiochemical purity was greater than 95%. \(^{99m}\text{Tc-HYNIC-H6F}\) displayed excellent HER2 binding specificity both \textit{in vitro} and \textit{in vivo}. SPECT/CT imaging revealed that the MDA-MB-453 tumors were clearly visualized (3.58 ± 0.01 %ID/g at 30 min p.i.), whereas the signals in HER2-negative MDA-MB-231 tumors were much lower (0.73 ± 0.22 %ID/g at 30 min p.i.). Tumor uptake of MDA-MB-453 was blocked by the co-injection of excess H6F but not by excess trastuzumab.

Conclusion: The \(^{99m}\text{Tc-HYNIC-H6F}\) peptide probe specifically accumulates in HER2-positive tumors and is therefore a promising probe for the diagnosis of HER2-positive cancers. Because \(^{99m}\text{Tc-HYNIC-}
H6F and trastuzumab target different regions of the HER2 receptor, this radiotracer also has great potential for monitoring the therapeutical efficacy of trastuzumab by rechecking the expression level of HER2 without blocking effect during therapy.

**Keywords** HER2, breast cancer, SPECT, targeting peptide, trastuzumab
INTRODUCTION

Breast cancer is the most frequent cancer and the second leading cause of cancer death among women worldwide. The great progress in breast cancer screening and early diagnosis in recent decades has significantly increased life expectancy and patient quality of life (1,2). The most well-studied tumor-associated antigen in breast cancer is HER2, which is positive in approximately 20-30% of all breast cancers (3-5). Moreover, overexpression of HER2 is also characterized as a major negative prognostic factor that is associated with higher mortality in early-stage disease, increased incidence of metastasis, and reduced time to relapse (6-8). Trastuzumab (Herceptin), the first approved HER2-targeted humanized monoclonal antibody, is the standard-of-care treatment for patients with HER2-positive breast cancer. In several trials, trastuzumab has proven effective in combination with or following standard chemotherapy (9-11). HER2 expression status in histopathological samples of primary cancer or metastatic tissue can be analyzed by immunohistochemistry or fluorescence in situ hybridization in clinical practice (12). However, the acquisition of biopsies is invasive, and tumor heterogeneity can lead to variable results (13). Intermetastatic, intrametastatic and even intratumoral heterogeneity in breast cancers also make indirect approaches like serum HER2 immunoassays insufficient to predict long-term responses or to appropriately change the treatment schedule. Thus, a more accurate and noninvasive method for the assessment of HER2 expression of whole-body tumors is needed (14).

Nuclear imaging may represent an appropriate tool to obtain real-time and quantifiable information about HER2 expression with high sensitivity and spatial resolution and remedy current deficiencies in the assessment of HER2 expression in vivo (15). In several preclinical and clinical studies, radiolabeled trastuzumab and pertuzumab have been shown high accumulation in tumor tissues (16,17). But the best time for assessment of antibody-based imaging with reasonable tumor-to-organ ratios is typically 3–5 days after administration, which may delay the treatment modification. Thus, small-molecule
alternatives such as ScFv-Fc, F(ab’)_2, minibody, diabody and affibody molecules were developed and radiolabeled as cancer diagnosis probes (18). The decreased molecular size of these molecules alters the clearance pathway to enable favorable imaging properties. As small targeting molecules, peptides exhibit relatively short circulatory half-lives, good penetration, low immunogenicity and ease of chemical modification and are thus more suitable for clinical imaging procedures (19,20). Although numerous peptide-based imaging agents have been synthesized and translated from bench to bed (21), HER2-targeted peptides for nuclear imaging remain underdeveloped.

We previously screened out a novel HER2-targeted peptide, H6F, using a one-bead one-compound combinatorial library approach. In vivo and ex vivo experiments demonstrated that the H6F peptide has high affinity and high specificity toward HER2 (22). In the present study, we developed this H6F peptide as a SPECT imaging probe for in vivo breast cancer HER2 detection and evaluated its targeting capability in subcutaneous human breast cancer xenografts.

MATERIALS AND METHODS

Cell Culture and Animal Models

The MDA-MB-453 and MDA-MB-231 human breast cancer cell lines were obtained from China Infrastructure of Cell Line Resources and were grown in Leibovitz L15 medium. Both cells lines were cultured in medium supplemented with 10% fetal bovine serum at 37 ºC in a humidified atmosphere containing 5% CO₂.

All animal experiments were performed in accordance with the guidelines of the Peking University Animal Care and Use Committee. To obtain MDA-MB-453 and MDA-MB-231 subcutaneous tumor models, MDA-MB-453 cells (1 × 10⁷ in 200 μL of phosphate-buffered saline) or MDA-MB-231 cells (6 × 10⁶ in 200 μL of phosphate-buffered saline) were inoculated subcutaneously into the right front flanks
of female Balb/C nude mice. The animals were used for in vivo studies when the tumor size reached 100–150 mm³ (2–3 wk after inoculation).

**Preparation of ⁹⁹ᵐTc-HYNIC-H6F**

The H6F peptide (TLFFVFER) and fluorescein isothiocyanate (FITC)-H6F were synthesized as previously described (22). The H6F peptide was then conjugated with SBZ-HYNIC using a standard procedure (23). A solution of 2 μmol of H6F peptide was mixed with 6 μmol of SBZ-HYNIC in 500 μL of DMF. After stirring at room temperature for 6 h, HYNIC-H6F was isolated by semipreparative high performance liquid chromatography (HPLC) and lyophilized to afford the final product. HYNIC-H6F was labeled with ⁹⁹ᵐTc using tricine and TPPTS as the co-ligands (23) and then purified with Sep-Pak C18 cartridges (Waters). After purification, the radiochemical purity of ⁹⁹ᵐTc-HYNIC-H6F was determined by radio-HPLC.

**Fluorescence Staining and Flow Cytometry**

To evaluate the expression status of HER2 in different breast cancer cells, fluorescence staining and flow cytometry of MDA-MB-453 and MDA-MB-231 cells were performed. In the cell staining experiment, approximately 1×10⁵ MDA-MB-453 and MDA-MB-231 breast cancer cells in 1 mL of medium were seeded into culture dishes, and incubated with FITC-trastuzumab solution (200 μg) with Hoechst 33342 (1 mmol/L) for 120 min at 4 ºC, and visualized using a confocal microscope (Wetzler, Heidelberg, Germany). In the flow cytometry experiment, the cells were harvested and suspended in PBS supplemented with 1% bovine serum albumin (BSA), followed by incubation with FITC-trastuzumab (30 μg) for 1 h at 4 ºC. Then the samples were analyzed using an LSR-II flow cytometer (Becton Dickinson, Germany). The HER2-targeting capability of the H6F peptide was investigated
using the same protocols. Instead of FITC-trastuzumab, FITC-H6F (50 μmol/L, 200 μL) was used in cell staining, and 15 μg of FITC-H6F was applied in the flow cytometry experiment.

**Binding Affinity of HYNIC-H6F to HER2**

The receptor-binding affinity of HYNIC-H6F was assessed *in vitro* (24). For binding assays, HYNIC-(125I)H6F was prepared in high specific activity (44.4 MBq/nmol). The *in vitro* HER2 binding affinity/specificity of HYNIC-H6F was compared with that of H6F and trastuzumab. High-HER2-positive MDA-MB-453 cells were incubated with 200,000 cpm of HYNIC-(125I)H6F and various concentrations of competitive inhibitors for 2 h at 4 °C. The cells were then washed with ice-cold 0.1 M PBS (pH 7.4) buffer to remove free radioactivity. The cells with bound radioactivity were collected, and the radioactivity was measured in a γ-counter (Wallac 1470-002, Perkin Elmer, Finland). The results were expressed as the percentage of the bound radioactivity versus total added radioactivity after decay correction. All experiments were performed twice with four samples for each.

**Small-Animal SPECT/CT Imaging**

A small-animal SPECT/CT imaging study was performed on Balb/C nude mice bearing MDA-MB-453 or MDA-MB-231 breast cancer xenografts. Each tumor-bearing nude mouse was injected via the tail vein with 37 MBq of 99mTc-HYNIC-H6F (n = 4 per group). The blocking study was also performed in MDA-MB-453 mice (n = 4 per group) by co-injection of 37 MBq of 99mTc-HYNIC-H6F with an excess dose of the H6F peptide (200 μg) or trastuzumab (500 μg). At 30 min, 1 h, and 2 h post-injection (p.i.), the mice were anesthetized by inhalation of 2% isoflurane and imaged using nanoScan (Mediso Inc., Hungary) following a standard protocol. The pinhole SPECT images (peak: 140 keV, 20% width; frame time: 25s) were acquired for 13.5 minutes and subsequently CT images were acquired (50 kVp,
0.67 mA, rotation 210°, exposure time: 300 ms). All SPECT images were reconstructed and further analyzed with Fusion (Mediso Ltd., Hungary) by drawing appropriate Volume of Interests on tumor and major organs such as liver (without gallbladder), heart, and kidneys.

**Biodistribution**

Female nude mice bearing MDA-MB-453 or MDA-MB-231 tumor xenografts were injected with 0.37 MBq of $^{99m}$Tc-HYNIC-H6F to evaluate the distribution of $^{99m}$Tc-HYNIC-H6F in tumors and major organs ($n = 4$ per group). The mice were sacrificed and dissected at 30 min, 1 h, 2 h after injection. Blood, tumor, liver (without gallbladder) and other major organs were collected and weighted. The radioactivity in the tissue was measured using a γ-counter. The results are presented as the percentage injection dose per gram of tissue (%ID/g). Blocking studies were also performed in 4 nude mice bearing MDA-MB-453 tumors. For the H6F peptide-blocking group, each mouse was co-injected with 200 μg of unlabeled H6F peptide and 0.37 MBq of $^{99m}$Tc-HYNIC-H6F. At 30 min after injection, all mice in the blocking group were sacrificed, and the organ biodistribution of $^{99m}$Tc-HYNIC-H6F was determined.

**Statistical Analysis**

Quantitative data are expressed as the mean ± SD. Statistical analysis of image quantification and biodistribution were performed with one-way analysis of variance and Student’s t-test with GraphPad Prism 5.0 (GraphPad Software, Inc.). $P < 0.05$ was considered statistically significant. The best-fit 50% inhibitory concentration (IC$_{50}$) values were calculated by fitting the data with nonlinear regression one-site competitive binding model using GraphPad Prism 5.0 (GraphPad Software, Inc.).

**RESULTS**
Chemistry and Radiochemistry

HYNIC-H6F was obtained in 68% yield with a retention time of 14.8 min using analytical HPLC. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry detected 1423.41 (m/z) (C_{71}H_{86}N_{14}O_{16}S, calculated molecular weight 1423.61). The product was confirmed by mass spectroscopy analysis (Supplemental Fig. 1A). The HPLC purity of HYNIC-H6F was greater than 95%, and thus, HYNIC-H6F was directly used for a receptor binding assay and ⁹⁹mTc radiolabeling. The ⁹⁹mTc labeling procedure was performed within 25 min, with a labeling yield of 92.6%. After purification, the radiochemical purity of ⁹⁹mTc-HYNIC-H6F was greater than 95% as determined by radio-HPLC (Supplemental Fig. 1B), and the specific activity of ⁹⁹mTc-HYNIC-H6F was > 35 MBq/nmol. The structure of ⁹⁹mTc-HYNIC-H6F is shown in Fig. 1A, and the analysis of the H6F peptide with HER2 using AutoDock (25) is shown in Fig. 1B.

Fluorescence Staining and Flow Cytometry

The HER2 targeting capability of the H6F peptide was evaluated in MDA-MB-453 and MDA-MB-231 cells in vitro. The results of incubation with FITC-trastuzumab clearly demonstrated that MDA-MB-453 cells were HER2-positive, whereas MDA-MB-231 cells were HER2-negative (Figs. 2A and 2B), consistent with the results of previous studies (26,27). The FITC-labeled H6F peptide exhibited similar binding to HER2-overexpressing breast cancer cells (Fig. 2A). Double fluorescent staining revealed that both Cy3-trastuzumab and FITC-H6F accumulated on the cell membrane in MDA-MB-453 cells (Supplemental Fig. 2).

SPECT/CT Imaging with ⁹⁹mTc-HYNIC-H6F

Tumor imaging using ⁹⁹mTc-HYNIC-H6F was evaluated in Balb/C nude mice bearing MDA-MB-
453 and MDA-MB-231 tumors. SPECT images were acquired at 30 min, 1 h, and 2 h after the administration of $^{99m}$Tc-HYNIC-H6F. HER2-positive tumors were detected as early as 30 min post-injection (p.i.) with high contrast compared with the contralateral background (Fig. 3A). The biodistribution patterns of $^{99m}$Tc-HYNIC-H6F in normal organs were similar in both mouse groups (Figs. 3A-C). Gall bladder uptake of $^{99m}$Tc-HYNIC-H6F in both groups is high due to its high lipophilicity. However, the tumor uptake values of $^{99m}$Tc-HYNIC-H6F were significantly higher in MDA-MB-453 tumors than in MDA-MB-231 tumors (Fig. 3C), and orthotopic MDA-MB-453 tumors were clearly visualized by SPECT/CT imaging (Supplemental Fig. 3).

**H6F and Trastuzumab Blocking Studies**

Unlabeled H6F peptides were co-injected with $^{99m}$Tc-HYNIC-H6F to assess the binding specificity in MDA-MB-453 tumors, and images and quantification results clearly demonstrated that the radioactive signals of the imaging tracer were mostly blocked (Figs. 4A-B). However, MDA-MB-453 tumors were still clearly visible in the trastuzumab blocking group, which indicated that $^{99m}$Tc-HYNIC-H6F could be used to detect HER2-positive tumors during trastuzumab-based therapy without concern about the competitive binding of trastuzumab. The HER2 binding affinity of HYNIC-H6F to HER2-positive MDA-MB-453 cells was compared with that of H6F using a competition binding assay with HYNIC-(125I)H6F as a radioligand (Fig. 4C). The $IC_{50}$ (50% inhibitory concentrations) values for HYNIC-H6F and H6F were 11.25 ± 2.14 and 7.48 ± 3.26 nM, respectively, which confirmed that the conjugation of HYNIC had little impact on ligand-receptor binding affinity. Furthermore, excess unlabeled HYNIC-H6F significantly inhibited the binding of HYNIC-(125I)H6F to the HER2 protein (Supplemental Fig. 4). However, trastuzumab did not inhibit the binding of HYNIC-(125I)H6F *in vitro* (Fig. 4C).
Biodistribution

The biodistribution results for $^{99m}$Tc-HYNIC-H6F at 30 min, 1 h and 2 h p.i. are summarized in Figure 5. The uptake values of $^{99m}$Tc-HYNIC-H6F in MDA-MB-453 tumors were $2.47 \pm 0.12$, $0.66 \pm 0.24$ and $0.21 \pm 0.05\%$ID/g at 30 min, 1 h and 2 h p.i., respectively (Fig. 5A). The tumor uptake of $^{99m}$Tc-HYNIC-H6F was significantly higher than uptake in normal organs such as the heart, spleen, intestine and muscle at nearly all time points examined.

The biodistribution patterns of $^{99m}$Tc-HYNIC-H6F in normal organs were similar in HER2-negative MDA-MB-231 mice and HER2-positive MDA-MB-453 mice at 30 min p.i. (Fig. 5B), except liver. However, at the same time point, the uptake of $^{99m}$Tc-HYNIC-H6F was significantly higher in MDA-MB-453 tumors than in MDA-MB-231 tumors ($2.47 \pm 0.12$ vs $0.99 \pm 0.19\%$ID/g, $P < 0.05$ at 30 min p.i.). The specificity of $^{99m}$Tc-HYNIC-H6F was then examined in a separate group of mice ($n = 4$), whereas the co-injection of an excess dose of cold H6F peptide with $^{99m}$Tc-HYNIC-H6F resulted in significantly reduced tumor uptake at 30 min p.i. (from $2.47 \pm 0.12$ to $1.03 \pm 0.37\%$ID/g, $P < 0.05$).

DISCUSSION

HER2 amplification is correlated with aggressive tumor behavior and a significantly worse prognosis. Because of its prognostic and predictive value, HER2 protein expression is widely accepted as an examination item in all patients with newly diagnosed primary invasive breast cancer (28). Based on HER2 status in tumors, proper clinical treatment strategies with HER2-directed therapeutic drugs have been designed to improve clinical outcomes for millions of breast cancer patients. Trastuzumab alone or in combination with other chemotherapy drugs is approved for first-line treatment of HER2-positive breast cancer. However, the trastuzumab monotherapy response rate for metastatic breast cancer is less than 34% (29,30). Monitoring the response to therapies in vivo at early time points is of important
clinical significance for use as an individualized tool in precision medicine for breast cancer patients.

In this study, a HER2-targeting peptide, H6F, was modified with a bifunctional chelator and then labeled with $^{99m}$Tc to form a novel SPECT imaging agent, $^{99m}$Tc-HYNIC-H6F. $^{99m}$Tc is one of the most commonly used radionuclides in the clinic, and its appropriate half-life and excellent radiochemical properties make it suitable for peptide-based nuclear imaging. TPPTS was employed as a co-ligand and reductant in the radiolabeling protocol, and the whole procedure was rapid and efficient with a high labeling yield, consistent with the convenient formulation of a labeling kit (31). Moreover, $^{99m}$Tc-HYNIC-H6F exhibited excellent in vitro stability in saline for 6 h (Supplemental Fig. 5). In both cell staining and flow cytometry studies, FITC-H6F exhibited tumor cell binding features but a lower affinity for receptors than FITC-trastuzumab. In HER2-positive SKBR3 breast cells, FITC-H6F fluorescence signals decrease significantly upon transfection of the recombinant plasmid pRNAi-HER2 in HER2-positive SKBR3 breast cells, confirming that the HER2 protein is the specific target of the H6F peptide (22). Furthermore, the introduction of HYNIC to the original H6F peptide did not significantly affect its binding affinity to the HER2 protein (Fig. 4C). The easy labeling protocol, favorable stability, high specificity and good receptor-binding affinity support further application to tumor imaging in animal models.

SPECT/CT imaging and the biodistribution in mice injected with $^{99m}$Tc-HYNIC-H6F revealed rapid accumulation and relatively high uptake in subcutaneous HER2-positive MDA-MB-453 tumors and very low uptake in HER2-negative MDA-MB-231 tumors. The maximum uptake values of the radiotracer were observed at 30 min p.i., and the highest tumor-to-organ ratios were observed at 1 h p.i. The rapid clearance from normal organs reduces the wait time between administration and imaging and also avoids unnecessary radiation dose burden to surrounding tissues. In addition, SPECT imaging with $^{99m}$Tc-HYNIC-H6F accurately located small orthotopic tumors (< 5 mm in diameter) in the right mammary fat
pads of female Balb/C nude mice (Supplemental Fig. 3), indicating potential clinical applications of $^{99m}$Tc-HYNIC-H6F in whole-body screening of HER2-positive metastatic tumors. Co-injection of excess unlabeled H6F peptide effectively blocked the tumor uptake of $^{99m}$Tc-HYNIC-H6F in HER2-positive tumors, confirming that the binding of $^{99m}$Tc-HYNIC-H6F with HER2 was receptor-mediated.

Trastuzumab plays its anti-tumor function in HER2-positive breast cancers partially relying on the activation of antibody-dependent cellular cytotoxicity and increased intracellular degradation of HER2 via binding (32,33), which can induce the loss of HER2-positive cells and down-regulation of HER2 expression in tumors. Moreover, HER2-positive breast cancer tumors converted to HER2-negative in about one third of patients during trastuzumab-based neoadjuvant therapy (34). Monitoring HER2 expression without trastuzumab blocking during therapy is very important to determine the following treatment strategy. Ligand binding site analyses demonstrated that H6F targeted domain II of the extracellular segment of HER2, whereas trastuzumab bound HER2 in the C-terminal portion of domain IV (35,36). The binding of different domains of HER2 by the H6F peptide and trastuzumab permits real-time monitoring of HER2 expression with H6F-based imaging tracers during trastuzumab-based monotherapy and combined therapy. Radioligand competition binding assays demonstrated that excess trastuzumab did not block the binding of radiolabeled H6F to HER2 in HER2-positive MDA-MB-231 cells (Fig. 4C). Similarly, co-injection with trastuzumab did not inhibit the tumor accumulation of $^{99m}$Tc-HYNIC-H6F (Fig. 4A). Thus, $^{99m}$Tc-HYNIC-H6F recognizes HER2 on tumor cells and does not compete with trastuzumab for binding during therapy. Different tumor subtypes vary widely in sensitivity to trastuzumab inhibition, partly due to differences in HER2 expression and downregulation levels during therapeutic courses. Imaging with $^{99m}$Tc-HYNIC-H6F can supply real-time feedback on HER2 status and guide the adjustment of treatment strategies.

In the last decade, several HER2-targeted peptides have been identified and evaluated for
applications in tumor inhibition or drug delivery (37-40), and some specific agents have been used on NIRF imaging (41,42). However, very little research on peptide-based nuclear imaging of HER2 has been reported. In this study, we developed $^{99m}$Tc-HYNIC-H6F as a SPECT radiotracer for noninvasive imaging of HER2 expression in vivo. SPECT/CT with $^{99m}$Tc-HYNIC-H6F could effectively detect HER2-positive breast cancers with high tumor-to-muscle ratios. Due to different targeted domains between $^{99m}$Tc-HYNIC-H6F and trastuzumab, $^{99m}$Tc-HYNIC-H6F imaging can not only facilitate selection of patients for HER2-targeted therapy but also effectively monitor the therapeutical efficacy of trastuzumab by rechecking the expression level of HER2 without blocking effect during therapy.
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Figure 1. (A) Structure of $^{99m}$Tc-HYNIC-H6F. (B) The active site structure of the complex of HER2 (green line) with H6F (red line).
Figure 2. (A) Cell staining of MDA-MB-453 (HER2-positive) and MDA-MB-231 (HER2-negative) cells with FITC-trastuzumab and FITC-H6F. (B) Representative flow cytometry histograms of MDA-MB-453 and MDA-MB-231 cells without ligand (black-out spectrum). (C) Comparison of the binding rates of FITC-trastuzumab and FITC-H6F to MDA-MB-453 and MDA-MB-231 tumor cells.
Figure 3. (A-B) Representative SPECT/CT images obtained at 30 min, 1, and 2 h after injection of $^{99m}$Tc-HYNIC-H6F in MDA-MB-453 (A) and MDA-MB-231 (B) tumor-bearing mice. (C) Quantification of liver, kidney, heart and tumor (***$P < 0.001$) uptake from A and B. (T = tumor, GB = gall bladder, Ki = kidney)
Figure 4. (A) Representative SPECT/CT images obtained at 30 min after injection of $^{99m}$Tc-HYNIC-H6F in MDA-MB-453 tumor-bearing mice with or without blocking doses of cold H6F peptide or trastuzumab. (B) Quantification of the SPECT imaging results. (C) Inhibition of HYNIC-($^{125}$I)H6F binding to HER2 on MDA-MB-453 cells by H6F, HYNIC-H6F and trastuzumab. (Arrows indicate tumors)
Figure 5. (A) Biodistribution of $^{99m}$Tc-HYNIC-H6F in MDA-MB-453 tumor-bearing nude mice at 30 min, 1 h and 2 h after injection. (B) Biodistribution of $^{99m}$Tc-HYNIC-H6F in MDA-MB-453 and MDA-MB-231 tumor-bearing nude mice at 30 min after injection and in MDA-MB-453 tumor-bearing nude mice co-injected with cold H6F peptide as a blocking agent at 30 min after injection. Inset: $^{99m}$Tc-HYNIC-H6F tumor uptake values from B. **$P < 0.01$. ***$P < 0.001$. 
Supplementary Figure 1. (A) MS analysis of HYNIC-H6F. MALDI-TOF-MS: [M+H]^+ m/z 1423.4 (calc. 1423.4); MW: 1422.4; Yield: 68%. (B) HPLC analysis of ^{99m}Tc-HYNIC-H6F after purification with Sep-Pak C18 cartridges (Waters). HPLC analysis (C18 column): t_R = 14.80 min, purity > 95%.

MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight. MS: mass spectrometry.
Supplementary Figure 2. Double fluorescent staining of MDA-MB-453 cells (HER2-positive) with Cy3-trastuzumab and FITC-H6F.
Supplementary Figure 3. (A, B and C) SPECT/CT imaging of $^{99m}$Tc-HYNIC-H6F at 30 min p.i. in the MDA-MB-453 \textit{in situ} tumor model. Red arrows mark the location of the MDA-MB-453 tumor on the mouse mammary fat pad. (D) The tumor size was measured by a Vernier caliper.
Supplementary Figure 4. Inhibition of HYNIC-(^{125}I)H6F binding to the HER2 protein by HYNIC-H6F.
Supplementary Figure 5. Solution stability data for $^{99m}$Tc-HYNIC-H6F in 25 mM phosphate buffer (pH = 7.4) containing cysteine (1.0 mg/mL).