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Preparation and *In Vitro* and *In Vivo* Characterization of the Tumor-specific Antigen-derived Peptide as a Potential Candidate for Targeting Human Epidermal Growth Factor Receptor 2-positive Breast Carcinomas

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Abstract. *Background/Aim: The human epidermal growth factor receptor (HER2) is considered as one of the most well-characterized tumor-associated antigens for cancer therapy and plays an important role in the growth and progression of breast cancer. Overexpression of HER2 in various cancers and the availability of its extracellular region makes it a clinically useful target for the development of tumor-antigen specific agents. In this study, we have prepared a HER2-targeted hybrid peptide as a single-photon emission computed tomography (SPECT) imaging probe and evaluated its tumor targeting potential in subcutaneous HER2-positive breast cancer xenograft models. Materials and Methods: The HER2-targeted hybrid peptide was prepared by solid-phase peptide synthesis and radiolabeled with ^{99m}Tc by the ligand exchange method. In vitro tumor cell binding properties of ^{99m}Tc-HER2 were evaluated in HER2-positive (SKBR3) and ER-positive (MCF7 and T47D) breast cancer cell lines. In vivo tumor targeting characteristics were investigated in both SKBR3 (HER2-positive) and MDA-MB-231 (HER2-negative) xenografted animal models. Results: A high labeling efficiency of greater than 95% was achieved when HER2 peptide was radiolabeled with ^{99m}Tc by the standard ligand exchange method. ^{99m}Tc-HER2 displayed a high binding affinity (K_d=49.95±14.11 nM) to HER2-positive SKBR3 cell line whereas in the case of the ER-positive cell lines (MCF-7 and T47D), the binding affinity was found to be 2-3-fold lower than SKBR3. In vivo tumor uptake in nude mice with SKBR3 tumor xenografts was 2.81±0.79% ID/g as early as 60 min p.i. The uptake in SKBR3 tumors was always higher than the uptake in the blood and muscle, with good tumor-to-blood and tumor-to-muscle ratios. In contrast, low accumulation in ER-positive tumors (MCF7 and T47D) was observed compared to HER2-positive SKBR3 tumor mice. A low to moderate (less than 5% ID/g) accumulation and retention of ^{99m}Tc-HER2 was found in most of the major organs excluding the kidneys in both healthy and tumor-bearing mice. Conclusion: In view of its ability to detect HER2-positive breast cancer cells in vivo, ^{99m}Tc-HER2-targeted peptide may be a promising tumor imaging probe and warrants further investigation.*

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The human epidermal growth factor receptor (HER2) is the most studied tumor-associated antigen in breast cancer (1, 2). This receptor tyrosine kinase is part of the epidermal growth factor receptor (EGFR) family and is overexpressed in many cancers, such as breast, ovarian, endometrial, gastric, pancreatic, and prostate cancer. Particularly in breast cancer, HER2 is overexpressed in about 30% of primary and metastatic breast cancers, and about 90% of all breast cancers express HER2 to some extent. This overexpression can result in up to 200-fold increase in the concentration of HER2 in tumor *versus* normal tissues. Amplification/overexpression of HER2 in breast cancers is classified as HER2-positive breast cancers, which is one of the most aggressive forms of breast cancer since a high level of HER2 expression in breast cancer correlates with drug resistance, a higher tumor growth rate, high metastatic potential, and poor long-term patient survival rate (3-5). This suggests that HER2 overexpression may be an important tumor initiating event in breast cancer and that the overexpressed receptors promote the growth and spread of cancer cells (6). The high HER2 expression in many cancers and the availability of its extracellular region as a useful target for non-invasive detection of HER2-expressing tumors can provide a useful

insight for better patient management and create great clinical interest in developing HER2-specific agents.

Over the past few years, several monoclonal antibodies and tyrosine kinase inhibitors have been investigated for targeting HER2-expressing tumors (7-9). Trastuzumab (herceptin), a humanized recombinant monoclonal antibody, which binds to the extracellular region of HER2, was developed and approved by the FDA (U.S. Food and Drug Administration) for the treatment of HER2-positive cancer patients. Treatment outcome was found to be more beneficial when trastuzumab was used in combination with other drugs or after standard chemotherapy; and prognosis was improved in patients with metastatic HER2-positive cancer after trastuzumab treatment. However, patients with low and/or heterogeneous HER2 expression have shown a poor response to trastuzumab treatment; determination of HER2 status is, therefore, essential in selecting patients that would benefit from HER2-targeted therapy (5, 9-12). Molecular imaging especially "image and treat" is an attractive approach for achieving this task however, the use of full size monoclonal antibodies, such as trastuzumab for diagnostic imaging is not ideal because of their long retention in blood. Thus, a rapid and more efficient non-invasive method for the assessment of HER2 tumor expression is needed in nuclear oncology (5).

The use of radionuclide molecular imaging would enable detection of HER2 tumor expression by a noninvasive procedure in both primary and advanced tumors, without false-negative results due to biopsy sampling errors. Clinical utility of radionuclide imaging of HER2 expression using ^{111}In-DTPA-trastuzumab allows the identification of patients responding to trastuzumab treatment and patients who may suffer from toxicity associated with such treatment (12). In many studies, ^{111}In-DTPA-trastuzumab has shown high accumulation in tumor lesions (13); however, because of high background activity the optimal time of antibody-based imaging was ~72 h after the administration of radioantibody which somewhat limits its clinical efficiency (14-16). Consequently, attention has been focused towards the development of smaller HER2-specific targeting agents, such as peptides because of their rapid pharmacokinetics (*i.e.* rapid clearance from the blood and nontarget tissues) thus allowing imaging within an hour after the injection of the peptide, radiopharmaceutically making them more suitable for clinical imaging procedures (3, 17, 18).

The advancement of peptide phage display has facilitated the discovery of a variety of tumor targeting peptides against a wide range of tumor types. Numerous high affinity tumor target-specific peptides have been discovered through phage display (16). One such hexapeptide, Lys-Cys-Cys-Tyr-Ser-Leu, which bound the extracellular domain of human HER2, has shown the potential for targeting HER2-positive human breast and prostate cancers (19, 20). Nonetheless, this method is not optimal in terms of tumor targeting and its pharmacokinetic properties and requires improvement (16, 20). Another HER2 targeted peptide that was selected based on the E75 [369-377] sequence of the HER2 protein has been shown to be overexpressed in many breast cancer patients. The E75 [Lys-Ile-Phe-Gly-Ser-Leu-Ala-Phe-Leu; HER2, 369-377] sequence derived from HER2 protein's extracellular region also has been employed in the formulation of a peptide-based cancer vaccine (nelipepimut-S) to prevent breast cancer recurrence in high-risk patients (1, 4, 21). It seems logical to assume that this key sequence can be used to design a HER2-based peptide for targeting HER2-positive breast cancer. Although no prior information is available about the receptor affinity of this tumor-antigen derived peptide for HER2-positive breast cancer, there was an interest to investigate the tumor targeting potential of this HER2-derived peptide.

The main aim of this study was to construct a potent HER2-targeted peptide as a single-photon emission computed tomography (SPECT) imaging probe by combining the key amino acid sequences of the two important HER2 peptides and evaluate its tumor targeting potential *in vivo* in HER2-positive human breast cancer xenograft models. The HER2 hybrid peptide was prepared by solid-phase peptide synthesis and radiolabeled with technetium-99m (^{99m}Tc). ^{99m}Tc is a preferred radionuclide for radiolabeling tumor targeting peptides due to its wide availability, suitable half-life (6 h), and ideal γ-energy (140 keV) for medical diagnostic imaging (18). We present herein the synthesis, radiolabeling, and preclinical *in vitro* and *in vivo* characterization of a new HER2 peptide for the detection of HER2-positive breast cancer *in vivo*.

Materials and Methods

General. All standard reagents, solvents and Fmoc-amino acids for the peptide synthesis were purchased from commercial sources and used as received. ^{99m}Tc ($^{99m}TcO_4^-$) was obtained from a ^{99}Mo/^{99m}Tc generator (Elumatic III, CIS bio international, Cedex, France). The structure of the HER2 peptide was confirmed by mass spectrometry (Waters Micromass Quattro Premier XE, Manchester, UK). Reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with a UV-VIS detector (Shimadzu Corporation, Kyoto, Japan), set at 220 nm, a γ-radioactivity detection system and the Lauralite chromatogram analysis program (LabLogic Systems Ltd., Sheffield, UK). Radioactive samples from *in vitro* and *in vivo* studies were measured using a γ-counter (Mucha, raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany).

Preparation of HER2-derived hybrid peptide. HER2-derived hybrid peptide (Ac-Gly^{19}-Gly^{18}-Cys^{17}-ALA^{16}-Lys^{15}-Ile^{14}-Phe^{13}-Gly^{12}-Ser^{11}-Leu^{10}-Ala^{9}-Phe^{8}-Leu^{7}-Lys^{6}-Cys^{5}-Cys^{4}-Tyr^{3}-Ser^{2}-Leu^{1}-$CONH_2$) was synthesized manually by solid-phase peptide synthesis following standard Fmoc (9-fluorenylmethoxycarbonyl) chemistry,

using Rink amide MBHA (4-methylbenzhydrylamine) resin (100–200 mesh) on a 0.1 mmol scale according to a method described previously (22, 23). Briefly, the first Fmoc-amino acid (Fmoc-Leu-OH) was activated with HBTU (*O*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) in the presence of DIEA (diisopropylethylamine) and coupled to the resin. The peptide chain was then elongated in cycles of Fmoc deprotection followed by coupling of the subsequent Fmoc-amino acid to the resin. After stepwise addition of all the desired 19-amino acids to the sequence, the peptide was acetylated at the *N*-terminus with acetic anhydride/triethylamine. The crude peptide was cleaved from the resin by treating with 94% trifluoroacetic acid (TFA), 1% triisopropylsilane, 2.5% 1,2-ethanedithiol, and 2.5% water. The purity of the peptide was confirmed by HPLC and its structural identity by mass spectrometry.

Radiolabeling with ^{99m}Tc. A 50 μl sample of the peptide solution (1 mg/ml CH_3CN/H_2O) was mixed with 200 μl 0.2 M citrate-phosphate buffer (pH 9), 250 μl sodium potassium tartrate (40 mg/ml aqueous solution) and 20 μl 5% ascorbic acid. To this, freshly prepared 100 μl of $SnCl_2$ (20 mg $SnCl_2.2H_2O$ in 5 ml 0.05 N HCl) was added followed by 200 μl of 5-10 mCi $^{99m}TcO_4^-$. The labeling mixture was then heated at 90˚C for 10 min and allowed to cool prior to HPLC analysis.

HPLC purification and analysis. The HPLC analysis and purification of the peptide was performed on a Shimadzu HPLC system using Econosphere C18 reversed-phase column (10 μm, 250 × 4.6 mm). For HPLC experiments, a gradient solvent system of 0.1% (v/v) TFA in H_2O (solvent A) and 0.1% (v/v) TFA in CH_3CN (solvent B) at a flow rate of 1.1 ml/min was used (22). The main peak of the radiopeptide was isolated and reconstituted in sterile saline for *in vitro* and *in vivo* experiments.

In vitro stability in plasma. The HPLC-purified radiopeptide (100 μl) was incubated with human plasma (500 μl) in duplicate at 37˚C for up to 4 h. Following incubation at 1 and 4 h, the plasma proteins were precipitated with a mixture of CH_3CN /EtOH (1:1 v/v, 400 μl) and the sample was centrifuged (7,000 rpm, 7 min). The supernatant layer was removed, filtered through Millex GP filter (0.22 μm), and analyzed by radio-HPLC under the conditions described above in order to determine the proteolytic stability of the ^{99m}Tc-HER2.

In vitro tumor cell binding and internalization. The cell-binding and subsequent internalization of ^{99m}Tc-HER2 peptide into HER2-positive SKBR3 and ER-positive MCF7 and T47D breast cancer cell lines (2, 10, 24, 25) [American Type Culture Collection, Rockville, MD, USA] was performed according to the method described previously (22). In addition, cell binding was conducted on HER2-negative MDA-MB-231 breast cancer cell line (14, 25) to further confirm the receptor specificity of ^{99m}Tc-HER2. The binding data were analyzed by a nonlinear regression analysis program (GraphPad Software Inc., San Diego, CA, USA). In order to differentiate between cell-surface bound and cellular internalized radioactivity, cell pellet was treated with 300 μl of acidic buffer (0.02 M sodium acetate in saline, pH 5.0) for 10 min at 37˚C at the end of the binding experiment, followed by centrifugation and washing with cold acidic buffer. The amount of cell surface-bound (acid-wash) and internalized (acid-resistant) radioactivity was determined by measuring the radioactivity of the supernatant and the cell-pellet, respectively, in a γ-counter.

In vivo animal biodistribution. Approval for the animal protocol used in this study was obtained from the Institutional Animal Care and Use Committee. Animal studies were conducted according to the international regulations governing the safe and proper use of laboratory animals (26). *In vivo* biodistribution was performed on healthy Balb/c mice (n=3-5 in each group, body mass 19-22 g) at 1 and 4 h after *i.v.* injection of the HPLC-purified radioligand (100 μl, 10-15 μCi, total peptide dose ~1 μg) *via* the lateral tail vein as described previously (22). Uptake of radioactivity in the tissues and organs was expressed as the percent injected dose per gram (% ID/g) of tissue/organ, which was calculated by comparison with standard solutions representing 10% of the injected dose per animal. For the clearance studies, radioactivity in the collected urine (100-200 μl) was measured and expressed as the percent of the injected dose per organ (% ID/tissue).

In vivo tumor targeting and gamma camera imaging. HER2-positive SKBR3 breast tumor xenografts in nude mice models were used for *in vivo* tumor targeting. About 7 million SKBR3 cells were injected subcutaneously into each mouse and allowed to grow for 4-6 weeks. After sufficient growth of tumors, the animals were sacrificed and the uptake of ^{99m}Tc-HER2 from the tumors and other major organs was determined by γ-counting. In the γ-camera imaging study, the mouse was injected with ~200 μCi of ^{99m}Tc-HER2 through the tail vein. Mouse static planar images were then acquired at 1 h after the injection by a portable hand-held γ-camera equipped with CsI(Tl) scintillation detector, with intrinsic spatial resolution of 2.4 mm (IP guardian 2, Li-Tech, Italy).

Statistical analysis. Results are expressed as mean±S.D. where appropriate. For data comparisons, mean values were compared using the Student's *t*-test (GraphPad Software, Inc., San Diego, CA, USA). A probability value (p) less than 0.05 was considered statistically significant.

Results and Discussion

High expression of tumor-specific antigens, such as HER2, found on various malignant cells as compared to normal cells provides the basis for successful use of radiolabeled tumor-antigens-derived peptide for tumor targeting. One attractive tactic of developing probes for targeting HER2-positive tumors is the use of active peptide sequence derived from such tumor-specific-antigens. One distinctive benefit of such peptides is that they can be easily synthesized chemically and modified molecularly to afford desired pharmacokinetics (15, 23). The most well studied tumor-associated antigen in breast cancer is HER2, which is positive in about 30% of all human breast cancers (1). The HER2 protein of the epidermal growth factor receptor family plays a key role in aggressive human breast cancer (9). As HER2 is overexpressed at the surface of tumor cells, the accessibility of the receptor target makes it suitable for tumor diagnosis and therapy. Accurate and non-invasive methods for detection of HER2 expression in breast cancer can provide useful diagnostic information with direct impact on patient management (27). The use of radionuclide imaging would enable detection of HER2 by a noninvasive

procedure in both primary tumors and metastases, without false-negative results due to biopsy sampling errors (5, 27). Thus HER2-specific peptides have attracted great clinical attention recently. Here, we present the preparation and evaluation of a new HER2-derived hybrid peptide as a potential SPECT imaging probe for targeting HER2-positive breast cancer.

Synthesis of HER2 peptide conjugate. The HER2 peptide evaluated in this study was conveniently and successfully prepared by solid-phase peptide synthesis according to Fmoc-chemistry in reasonably good yield (~40%). We utilized a well-characterized Cys-Gly-Gly chelating sequence because of its convenience of direct coupling to the peptide molecule during solid-phase peptide synthesis and the ease of radiolabeling with ^{99m}Tc (23). Aminolevulinic acid (ALA) residue was chosen as a spacer because of its possible role in breast cancer (28) and inserted between the chelating sequence and the targeting peptide to keep the chelating sequence distant from the binding region. The structural identity of HER2 peptide was confirmed by mass spectrometry: ($[M+H]^+$ calculated=2064; $[M+2H]^{2+}$ found=1034).

Radiolabeling with ^{99m}Tc. Radiolabeling of HER2 peptide with ^{99m}Tc was achieved by the ligand exchange method using sodium potassium tartrate as a weak chelating agent. By the exchange labeling approach, the radiolabeling efficiency of ^{99m}Tc-HER2 complex was greater than 95% with a specific radioactivity greater than 250 Ci/mmol. Radio-HPLC analysis revealed the formation of one main peak corresponding to ^{99m}Tc-HER2 at 20 min (Figure 1). Radiochemical purity was determined by evaluating radioactivity peak eluted for ^{99m}Tc-HER2 from the RP-HPLC column and calculating the area under the peak (region of interest). The optimal radiolabeling with ^{99m}Tc was achieved in the presence of 0.2 M citrate phosphate buffer pH 9. Generally, an alkaline pH enhances the formation of a complex between triamide thiol Cys-Gly-Gly and ^{99m}Tc by deprotonation of three-amide nitrogens of the N3S type chelating system (22, 23). It was found that the ^{99m}Tc-HER2 complex remained stable (>75%) at room temperature for up to 18 h post-labeling.

In vitro metabolic stability in plasma. The metabolic stability of ^{99m}Tc-HER2 was studied in human plasma *in vitro*. Following incubation of ^{99m}Tc-HER2 in plasma at 1 and 4 h, the plasma proteins were precipitated with a mixture of acetonitrile/ethanol and the supernatant layer was analyzed by radio-HPLC, using the experimental conditions described above, to test the stability of the radiolabeled peptide in human plasma. The results indicated that up to 80% radioactivity was remained bound to the radiopeptide after 4 h incubation, with a slow reformation to free $^{99m}TcO_4^-$ (up to 20%), indicating a reasonably high proteolytic stability and a low binding to plasma proteins of the ^{99m}Tc-HER2. The percent of peptide that remained intact in the plasma was 89% at 1 h, and 80% at 4 h, signifying a low enzymatic degradation of ^{99m}Tc-HER2 by plasma proteases. The high metabolic stability is in agreement with the high radiochemical stability obtained for ^{99m}Tc-HER2, suggesting a good correlation between the two. It is generally believed that the high metabolic stability of a radiopeptide in human plasma is important for tumor targeting, as the radiolabeled peptide has to reach the desired target intact to exert a maximum effect.

In vitro tumor cell binding and cellular internalization. In view of the variable degrees of HER2 expression found in different human breast cancer cell lines (2, 5, 24, 29), it is reasonable to explore the binding ability of ^{99m}Tc-HER2 peptide not only on the HER2-positive and estrogen receptor (ER) independent SKBR3 breast cancer cell line, but also on the ER-positive MCF7 and T47D breast cancer cell lines, which show low to moderate expression of HER2 (10, 29). The binding affinity (K_d) of ^{99m}Tc-HER2 to the respective breast cancer cell lines was determined by saturation binding assays. In addition, the maximum number of binding sites (B_{max}) was estimated (GraphPad Software Inc., San Diego, CA, USA).

The cell binding data (Table I) showed that ^{99m}Tc-HER2 displayed a high binding affinity to HER2-positive SKBR3 breast cancer cell line (K_d=49.95±14.11 nM). However, in the case of ER-positive breast cancer cell lines (MCF7 and T47D), ^{99m}Tc-HER2 exhibited a significantly lower binding affinity, with K_d values of 157.29±24.56 nM, p=0.002, and 169.11±22.09 nM, p=0.001, respectively. These results demonstrate that the binding affinity of ^{99m}Tc-HER2 was found to be more than 3-times higher for HER2 overexpressing SKBR3 cells than the binding values obtained for low-expressing HER2-positive and ER-positive MCF7 and T47D cell lines. It is worth mentioning that the binding affinity of ^{99m}Tc-HER2-derived peptide to SKBR3 cell line was about 6-fold higher than the one reported for HER2-targeted peptide (49.95 nM *versus* 295 nM) (20). Tumor receptor specificity of ^{99m}Tc-HER2 was further confirmed by results showing low affinity binding to HER2-negative MDA-MB-231 breast cancer cells, with a K_d value of 962.74±113.51 nM. In addition to its higher binding affinity, the HER2-positive SKBR3 cell line also displayed higher number of binding sites (B_{max}=9.2×10^4 receptors/cell, when compared with ER-positive MCF7 (B_{max}=4.5×10^4 receptors/cell) and T47D (B_{max}=4.2×10^4 receptors/cell) cell lines.

To differentiate between the tightly cell-surface bound and cellular internalization of ^{99m}Tc-HER2 peptide, the cell-bound radioactivity was treated with acidic buffer (30) for 10 min at the end of the cell binding experiments. A fast and significant internalization was noticed for ^{99m}Tc-HER2 since 19.89±5.34% of the cell-surface bound radiopeptide was internalized into

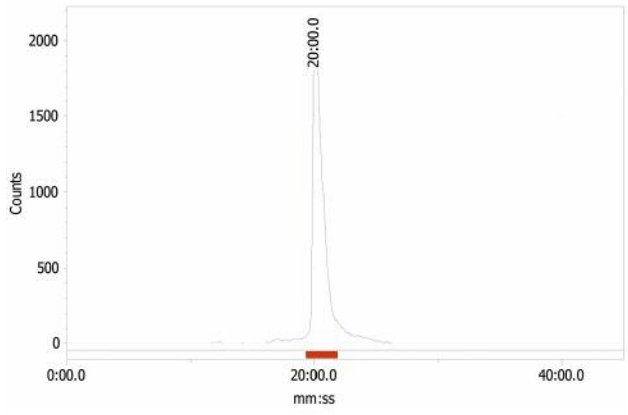

Figure 1. *Radio-HPLC elution profile of ^{99m}Tc-HER2 peptide.*

Table I. *In vitro tumor cell binding characteristics and cellular internalization of ^{99m}Tc-HER2 peptide to SKBR3, MCF7, T47D human breast cancer cell lines.*

Cell line	K_d (nM)	B_{max} (Sites/cell)	% Internalization
SKBR3	49.95±14.11	92,450±6731	19.89±5.34
MCF7	157.29±24.56	45,815±5093	12.15±3.19
T47D	169.11±22.09	42,100±5469	11.86±2.82

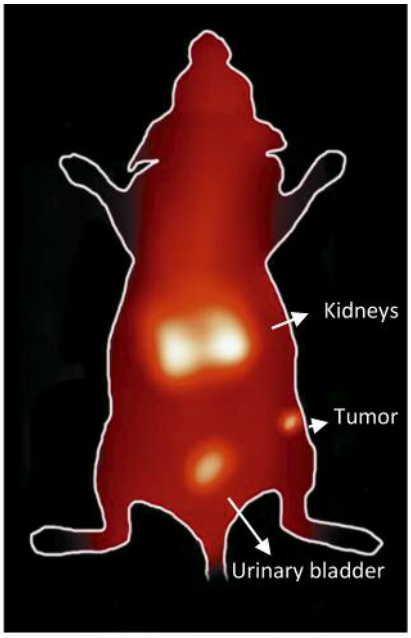

Figure 2. *Gamma camera image of nude mouse model bearing SKBR3 tumor xenografts at 1 h after tail vein injection of 200 μCi of ^{99m}Tc-HER2 hybrid peptide.*

SKBR3 cells after incubation in acidic buffer for 10 min at 37˚C. The internalization capacity of two ER-positive cell lines was found to be relatively lower than HER2-positive SKBR3 cell line as only 12.15±3.19% and 11.86±2.82% of the cell bound radioactivity was internalized into MCF7 and T47D cells, respectively (Table I). The results of cell-binding and internalization into various breast cancer cells demonstrate that in spite of the modifications in the peptide sequence, such as the introduction of a chelating sequence for radiolabeling and the addition of spacer function, the HER2-derived peptide retained its potency and held high affinity and specificity for breast cancer cell lines. This highlights the potential of ^{99m}Tc-HER2 for targeting human breast carcinomas.

In vivo biodistribution and tumor uptake studies. The findings of the biodistribution studies in normal Balb/c mice at 1 and 4 h post-injection (*p.i.*) of ^{99m}Tc-HER2-derived peptide are summarized in Table II. The biodistribution of ^{99m}Tc-HER2 was first studied in healthy mice to determine normal tissue uptake patterns and clearance kinetics. The results show that the ^{99m}Tc-HER2 exhibited a rapid and efficient clearance from the blood as less than 2% ID/g was found in the blood at 1 and 4 h *p.i.* The initial uptake taken by the liver was 3.74±0.70% ID/g at 1 h which decreased to 2.18±0.39% ID/g at 4 h *p.i.* The reasonable uptake of ^{99m}Tc-HER2 peptide in the liver is possibly related to its modest lipophilicity (log *P*=1.38±0.06). The uptake in the intestines (without the contents) was also found to be low (up to 2.41±0.42% ID/g) both at 1 and 4 h *p.i.* The ^{99m}Tc-HER2 peptide revealed a moderate accumulation and retention in the kidneys (5.96±1.02% ID/g at 1 h and, 4.85±0.86% ID/g at 4 h *p.i.*). Though the observed kidney uptake is not very high but still a low uptake and retention by the kidneys is desired for diagnostic imaging in general and for radionuclide therapy in particular, because of potential kidney toxicity (31). A low to moderate uptake of radioactivity was observed in the stomach (range=2.66-2.0 % ID/g) (Table II), indicating a low breakdown of the ^{99m}Tc-HER2 complex *in vivo* and minimal reformation of free $^{99m}TcO_4^-$. Nonetheless, the high *in vivo* stability for ^{99m}Tc-HER2 peptide correlates well with the high *in vitro* metabolic stability obtained in plasma. The uptake in the lungs was also low (below 2% ID/g both at 1 and 4 h *p.i.*) indicating low colloidal particles formation by lungs. Urinary excretion values were estimated using the radioactivity associated with the excreted urine and bladder contents at the time of sacrifice. As anticipated, the clearance of the radiopeptide from the whole body occurred mainly through the urinary tract as up to 40.0±5.10% ID was found in the urine at 1 h *p.i.* In general, a rapid and efficient clearance of the radioactivity was observed from all the major organs, excluding the kidneys for ^{99m}Tc-HER2-peptide.

^{99m}Tc-HER2 peptide was further investigated in nude mice with HER2-positive SKBR3 tumor xenografts in order to determine its ability to target human breast cancer *in vivo* (Table III). Also, in nude mice, a fast and efficient clearance from the blood was observed as it was the case with Balb/c mice. A moderate uptake of 2.81±0.79% ID/g was found in the HER2-positive SKBR3 tumors as early as 1 h *p.i.*, which was reduced to 1.22±0.25% ID/g at 4 h *p.i.* (with 57% washout from the tumors over 4 h). The uptake value in the tumor was always higher than the radioactivity found in the blood and muscle. Tumor-to-nontumor uptake ratios were also calculated and are shown in Table III. A trend of slight decreased tumor-to-blood and tumor-to-muscle uptake ratios over time was obtained for ^{99m}Tc-HER2 peptide. The tumor-to-blood ratio obtained was 1.54 at 1 hand was dropped to 1.34 at 4 h *p.i.* The tumor-to-muscle ratio was found to be 9.37 at 1 h and was reduced to 8.71 at 4 h *p.i.* Uptake of radioactivity in the stomach was found to be lower (Table III) in nude mice than that in Balb/c mice. The kidneys showed the highest accumulation and retention of radioactivity (up to 10% ID/g) and these values are about 2-times higher than the values found in the Balb/c mice. A high amount of radioactivity was excreted in the urine (up to 41% ID) whereas, the hepatobiliary excretion (liver + intestines) of this radiopeptide was below 15% ID. The results suggest that the good tumor targeting properties of this tumor-specific antigen peptide may make it useful for targeting human breast carcinomas.

In a receptor blocking study, administration of 100 μg of HER2 peptide 30 min before the injection of ^{99m}Tc-HER2 reduced its uptake from the tumors by approximately 62% (1.07±0.24% ID/g blocked *vs.* 2.81±0.79% ID/g unblocked, p=0.02), highlighting the specificity of the ^{99m}Tc-HER2-derived peptide for HER2-positive SKBR3 breast cancer cell line. No marked influence of the blocking dose was observed in other major organs including lungs and kidneys suggesting nonspecific uptake of ^{99m}Tc-HER2 by these organs (data not shown).

Tumor targeting potential of ^{99m}Tc-HER2-derived peptide was also determined in mice with ER-positive MCF7 and T47D xenografts (Table IV). The ER-positive cell lines, MCF7 and T47D are classified as low HER2 expressing breast cancer cell lines (2, 10, 29). Comparable tumor uptake and tissue biodistribution profiles were obtained between the nude mice models carrying ER-positive MCF7 and T47D breast cancer xenografts. The uptake of ^{99m}Tc-HER2 in the MCF7 and T47D tumors was 1.33±0.41% ID/g and 1.25±0.32 at 1 h and 0.91±0.22% ID/g and 0.80±0.17% ID/g at 4 h *p.i.*, respectively. These results demonstrate that the uptake by ER-positive tumors was about 2-fold lower than the uptake found in HER2-positive SKBR3 breast carcinoma. It is obvious from the biological evaluation that the ^{99m}Tc-HER2 displayed comparable tumor targeting characteristics in the species carrying two pharmacologically different breast cancer cell lines highlighting the ability of ^{99m}Tc-HER2-derived peptide to target not only HER2-positive but also ER-positive breast cancer lines.

Furthermore, tumor-targeting behavior of ^{99m}Tc-HER2 was evaluated in nude mice carrying HER2-negative MDA-MB-231 breast xenografts in order to further examine the tumor specificity of ^{99m}Tc-HER2 peptide by comparing the extent of tumor uptake in HER2-positive and HER2-negative cancer cell lines (Table IV). MDA-MB-231 is considered as HER2-negative breast cancer cell line because of its low-expression for HER2 (14, 25). The tumor uptake value of 0.66±0.13% ID/g *vs.* 2.81±0.79% ID/g (p=0.009) at 1 h, and 0.31±0.10%

Table II. *In vivo tissue biodistribution studies of ^{99m}Tc-HER2 peptide in Balb/c mice at 1 and 4 h post-injection.*

	1 h	4 h
Blood	1.94±0.42	1.49±0.31
Lungs	1.89±0.50	1.21±0.23
Stomach	2.66±0.67	2.0±0.41
Pancreas	1.04±0.15	0.77±0.11
Intestines[a]	2.41±0.42	1.18±0.19
Liver	3.74±0.70	2.18±0.39
Kidneys	5.96±1.02	4.85±0.86
Muscle	0.63±0.10	0.25±0.03
Urine & bladder[b]	40.0±5.10	37.0±4.71

Data are expressed as % injected dose per gram of tissue/organ (n=3-5, mean values±SD). [a]Part of the intestines was measured without their contents. The radioactivity in the [b]urine + bladder is expressed as %-injected dose per tissue.

Table III. *In vivo tumor targeting studies of ^{99m}Tc-HER2 peptide in subcutaneous HER2-positive SKBR3 breast cancer xenografts mice models.*

	1 h	4 h
Blood	1.82±0.35	0.91±0.15
Lungs	2.75±0.41	0.84±0.14
Stomach	1.81±0.28	1.05±0.14
Pancreas	1.47±0.09	0.67±0.06
Intestines[a]	1.73±0.32	1.32±0.18
Liver	3.62±0.53	2.28±0.46
Kidneys	9.94±2.11	8.90±2.28
Urine & bladder[b]	41.0±4.21	37.0±5.10
Muscle	0.30±0.06	0.14±0.02
Tumor	2.81±0.79	1.22±0.25
Ratios		
Tumor/blood	1.54	1.34
Tumor/muscle	9.37	8.71

Data are expressed as % injected dose per gram of tissue/organ (n=3-5, mean values±SD). [a]Part of the intestines was measured without their contents. The radioactivity in the [b]urine + bladder is expressed as % injected dose per tissue.

Table IV. *In vivo tumor-targeting properties of ^{99m}Tc-HER2 peptide in ER-positive MCF7 and T47D as well as in HER2-negative MDA-MB-231 breast cancer xenografts mice models.*

	MCF7 tumor		T47D tumor		MDA-MB-231 tumor	
	1 h	4 h	1 h	4 h	1 h	4 h
Blood	2.12±0.36	0.88±0.21	2.51±0.12	0.75±0.10	2.40±0.31	1.45±0.22
Lungs	2.57±0.10	0.62±0.05	3.53±0.10	0.81±0.10	2.05±0.23	1.29±0.12
Stomach	1.42±0.16	1.0±0.14	1.64±0.30	1.14±0.40	4.59±0.48	3.26±0.33
Pancreas	1.19±0.10	0.79±0.06	0.57±0.05	0.35±0.03	1.35±0.09	0.95±0.07
Intestines[a]	1.57±0.21	1.22±0.14	1.39±0.14	0.90±0.12	1.63±0.30	1.13±0.15
Liver	3.62±0.17	2.32±0.16	3.12±0.15	1.98±0.11	4.22±0.18	3.54±0.10
Kidneys	9.90±0.96	8.74±1.07	10.35±1.50	8.36±0.36	8.15±1.02	4.96±0.95
Urine & bladder[b]	31.00±8.90	25.00±4.16	28.00±5.00	23.00±3.17	25.00±10.00	24.00±9.510
Muscle	0.36±0.07	0.15±0.03	0.25±0.04	0.13±0.02	0.19±0.03	0.10±0.02
Tumor	1.33±0.41	0.91±0.22	1.25±0.32	0.80±0.17	0.66±0.13	0.31±0.10
Ratios						
Tumor/blood	-	1.03	-	1.07	-	-
Tumor/muscle	3.70	6.01	5.0	6.15	3.47	3.10

Data are expressed as % injected dose per gram of tissue/organ (n=3-5, mean values±SD). [a]Part of the intestines was measured without their contents. The radioactivity in the [b]urine + bladder is expressed as % injected dose per tissue.

ID/g *vs*. 1.22±0.25% ID/g (p=0.004) at 4 h was found in HER2-negative MDA-MB-231 *versus* HER2-positive SKBR3 tumors, respectively. These results validate the significant tumor uptake specificity by ^{99m}Tc-HER2 peptide for HER2-positive SKBR3 cell line over HER2-negative MDA-MB-231 tumor xenografts. No marked difference in uptake patterns by the major organs was seen between the HER2-positive and HER2-negative tumor xenograft models (Table IV). In general, a fast clearance from all the organs and tissues was achieved, with the exception of the kidneys.

In vivo planar scintigraphic imaging. The tumor imaging property of ^{99m}Tc-HER2-derived peptide was examined in a nude mouse bearing HER2-positive SKBR3 tumor xenografts at 1 h after the administration of radiopeptide. Although high accumulation of the radioactivity in the kidneys and urinary bladder is seen, the SKBR3 xenografted tumors are detectable in the image at 1 h *p.i*. (Figure 2), highlighting the potential use of tumor-antigen derived HER2 peptide for human breast cancer imaging. Immediately after the imaging, the mouse was sacrificed and quantitative tissue biodistribution was performed in order to confirm the findings of γ-imaging. The data from the *in vivo* imaging were in agreement with the *in vivo* biodistribution studies (Table III). Kidneys have been found to be the organs with the highest radioactivity uptake in the biodistribution and gamma image. So, to make ^{99m}Tc-HER2-derived peptide more suitable for tumor imaging, besides the strategy to decrease kidney uptake, it is also important to increase the tumor uptake in future studies.

Conclusion

In the current study, we present a new ^{99m}Tc-labeled HER2-targeted peptide with high metabolic stability and high affinity and specificity towards HER2-expressing tumor receptors, reflecting important properties of tumor-antigen derived peptides for molecular imaging of breast carcinoma. Further investigations will focus on the strategies to enhance tumor targeting characteristics and to reduce kidney retention of HER2-targeted peptide in order to make this emerging class of tumor-antigen peptides more suitable for breast cancer imaging.

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