

University of Groningen

Cardiotoxicity after anticancer treatment

Perik, Patrick Jozef

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2005

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Perik, P. J. (2005). *Cardiotoxicity after anticancer treatment: clinical investigations and molecular mechanisms*. s.n.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

4

PRECLINICAL CHARACTERIZATION OF ¹¹¹IN-DTPA-TRASTUZUMAB

MARJOLIYN N. LUB-DE HOOGHE ^{1,2}

JOS G.W. KOSTERINK ¹

PATRICK J. PERIK ³

HUGO NIJNUIS ²

LY TRAN ¹

JOOST L. BART ²

ALBERT J. SUURMEIJER ⁴

STEVEN DE JONG ³

PIETER L. JAGER ²

ELISABETH G.E. DE VRIES ³

¹ HOSPITAL PHARMACY,

² DEPARTMENT OF NUCLEAR MEDICINE,

³ DEPARTMENT OF MEDICAL ONCOLOGY,

⁴ DEPARTMENT OF PATHOLOGY,

UNIVERSITY MEDICAL CENTER GRONINGEN, THE NETHERLANDS

BRITISH JOURNAL OF PHARMACOLOGY 2004;143:99-106

ABSTRACT

Background

Trastuzumab (Herceptin[®]) is a recombinant humanized IgG1 monoclonal antibody against the human epidermal growth factor receptor 2 (HER2), used for metastatic breast cancer treatment. Radiolabeled trastuzumab may have several future applications for diagnostic use. The aim of the present study was to develop clinical grade ¹¹¹Indium (¹¹¹In) radiolabeled trastuzumab, to evaluate the stability and immunoreactivity of the tracer and to perform a biodistribution study in human tumor-bearing mice.

Methods and results

Trastuzumab was radiolabeled with ¹¹¹In using DTPA as a chelator. ¹¹¹In-DTPA-trastuzumab (labeling yield $92.3 \pm 2.3\%$, radiochemical purity $97.0 \pm 1.5\%$) is stable in PBS when stored at 4°C for more than 14 days. The immunoreactive fraction determined by cell-binding assays, using the HER2-overexpressing human ovarian SK-OV-3 tumor cell line, was 0.87 ± 0.06 . Biodistribution and tumor targeting were studied in HER2 receptor-positive and -negative tumor-bearing athymic mice. The HER2-positive tumor showed ($9.77 \pm 1.14\%$ injected dose per gram (ID g⁻¹)) substantial uptake of the labeled antibody already after 5 hours. The difference in uptake between HER2-positive versus -negative tumors was even more pronounced 3 days after injection ($16.30 \pm 0.64\%$ ID g⁻¹), and was visualized by radioimmunoscinigraphy. Liver, spleen and kidney showed marked tracer uptake.

Conclusion

Trastuzumab can be efficiently radiolabeled with ¹¹¹In with high labeling yields and high stability. ¹¹¹In-DTPA-trastuzumab selectively binds to the human HER2 receptor both *in vitro* and *in vivo* in animals. Therefore, ¹¹¹In-DTPA-trastuzumab appears suitable for clinical use.

INTRODUCTION

Trastuzumab, a recombinant humanized IgG1 monoclonal antibody against the human epidermal growth factor receptor 2 (HER2), is currently used for the treatment of patients with metastatic breast cancer, whose tumor overexpresses HER2. The human epidermal growth factor receptors (HER/erbB) constitute a family of four cell surface receptors (HER1–4) with tyrosine kinase activity, involved in transmission of signals controlling normal cell growth and differentiation. The HER2 receptor is encoded by the human gene *HER2/c-erbB2* (*HER2/neu*). HER2 functions as a ligandless receptor and is overexpressed in a wide variety of human cancers, including 20–30% of primary breast cancers.¹ Overexpression of the HER2 receptor is a predictor of poor prognosis, because it is associated with aggressive tumor growth and metastatic activity.¹ Several trials investigated the role of trastuzumab alone and in combination with chemotherapeutic drugs, especially in metastatic breast cancer.² Unfortunately, the use of trastuzumab is associated with cardiotoxicity. The risk of cardiotoxicity is greatest when trastuzumab is used in combination with anthracycline-based chemotherapy regimens namely 28 versus 7% for trastuzumab alone.¹

The exact mechanism of trastuzumab-induced cardiotoxicity is still unknown, but is suggested to result from a direct action on the HER2 receptor of cardiomyocytes. HER2 signalling in cardiomyocytes is essential for the prevention of dilated cardiomyopathy.³ The HER2 receptor plays a critical role in cardiac development. Conditional *ErbB2* mutants developed severe dilated cardiomyopathy.⁴ In addition, HER2 is thought to participate in an important pathway for growth, repair and survival of adult cardiomyocytes, as part of a signalling network that also involves neuregulins and the neuregulin receptor HER4. However, HER2 levels in the healthy adult heart are low compared to the levels in HER2-overexpressing breast cancer cells that are the intended targets of trastuzumab therapy.^{5,6}

The risk of developing cardiotoxicity makes patient selection for trastuzumab therapy of great importance. A reliable test to predict tumor response and the risk of developing cardiac failure is therefore needed. Currently, there is no noninvasive method to visualize and stage HER2-overexpressing tumor localizations. Radionuclide imaging using radiolabeled trastuzumab might be useful for the detection of tumor localizations, to determine the degree of trastuzumab uptake as well as for the selection of patients who should not receive trastuzumab, because they are likely to develop cardiotoxicity. In a preliminary report, Behr et al recently suggested that pretreatment scanning with a tracer dose of radiolabeled trastuzumab might predict therapy response and cardiotoxicity.⁷

The aim of the present study was to develop adequate radiolabeling of trastuzumab and to characterize and validate ¹¹¹In radiolabeled trastuzumab as a new tracer intended for future clinical use. The optimization of the labeling method, the *in vitro* characterisation and the biodistribution of ¹¹¹In-DTPA-trastuzumab in tumor-bearing athymic mice were studied.

METHODS

DTPA conjugation

Following the instructions of the manufacturer, trastuzumab (Herceptin[®], Roche, Welwyn Garden City, Hertfordshire, U.K.) was reconstituted in water for injection to obtain a 21 mg ml⁻¹ solution of trastuzumab. Trastuzumab was purified from other excipients (histidine, polysorbate and α,α -trehalose) by ultrafiltration (Centricon[®] filter 30 kDa, Millipore, Etten-Leur, The Netherlands; 30 min at 2684 x g). The chelator diethylenetriamine penta-acetic acid dianhydride (DTPA dianhydride, Sigma Chemical Co., St Louis, MO, U.S.A.) was then conjugated to the antibody using a small modification of the well-known cyclic anhydride method.^{8,9} Conjugation was performed at a 1 : 1 molar ratio. In brief, 20 μ l of a 1 mg ml⁻¹ suspension of DTPA anhydride in dry chloroform (Merck, Darmstadt, Germany) was pipetted under ultrasonication and transferred to a glass tube. The chloroform was evaporated under a gentle stream of nitrogen. Purified trastuzumab (10 mg = 0.5 ml) and 1.4% sodium bicarbonate (pH 8; 200 μ l) were subsequently added and gently mixed at room temperature for 5 min. Unbound DTPA was then removed by ultrafiltration (twice 30 min at 2684 x g). The purified immunoconjugate DTPA-trastuzumab was either stored at -20 °C or immediately used for radiolabeling.

Radiolabeling

¹¹¹InCl₃ (DRN 4901; 370 MBq ml⁻¹ in 0.05 M HCl, pH 1.5–1.9) was obtained from Tyco Health (Petten, The Netherlands). An equal volume of a 0.1 M sodium acetate buffer (sodium acetate 99.995%, Aldrich Chem. Co., Milwaukee, WI, U.S.A.) was added to the ¹¹¹InCl₃ stock solution and carefully mixed, resulting in pH 5.5. The ¹¹¹InCl₃ was then added to the conjugated DTPA-trastuzumab and the reaction mixture was incubated for 5 min. After incubation, 20 mM DTPA in 0.1 M sodium acetate solution was added in order to bind free ¹¹¹In. The resulting ¹¹¹In-DTPA was then removed by ultrafiltration. The product was diluted in normal saline and sterilized by filtration through a 0.2 μ m Millex GV filter (Millipore).

The complete labeling procedure was optimized by subsequently varying reaction pH, incubation times, molar DTPA to trastuzumab ratios, amounts of ¹¹¹InCl₃ added per mg conjugate and purification methods with a Sephadex G25 column (PD-10 column, Amersham Biosciences AB, Uppsala, Sweden) versus ultrafiltration.

The optimal labeling procedure, described above, was then validated and performed under good manufacturing practice (GMP) conditions. The Departments of Nuclear Medicine (including the Radiopharmacy) and Hospital Pharmacy have a Quality Management System and are ISO 9001 : 2000 certified. Personnel are appropriately trained with respect to GMP and radiation safety aspects. The labeling procedure is conducted in a validated biohazard Laminar Air Flow hood that is placed in a background environment conforming to GMP grade C. The final product meets Ph. Eur. criteria. Protein content, pyrogen content and sterility are therefore measured. (Buffer) solutions are produced in the production facility of the Department of

Hospital Pharmacy and are sterilized (15 min at 121 °C). Glassware, materials and solutions for the labeling procedure were sterilized, pyrogen-free and metal-free. Centricon filters were not sterilized.

Quality control of ^{111}In -DTPA-trastuzumab

Radiochemical purity was determined by size exclusion-high-performance liquid chromatography (SE-HPLC) and instant thin-layer chromatography (ITLC). The HPLC system used consisted of a Waters 1500 series manual injector with 20 μl injection loop (RheodyneTM 7725i Injector, Milford, MA, USA), a Waters 1525 Binary HPLC pump, a Waters 2487 dual-wavelength absorbance detector and an in-line radioactivity detector made of a sodium iodide crystal coupled to a multichannel analyzer (Ortec, Nieuwegein, The Netherlands). Chromatograms were analyzed using the Breeze software (Waters, Etten-Leur, The Netherlands). The size exclusion column used was a Bio Silect SEC 250-5, 300 x 7.8 mm² column from Bio-Rad Laboratories BV (Veenendaal, The Netherlands). The mobile phase was phosphate-buffered saline (PBS; NaCl 140 mmol l⁻¹, Na₂HPO₄ 9.0 mmol l⁻¹ and NaH₂PO₄ 1.3 mmol l⁻¹; pH = 7.4). The flow was 1.0 ml min⁻¹ and the UV detector wavelengths were set at 220 and 280 nm. The column performance was tested using a reference Bio-Rad Gel Filtration standard. The retention time of trastuzumab is 7.8 min, ^{111}In -DTPA elutes at 11.7 min. Recovery from the HPLC column was assessed by collecting fractions and counting for radioactivity (well-type LKB-1282-Compu-gamma system (LKB Wallac, Turku, Finland)).

ITLC was performed on silica-impregnated glass fibre sheets (ITLC-SG 2.5 x 10 cm², Pall Gelman Sciences, Ann Arbor, MI, U.S.A.). From the final product, 5 μl was applied to the ITLC strip that was developed with 0.9% NaCl for 5 min. Radioactivity was determined by an instant chromatography scanner (VCS-101, Veenstra Instruments, Joure, The Netherlands) equipped with an NaI crystal. ^{111}In -DTPA will move to the front, the ^{111}In -labelled monoclonal antibody remains at the starting position.

Stability testing of the radiolabeled compound

The stability of the labeled compound was evaluated in PBS and in human serum. Stability of the ^{111}In -DTPA-trastuzumab in PBS was determined by storing the final solution at 4 °C for 14 days and performing frequent SEC-HPLC analysis to determine radiochemical purity. Serum stability during 7 days was assessed after the addition of 1 mg ^{111}In -DTPA-trastuzumab (20 MBq) to 1 ml serum and storage at 37 °C. Frequent SEC-HPLC analysis was performed. Furthermore, the stability of the conjugated DTPA-trastuzumab stored at -20 °C for more than 1 year was investigated. HPLC-UV analysis of the conjugated product was performed to monitor for degradation products or other impurities. After subsequent ^{111}In -labelling of the stored conjugated product, both labeling efficiency and radiochemical purity were determined.

Iodination of trastuzumab

Direct iodination of purified trastuzumab with ^{125}I ($^{125}\text{I-NaI}$ in NaOH 0.05 M; 185 MBq = 0.05 ml, Amersham Health, Eindhoven, The Netherlands), at a specific activity of 15 MBq mg^{-1} (0.4 mCi mg^{-1}), was performed using the Iodogen[®] method. Nonbound ^{125}I was removed by gel filtration chromatography (PD-10 column, Sephadex[™] G-25M, Amersham Biosciences AB, Uppsala, Sweden). The radiochemical purity was determined by trichloroacetic acid (TCA) precipitation and ITLC using the method described above.

Determination of the immunoreactive fraction

The immunoreactive fraction of radiolabeled trastuzumab was essentially determined by cell-binding assays at infinite antigen excess, as described by Lindmo et al.¹⁰ For this assay, the human breast cancer cell line SK-BR-3 and the human ovarian cancer cell line SK-OV-3, both overexpressing HER2, were used. These cell lines were used previously in studies with trastuzumab.² The human, small-cell lung cancer cell line GLC4, with low HER2 expression (flow cytometry analyses), served as a control and is further referred to as 'negative' control. SK-OV-3 was cultured in DME high glucose/10% fetal calf serum (FCS). GLC4 was cultured in RPMI 1640/10% FCS, both in a humidified atmosphere with 5% CO_2 at 37 °C. SK-OV-3 or SK-Br-3 cells were washed with PBS and detached from the flask using a trypsin solution. After 15 min incubation at 37°C, the cell suspension was transferred into a Falcon tube and medium was added. Cells were harvested by centrifugation (5 min, 167 x g) and resuspended in fresh medium to 40×10^6 cells ml^{-1} . A fixed amount of $^{111}\text{In-DTPA-trastuzumab}$ (50 ng, 10,000 c.p.m.) or $^{125}\text{I-trastuzumab}$ was added to increasing numbers of cells (ranging from 0.3×10^6 to 10×10^6 in 200 μl) and incubated at 4 °C for 1 hour. To determine uptake, cell suspensions were centrifuged (10 min, 167 x g) and washed three times with PBS containing 5% FCS. Specific binding was calculated as the ratio of cell-bound (pellet obtained after the last centrifugation step) to total radioactivity applied minus nonspecific binding, determined by the same procedure after adding a 500-fold excess of unlabelled trastuzumab.

Internalization assay

This assay was performed as described by Zalutsky et al.^{11,12} About 5–10 ng $^{125}\text{I-trastuzumab}$ or $^{111}\text{In-DTPA-trastuzumab}$ was added to 1.4×10^6 SK-OV-3 cells and incubated at 4 °C for 1 hour. After washing twice with cold PBS, cells were incubated at 37 °C in medium. After 1, 2, 4, 20 and 24 hours incubation, cells were assayed in duplicate for surface-bound, intracellular and supernatant activity. Cell suspensions were centrifuged and the supernatant removed. Membrane-bound activity was determined by elution from the cell surface after a 5 min exposure of the cells to 0.1 M sodium citrate buffer (pH 1–2). The cell suspension was centrifuged to separate the acid-soluble cell surface activity (supernatant) and the intracellular acid-resistant radioactivity (cell pellet). The acid-soluble cell surface

radioactivity, intracellular acid-resistant radioactivity and supernatant fractions were counted in a well-type LKB-1282-Compu-gamma system (LKB Wallac, Turku, Finland). Results were expressed as percentage of the total activity that was present in each of these three fractions as a function of time.

Biodistribution, radioimmunoscinigraphy and immunohistochemistry in tumor-bearing mice

The *in vivo* behavior of the radioimmunoconjugate was assessed using athymic mice bearing human SK-OV-3 or GLC4 xenografts. Male athymic mice (Hsd:Athymic Nude-*nu*) obtained from Harlan Nederland (Horst, The Netherlands) at 4–6 weeks of age (30 g) were injected subcutaneously with either 1×10^6 SK-OV-3 cells or 1×10^6 GLC4 cells mixed equally with 0.1 ml MatrigelTM (Becton Dickinson, Bedford, MA, U.S.A.). Animals were used for *in vivo* studies approximately 2 weeks after inoculation, when the tumor measured between 0.5 and 0.8 cm in maximal diameter.

¹¹¹In-DTPA-trastuzumab was injected intravenously (I.V.) in the penile vein (450 ± 25 kBq, 25 μ g, 0.2 ml). At six time points ($t = 5$ hours, $t = 1, 2, 3, 4, 7$ days), groups of mice ($n = 3 - 6$) were killed and several organs and tissues were excised, rinsed for residual blood and weighed. Group size is variable because the biodistribution was performed in two series. Samples were counted for radioactivity in a calibrated well-type LKB-1282-CompuGamma gammacounter. Tissue activity is expressed as percentage of the injected dose per g tissue (% ID g^{-1}). Tumor-to-tissue ratios were also calculated. All data were corrected for physical decay and compared with a known standard sample. The mean and the standard error of the mean (SEM) for each tissue, at every time point, were determined. The animal studies were conducted in accordance with the Law on Animal Experimentation and local guidelines, and were approved by the local ethical committee.

Expression of HER2 was confirmed immunohistochemically. Formalin-fixed, paraffin-embedded tumors were stained with antibodies against HER2/neu (HercepTestTM, DAKO, Copenhagen, Denmark). Immunohistochemical results were scored semiquantitatively, according to the system used in clinical testing (0, 1, 2 and 3 + = no/weak/moderate/strong circumferential, membranous staining).

Radioimmunoscinigraphy was performed with tumor-bearing mice to further demonstrate the tumor localization of ¹¹¹In-DTPA-trastuzumab, as well as illustrate the potential use of the radioimmunoconjugate in nuclear medicine applications. The mice were anaesthetized with 0.1 ml ketamine (25 $mg\ ml^{-1}$)/medetomidine HCl (1 $mg\ ml^{-1}$) (2 : 1) and scanned for 10 min with a gammacamera (Diacam, Siemens, The Netherlands) equipped with a medium energy collimator. In the absence of an animal SPECT, for practical purposes, images were only acquired 72 hours after administration of ¹¹¹In-DTPA-trastuzumab.

Statistical analysis

Data are presented as means \pm SEM. Statistical analysis was performed using a Student's t-test (SPSS version 10.0.7). A P-value of < 0.05 was considered significant.

RESULTS

¹¹¹In-DTPA-trastuzumab radiolabeling

First, both the DTPA conjugation step and the final labeling step were optimized by varying pH, incubation times, molar DTPA-to-trastuzumab ratio and methods of purification. The best yield was obtained by carrying out all reaction steps under absolute metal-free circumstances and by removing any unreacted DTPA by ultracentrifugation. Purification with a Sephadex G25 column reduced the labeling yields considerably. In the conjugation step, the optimal pH is 8. Incubation times longer than 5 min did not increase the labeling efficiency. The optimal molar DTPA-to-trastuzumab conjugation ratio was 1 : 1. At higher amounts of DTPA, aggregation increased. Lower amounts of DTPA limited the labeling efficiency. The conjugated trastuzumab was radiolabeled with a specific activity of 18.5 MBq (0.5 mCi) per mg protein. Labeling with ¹¹¹InCl₃ resulted in labeling yields of $92.3 \pm 2.3\%$ (n = 4). After purification of the final product by ultrafiltration, the radiochemical purity of the final product ¹¹¹In-DTPA-trastuzumab was $97.0 \pm 1.5\%$, as determined by HPLC and ITLC. The only impurities detected were unbound ¹¹¹In-DTPA and some labeled aggregates ($3.35 \pm 0.26\%$). The recovery of the HPLC column was always $> 85\%$. A typical HPLC chromatogram of ¹¹¹In-DTPA-trastuzumab is shown in Figure 1.

The stability of the conjugated DTPA-trastuzumab stored at $-20\text{ }^{\circ}\text{C}$ was investigated over a period of 15 months. HPLC-UV analysis of the stored conjugated product showed no degradation products or other impurities. ¹¹¹In labeling of the stored conjugated product resulted in a labeling efficiency of $89.4 \pm 6.3\%$ (77 – 96%, n = 20) and a radiochemical purity of $97.2 \pm 1.3\%$.

¹¹¹In-DTPA-trastuzumab *in vitro* stability

¹¹¹In-DTPA-trastuzumab stored at $4\text{ }^{\circ}\text{C}$ was highly stable in PBS over 14 days, with minimal decrease of protein-bound radioactivity (6%). In serum stored at $37\text{ }^{\circ}\text{C}$, a significant source of label instability was transcomplexation to transferrin. The transcomplexation as estimated by SEC-HPLC analysis was about 7% per day, which is similar to 35 – 40% in 5 days.

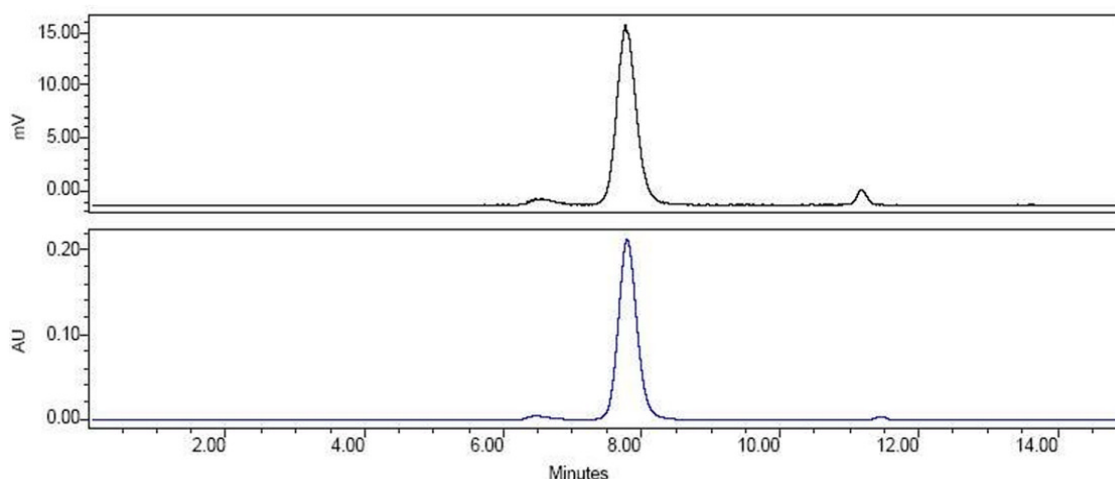


Figure 1. Typical SEC-HPLC-chromatogram of ^{111}In -DTPA-trastuzumab. Retention time for ^{111}In -DTPA-trastuzumab is 7.8 min and for ^{111}In -DTPA 11.7 min.

^{111}In -DTPA-trastuzumab immunoreactivity

The immunoreactive fraction of the purified, radiolabeled product ^{111}In -DTPA-trastuzumab was $0.87 (\pm 0.06, n = 3)$, determined by an SK-OV-3 cell-binding assay. The same results were achieved using HER2-overexpressing SK-BR-3 tumor cells instead of SK-OV-3 cells. The nonspecific binding was less than 3%, determined by adding a 500-fold excess of unlabelled trastuzumab. This is similar to the binding of ^{111}In -DTPA-trastuzumab to the HER2-negative GLC4. The immunoreactivity of the radioiodinated trastuzumab was 0.78.

^{111}In -DTPA-trastuzumab internalization

The internalization was 25% after a 1 hour and 45% after a 4 hours incubation period (Figure 2). This indicates that there is rapid internalization of the HER2 receptor. With ^{125}I -trastuzumab, radioactivity was released from the cell already after 4 hours, whereas ^{111}In -labelled trastuzumab was retained in the cell for at least 24 hours. Iodine conjugated to trastuzumab was thus released rapidly from the cell, apparently caused by intracellular rapid catabolism of the radioiodinated antibody.

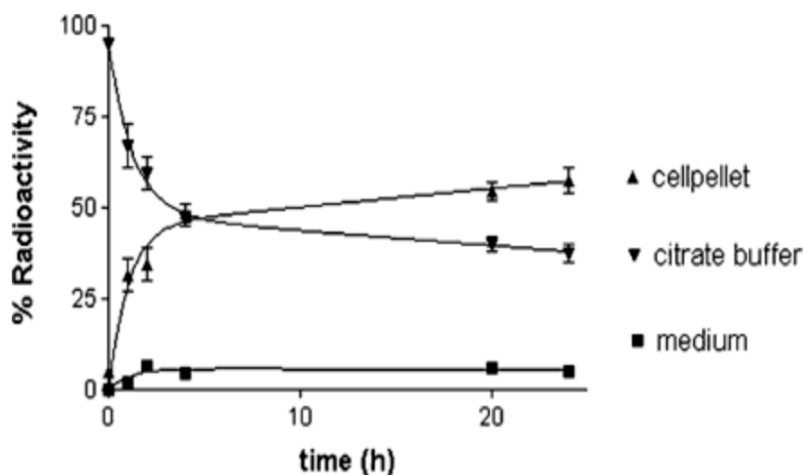


Figure 2. ^{111}In -DTPA-trastuzumab internalization assay. The acid-soluble cell surface radioactivity (citrate buffer), intracellular acid-resistant radioactivity (cell pellet) and supernatant (medium) fractions, expressed as the percentage of the total activity \pm SD that was present in each of the fractions as a function of time.

¹¹¹In-DTPA-trastuzumab biodistribution

Tables 1 and 2 show the biodistribution of ¹¹¹In-DTPA-trastuzumab in HER2-positive SK-OV-3 and HER2-negative GLC4 tumor-bearing mice, respectively. Uptake in the SK-OV-3 tumor could be demonstrated already 5 hours after injection ($9.77 \pm 1.14\%$ ID g⁻¹) and this uptake is even higher 2 days after injection ($16.30 \pm 0.64\%$ ID g⁻¹). The percentage of ¹¹¹In-DTPA-trastuzumab circulating in the blood meanwhile declined from 12.75 ± 1.50 to $2.33 \pm 0.46\%$ ID g⁻¹. In contrast with the marked uptake in the HER2-positive tumor, the uptake in the HER2-negative GLC4 tumor remained nearly constant around a mean of 4.3% ID g⁻¹. There is a higher tumor uptake in the SK-OV-3 versus GLC4 tumor at 24 hours ($P < 0.001$), 48 hours ($P < 0.001$) and 72 hours ($P < 0.02$) after injection (Figure 3). The 72 hours gamma camera scintigram of the two mice also clearly shows tracer uptake in the HER2 receptor-positive tumor in contrast to the HER2-negative tumor (Figure 4). Expected HER2 presence and absence were confirmed immunohistochemically.

Metastases were present in the abdominal cavity of at least two of the SK-OV-3 tumor-bearing mice. Uptake of labeled trastuzumab in these metastases was even more pronounced than the uptake in the primary tumor itself (15.4 versus 13.3% ID g⁻¹ at 24 hours and 11.2 versus 8.8% ID g⁻¹ at 96 h). Whole body distribution in the SK-OV-3 tumor-bearing mice showed at $t = 5$ hours uptake in the liver ($13.97 \pm 2.52\%$ ID g⁻¹), spleen ($8.40 \pm 1.67\%$ ID g⁻¹), kidney ($6.46 \pm 0.47\%$ ID g⁻¹), lung ($3.87 \pm 1.00\%$ ID g⁻¹) and heart ($3.83 \pm 0.15\%$ ID g⁻¹), all well-circulated organs. At 5 hours the blood contains $12.75 \pm 1.50\%$ ID g⁻¹. With time the amount of the injected dose per gram tissue in nearly all organs decreases, while the amount in the SK-OV-3 tumor initially increases. Uptake in the organs is not significantly different in the SK-OV-3 versus GLC4 tumor-bearing mice. As expected, no uptake is seen in the brain. The pancreas, stomach, bowel and skeletal muscle all demonstrate low uptake. The uptake in the spleen is considerable after 5 hours ($8.40 \pm 1.67\%$ ID g⁻¹) and still $4.26 \pm 0.84\%$ ID g⁻¹ at $t = 72$ hours. A minimal uptake of around 2% of the injected dose, remaining constant in time, is detected in bone, suggesting only limited leakage of ¹¹¹In from ¹¹¹In-DTPA-trastuzumab.

DISCUSSION

In the present study, trastuzumab, a humanized monoclonal antibody against the HER2 receptor, was radiolabeled with ¹¹¹In, a residualising radiolabel that is trapped in the cell after internalization of the antibody. We wanted to characterize this new tracer both *in vitro* and *in vivo* by performing a biodistribution study in mice bearing HER2-overexpressing tumor, before its use in the clinic.

Table 1. Biodistribution of ¹¹¹In-DTPA-trastuzumab in SK-OV-3 tumor bearing mice.

| | 5 h (n=3) | 24 h (n=6) | 48 h (n=3) | 72 h (n=3) | 96 h (n=3) | 168 h (n=7) |
|------------------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Brain | 0.15 (0.06) | 0.15 (0.03) | 0.11 (0.04) | 0.10 (0.03) | 0.04 (0.02) | 0.05 (0.01) |
| Kidney | 6.46 (0.47) | 5.42 (0.58) | 6.47 (0.21) | 6.20 (0.60) | 6.20 (0.59) | 4.29 (0.48) |
| Spleen | 8.40 (1.67) | 2.68 (0.48) | 2.45 (0.74) | 4.26 (0.84) | 5.84 (1.77) | 4.02 (0.96) |
| Pancreas | 0.59 (0.17) | 1.12 (0.26) | 0.73 (0.31) | 0.71 (0.18) | 4.24 (2.76) | 0.56 (0.11) |
| Bladder | 1.50 (1.28) | 2.23 (0.28) | 2.17(0.78) | 6.07 (0.80) | 1.48 (0.13) | 0.73 (0.30) |
| Heart | 3.83 (0.15) | 1.90 (0.05) | 1.64 (0.14) | 1.24 (0.15) | 0.94 (0.24) | 0.92 (0.15) |
| Bone | 2.01 (0.41) | 1.10 (0.32) | 0.94 (0.34) | 1.84 (0.29) | 1.55 (0.90) | 1.84 (0.84) |
| Small intestine | 2.91 (0.30) | 1.00 (0.25) | 1.54 (0.37) | 4.54 (1.97) | 0.88 (0.38) | 1.75 (0.61) |
| Colon | 2.26 (1.23) | 0.80 (0.23) | 1.12 (0.43) | 3.06 (1.39) | 1.63 (1.37) | 0.66 (0.11) |
| Blood | 12.75(1.50) | 8.76 (0.46) | 7.15 (0.69) | 2.33 (0.46) | 1.36 (0.51) | 1.63 (0.83) |
| Lung | 3.87 (1.00) | 4.09 (0.69) | 5.19 (1.63) | 2.42 (0.27) | 1.32 (0.06) | 1.30 (0.42) |
| Stomach | 1.51 (0.73) | 0.79 (0.22) | 1.48 (0.23) | 2.07 (1.12) | 0.86 (0.36) | 0.56 (0.11) |
| Muscle | 0.72 (0.14) | 0.50 (0.19) | 0.74 (0.14) | 0.36 (0.05) | 0.29 (0.21) | 0.18 (0.05) |
| Liver | 13.97(2.52) | 7.89(1.80) | 10.76(1.36) | 9.57 (0.69) | 7.35 (0.37) | 7.04 (1.09) |
| Tumor | 9.77 (1.14) | 13.71(0.57) | 16.30(0.64) | 15.29(1.57) | 8.31 (1.66) | 9.80 (3.22) |

The results are expressed as mean percentage of the injected dose per g tissue (%ID g⁻¹) with the standard error of the mean (SEM).

Table 2. Biodistribution of ¹¹¹In-DTPA-trastuzumab in GLC-4 tumor bearing mice.

| | 5 h (n=3) | 24 h (n=6) | 48 h (n=3) | 72 h (n=3) | 96 h (n=3) | 168 h(n=6) |
|------------------------|--------------|-------------|-------------|--------------|-------------|-------------|
| Brain | 0.22 (0.08) | 0.19 (0.09) | 0.09 (0.01) | 0.12 (0.08) | 0.23 (0.07) | 0.09 (0.03) |
| Kidney | 8.33 (2.59) | 5.72 (1.31) | 7.00 (0.20) | 6.85 (1.52) | 7.31 (0.64) | 3.85 (0.76) |
| Spleen | 13.15(7.93) | 5.68 (2.27) | 3.09 (1.38) | 7.81 (2.30) | 5.23 (2.23) | 3.08 (0.44) |
| Pancreas | 1.31 (0.79) | 1.13 (0.41) | 4.39 (4.00) | 1.02 (1.84) | 1.31 (0.24) | 0.85 (0.17) |
| Bladder | 6.45 (0.69) | 1.88 (0.52) | 1.92 (0.75) | 5.77 (1.32) | 2.53 (0.06) | 1.61 (0.42) |
| Heart | 4.00 (2.11) | 2.12 (0.39) | 1.61 (0.10) | 1.63 (0.52) | 1.75 (0.21) | 0.90 (0.20) |
| Bone | 2.69 (0.61) | 2.37 (0.52) | 1.85 (1.02) | 2.61 (0.61) | 2.44 (0.32) | 1.60 (0.30) |
| Small intestine | 3.64 (0.59) | 2.52 (0.90) | 2.49 (0.75) | 7.15 (2.30) | 4.36 (2.34) | 1.92 (0.67) |
| Colon | 1.95 (0.45) | 1.31 (0.37) | 2.58 (0.98) | 6.88 (1.90) | 2.28 (0.15) | 1.15 (0.25) |
| Blood | 17.92 (3.83) | 7.20 (2.18) | 4.83 (1.20) | 4.74 (2.20) | 4.85 (1.57) | 1.48 (0.57) |
| Lung | 7.43 (0.93) | 3.56 (0.93) | 2.38 (0.08) | 7.70 (1.94) | 2.83 (0.56) | 1.41 (0.36) |
| Stomach | 3.28 (1.11) | 0.90 (0.39) | 0.83 (0.34) | 1.57 (0.52) | 1.54 (0.13) | 0.86 (0.17) |
| Muscle | 2.45 (1.58) | 0.72 (0.28) | 0.44 (0.23) | 0.45 (0.28) | 0.64 (0.14) | 0.44 (0.15) |
| Liver | 20.70 (3.58) | 10.13(1.84) | 7.19 (1.45) | 11.62 (2.41) | 9.61 (0.83) | 8.24 (2.18) |
| Tumor | 4.70 (1.90) | 4.49 (1.24) | 4.40 (0.70) | 3.17 (1.19) | 5.70 (1.11) | 3.57 (0.96) |

The results are expressed as mean percentage of the injected dose per g tissue (%ID g⁻¹) with the standard error of the mean (SEM).

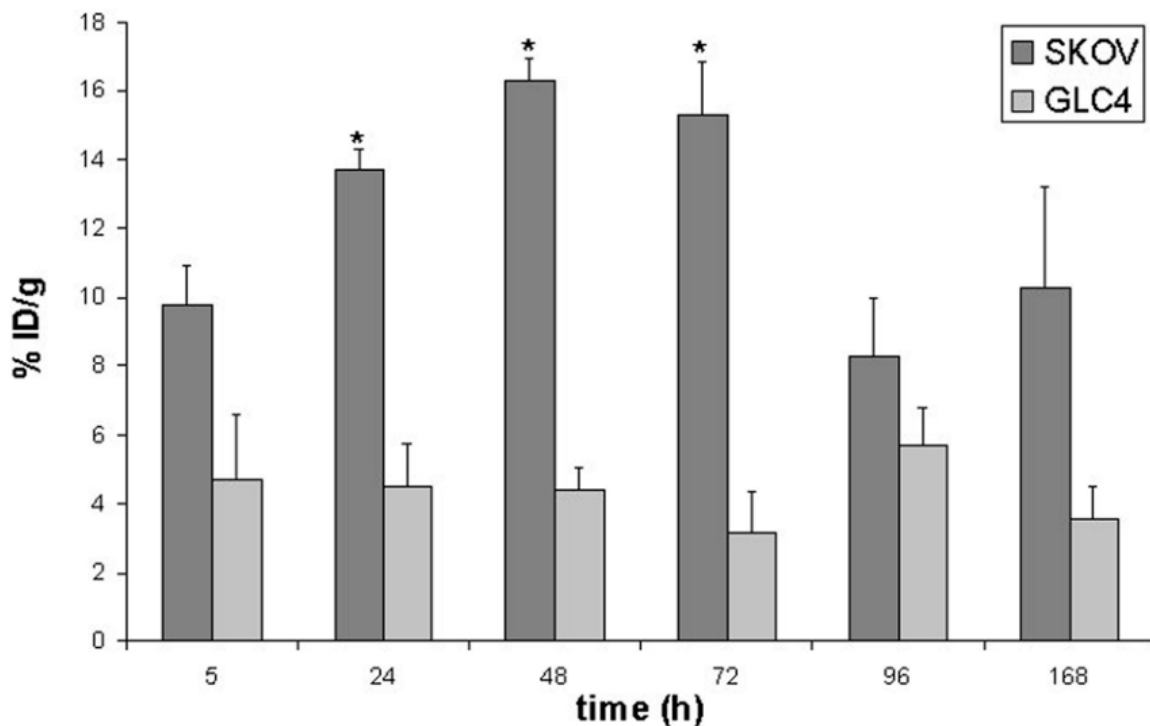


Figure 3. Uptake in the SK-OV-3 and GLC4 tumor at $t = 5, 24, 48, 72, 96$ and 168 hours after injection of $450 \text{ kBq } ^{111}\text{In-DTPA-trastuzumab}$. Data are represented as $\% \text{ID g}^{-1} \pm \text{SEM}$. * $P < 0.05$.

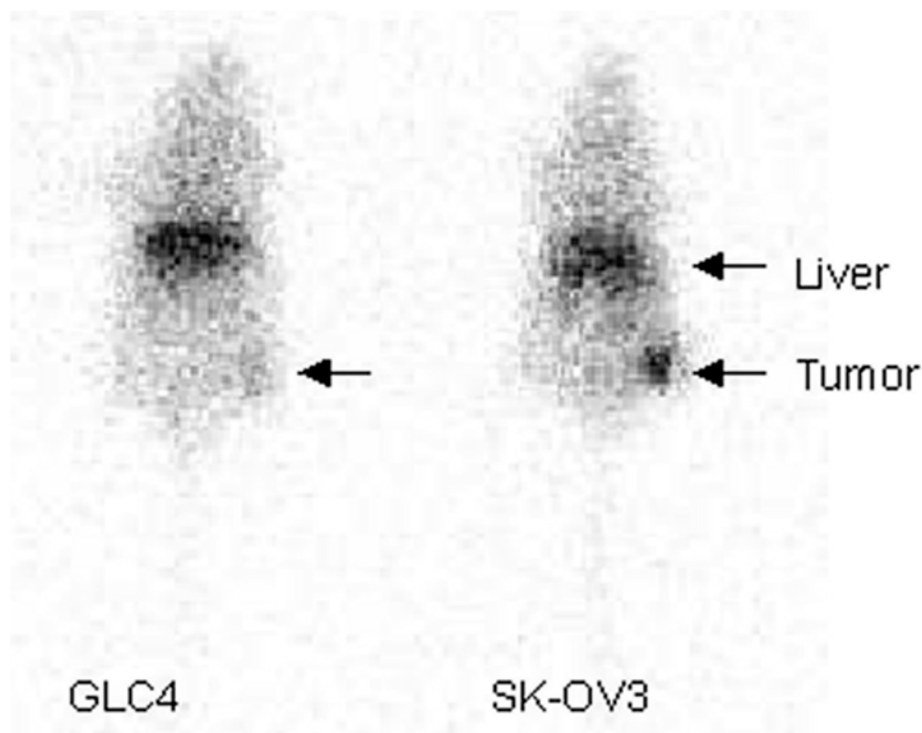


Figure 4. Typical scintigram of mice bearing HER2 positive human SK-OV-3 tumor (right, tumor weight 232 mg) and HER2 negative control GLC4 tumor (left, tumor weight 276 mg) in the right flank, imaged 72 hours after injection of $450 \text{ kBq } ^{111}\text{In-DTPA-trastuzumab}$

A number of murine monoclonal antibodies (muMAbs) directed against the HER2 receptor have been developed and radiolabeled in the past.¹¹⁻¹⁶ Before 10 years, De Santes et al. pioneered the feasibility of targeting the HER2 receptor with radioiodinated muMAbs.¹³ They concluded that radioiodinated anti-HER2/neu muMAbs are attractive agents for radioimmunodiagnosis and radioimmunotherapy of aggressive HER2/neu-positive breast and ovarian carcinomas. However, further development to the clinic was hampered by the absence of effective strategies for retarding intratumoral catabolism. This issue is now solved by the availability of trastuzumab. In the ongoing clinical study, we are interested in predicting cardiotoxicity.

Owing to the relative long elimination half-life of trastuzumab, we expect to be able to image the myocardium no earlier than at day 5 after sufficient reduction of bloodpool activity. Owing to the suitable half-life of 3 days, we therefore chose ¹¹¹In as radioisotope instead of shorter lived ^{99m}Tc or ¹²³I, despite their better imaging qualities. Furthermore, trastuzumab is an antibody with internalizing properties. Residualising radionuclides (like ¹¹¹In) are preferred over the use of ¹³¹I, which will not be trapped in the cell. Labeling of trastuzumab with PET radionuclides with middle long half-lives (⁸⁹Zr) is interesting because of the advantages of PET imaging (e.g. better resolution and quantification), and will be considered for future studies. The disadvantage of the choice for a residualising radionuclide is the considerable liver uptake, which might hamper quantification of uptake in the heart. However, in day-to-day practice, the heart area and the left liver lobe can be reliably separated with SPECT. Myocardial uptake is also well distinguishable from liver uptake, due to typical 'horse-shoe'-like shape of the left ventricle. This is similar to every day myocardial perfusion imaging.

¹¹¹In-DTPA-trastuzumab could be efficiently labeled with high yields and high stability, especially in buffer solution. The best yield was obtained by carrying out all reaction steps under absolute metal-free circumstances and by removing any unreacted DTPA by ultracentrifugation. Purification with a Sephadex G25 column reduced the labeling yields considerably. In serum, the only significant source of instability was the 35–40% transchelation in 5 days, most likely to transferrin (Mw 80 kDa). This could be considered a disadvantage of our choice for DTPA as chelator and could result in faster plasma clearance and higher uptake and radiation dose in the liver *in vivo*. Perhaps use of other chelators, such as 1B4M-DTPA or DOTA, will result in a tracer that is even more stable.

The immunoreactivity was highly preserved and in the internalization assay the prolonged cellular retention of ¹¹¹In as compared to ¹²⁵I is shown. Therefore, the use of ¹¹¹In-DTPA-trastuzumab is preferred over the use of iodinated trastuzumab. As also described by Lotti, internalization of erbB-2/p185 is fast and resembles that of epidermal growth factor receptor 1 (EGFR1).¹⁷

The biodistribution study in mice showed well the *in vivo* targeting potential of radiolabeled trastuzumab of a tumor overexpressing the human HER2 receptor.

Since mice lack the human HER2 receptor and trastuzumab is a humanized monoclonal antibody, the biodistribution of ^{111}In -DTPA-trastuzumab over organs other than the tumor must be interpreted with care. Uptake in the liver, spleen and kidney was expected and comparable with other ^{111}In -labelled antibodies. No uptake was seen in the brain, which can be explained by its size. An antibody normally does not pass the intact blood–brain barrier. Low uptake related to some blood pool activity was seen in the heart. Unfortunately, targeting human HER2 in the heart is not possible in this animal model. A transgenic animal model is more appropriate for this purpose.

The tumor-to-tissue ratio, together with the absolute uptake in the tumor, is important to evaluate the potential future use of the labeled antibody for HER2 receptor visualization. The tumor-to-tissue ratio was especially high for brain and muscle and was above 3 at most time points for all organs except the liver, kidney and spleen (Figure 5).

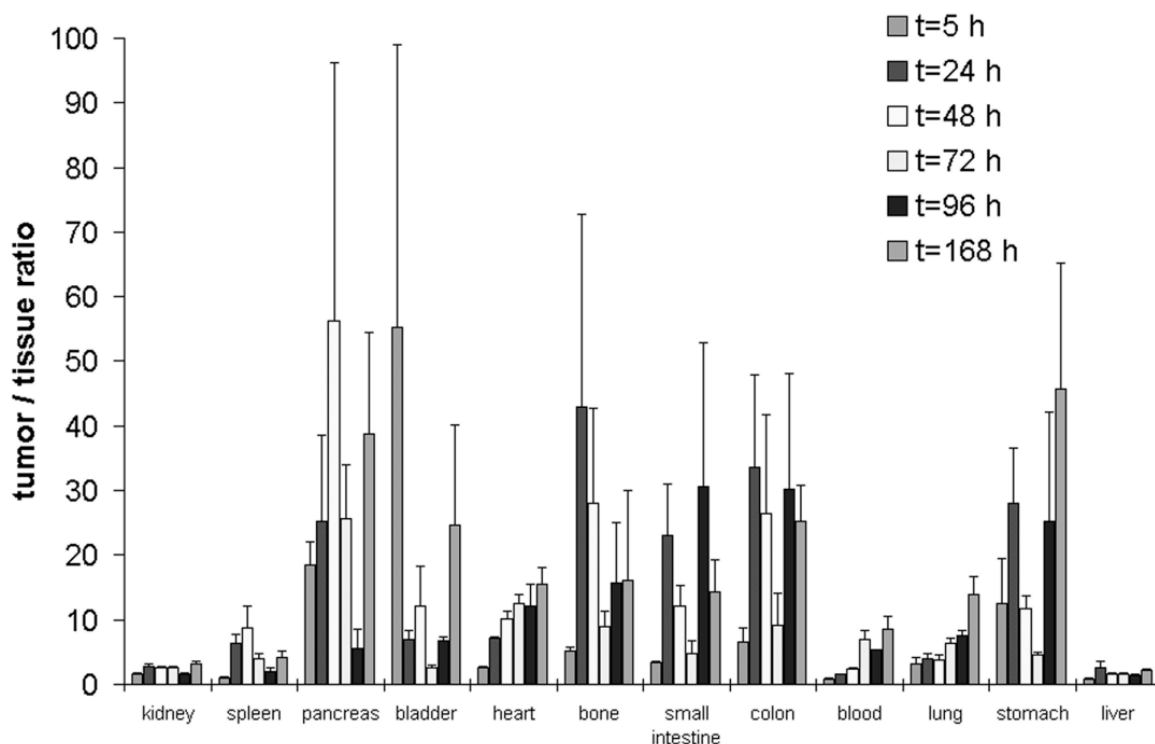


Figure 5. Tumor-to-tissue-ratio (\pm SEM) for several mice tissues at $t = 5, 24, 48, 72, 96$ and 168 hours after injection. Data presented are for the SK-OV-3 tumor bearing mice.

Not only did the primary tumor show marked uptake of ^{111}In -DTPA-trastuzumab, but also the tracer targeted to HER2-positive metastases showed a higher uptake than the primary tumor. Therefore, in the clinical situation, ^{111}In -DTPA-trastuzumab might be able to detect new tumor localizations and might predict tumor response to trastuzumab therapy.

Less extensive biodistribution studies of radiolabeled trastuzumab in different animal models and using a different chelator have been described earlier.^{18,19} The aim of Garmestani et al. was to develop a simple and rapid procedure for purification of cyclotron produced ^{86}Y .¹⁸ To assess whether the use of ^{86}Y might be a more accurate dosimetric tool than imaging with ^{111}In , they compared biodistribution of ^{111}In -trastuzumab and ^{86}Y -trastuzumab. Kobayashi et al. evaluated a novel macromolecular contrast agent (G6-(1B4M-Gd)₂₅₆) and used radio-labeled trastuzumab (^{125}I and ^{111}In) for comparison.¹⁹ Our data are largely in accordance with the data described in these studies. The most distinct difference is their lower liver uptake and more prolonged blood circulation and our observation that metastases also take up labeled trastuzumab at even higher concentrations than the primary tumor. Lower liver uptake and prolonged blood circulation could be the result of their choice for 1B4M-DTPA instead of DTPA as chelator.

As a result of the formation of immune complexes in the circulation, circulating antigen can restrict effective localization of radiolabeled muMAbs in human tumors growing as xenografts in nude mice.²⁰ Shedding of the extracellular domain (ECD) of the HER2 receptor is a well-described phenomenon and serum HER2 levels normally rise as tumors enlarge.²¹ In our animal model, a correlation existed between tumor size and tumor uptake at 168 hours. Larger tumors showed less uptake per gram tumor than smaller tumors. This is most likely the result of necrosis inside the tumor, although shedding is a possibility that cannot be ignored. Since trastuzumab is a humanized monoclonal antibody, shedding and the resulting formation of immune complexes should be taken into account in future radioimmunoscintigraphy in humans.

CONCLUSION

Trastuzumab can be efficiently labeled with ^{111}In using DTPA as chelator. ^{111}In -DTPA-trastuzumab is produced with high yield and great stability. The immunoreactivity and internalization properties are largely preserved. Specific tumor targeting was proven in an animal model using human HER2 tumor-bearing mice. From the preclinical characterisation, it is therefore concluded that ^{111}In -DTPA-trastuzumab appears suitable for clinical use in humans to visualize HER2 receptor presence.

REFERENCES

1. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. *N Engl J Med* 2001; 344: 783-792.
2. McKeage K, Perry CM. Trastuzumab: a review of its use in the treatment of metastatic breast cancer overexpressing HER2. *Drugs* 2002; 62: 209-243.
3. Crone SA, Zhao YY, Fan L, et al. ErbB2 is essential in the prevention of dilated cardiomyopathy. *Nat Med* 2002; 8: 459-465.
4. Ozcelik C, Erdmann B, Pilz B, et al. Conditional mutation of the ErbB2 (HER2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proc Natl Acad Sci U S A* 2002; 99: 8880-8885.
5. Keefe DL. Trastuzumab-associated cardiotoxicity. *Cancer* 2002; 95: 1592-1600.
6. Schneider JW, Chang AY, Rocco TP. Cardiotoxicity in signal transduction therapeutics: erbB2 antibodies and the heart. *Semin Oncol* 2001; 28: 18-26.
7. Behr TM, Behe M, Wormann B. Trastuzumab and breast cancer. *N Engl J Med* 2001; 345: 995-996.
8. Hnatowich DJ, Layne WW, Childs RL, et al. Radioactive labeling of antibody: a simple and efficient method. *Science* 1983; 220: 613-615.
9. Hnatowich DJ, Childs RL, Lanteigne D, Najafi A. The preparation of DTPA-coupled antibodies radiolabeled with metallic radionuclides: an improved method. *J Immunol Methods* 1983; 65: 147-157.
10. Lindmo T, Boven E, Cuttitta F, Fedorko J, Bunn PA, Jr. Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J Immunol Methods* 1984; 72: 77-89.
11. Xu FJ, Yu YH, Bae DS, et al. Radioiodinated antibody targeting of the HER-2/neu oncoprotein. *Nucl Med Biol* 1997; 24: 451-459.
12. Zalutsky MR, Xu FJ, Yu Y, et al. Radioiodinated antibody targeting of the HER-2/neu oncoprotein: effects of labeling method on cellular processing and tissue distribution. *Nucl Med Biol* 1999; 26: 781-790.
13. De Santes K, Slamon D, Anderson SK, et al. Radiolabeled antibody targeting of the HER-2/neu oncoprotein. *Cancer Res* 1992; 52: 1916-1923.
14. Goldenberg A, Masui H, Divgi C, et al. Imaging of human tumor xenografts with an indium-111-labeled anti-epidermal growth factor receptor monoclonal antibody. *J Natl Cancer Inst* 1989; 81: 1616-1625.
15. Rusckowski M, Qu T, Chang F, Hnatowich DJ. Technetium-99m labeled epidermal growth factor-tumor imaging in mice. *J Pept Res* 1997; 50: 393-401.
16. Tsai SW, Sun Y, Williams LE, et al. Biodistribution and radioimmunotherapy of human breast cancer xenografts with radiometal-labeled DOTA conjugated anti-HER2/neu antibody 4D5. *Bioconjug Chem* 2000; 11: 327-334.
17. Lotti LV, Di Lazzaro C, Zompetta C, Frati L, Torrasi MR. Surface distribution and internalization of erbB-2 proteins. *Exp Cell Res* 1992; 202: 274-280.
18. Garmestani K, Milenic DE, Plascjak PS, Brechbiel MW. A new and convenient method for purification of ⁸⁶Y using a Sr(II) selective resin and comparison of biodistribution of ⁸⁶Y and ¹¹¹In labeled Herceptin. *Nucl Med Biol* 2002; 29: 599-606.
19. Kobayashi H, Shirakawa K, Kawamoto S, et al. Rapid accumulation and internalization of radiolabeled herceptin in an inflammatory breast cancer xenograft with vasculogenic mimicry predicted by the contrast-enhanced dynamic MRI with the macromolecular contrast agent G6-(1B4M-Gd)(256). *Cancer Res* 2002; 62: 860-866.
20. Pimm MV. Circulating antigen: bad or good for immunoscintigraphy? *Nucl Med Biol* 1995; 22: 137-145.
21. Colomer R, Montero S, Lluch A, et al. Circulating HER2 extracellular domain and resistance to chemotherapy in advanced breast cancer. *Clin Cancer Res* 2000; 6: 2356-2362.