British Journal of Pharmacology (2004) 143, 99–106
www.nature.com/bjp

Preclinical characterisation of ^{111}In-DTPA-trastuzumab

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Trastuzumab (Herceptin®) is a recombinant humanised IgG1 monoclonal antibody against the human epidermal growth factor receptor 2 (HER2), used for metastatic breast cancer treatment. Radiolabelled trastuzumab may have several future applications for diagnostic use. The aim of the present study was to develop clinical grade ^{111}Indium (^{111}In) radiolabelled trastuzumab, to evaluate the stability and immunoreactivity of the tracer and to perform a biodistribution study in human tumour-bearing mice. Trastuzumab was radiolabelled with ^{111}In using DTPA as a chelator. ^{111}In-DTPA-trastuzumab (labelling yield 92.3±2.3%, radiochemical purity 97.0±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 tumour cell line, was 0.87±0.06. Biodistribution and tumour targeting were studied in HER2 receptor-positive and -negative tumour-bearing athymic mice. The HER2-positive tumour showed (9.77±1.14% injected dose per gram (ID g^{-1})) substantial uptake of the labelled antibody already after 5 h. The difference in uptake between HER2-positive *versus* -negative tumours was even more pronounced 3 days after injection (16.30±0.64% ID g^{-1}), and was visualised by radioimmunoscintigraphy. Liver, spleen and kidney showed marked tracer uptake. In summary, trastuzumab can be efficiently radiolabelled with ^{111}In with high labelling yields and high stability. ^{111}In-DTPA-trastuzumab selectively binds to the human HER2 receptor both *in vitro* and *in vivo* in animals. Therefore, ^{111}In-DTPA-trastuzumab appears suitable for clinical use.
British Journal of Pharmacology (2004) **143,** 99–106. doi:10.1038/sj.bjp.0705915

Keywords: Trastuzumab; ^{111}Indium; HER2; preclinical characterisation; biodistribution

Abbreviations: DME medium, Dulbecco's modified Eagle's medium; DTPA, diethylenetriamine pentaacetic acid; ECD, extracellular domain; EGFR1, epidermal growth factor receptor 1; FCS, foetal calf serum; GMP, good manufacturing practice; HER2, human epidermal growth factor receptor 2; HPLC, high-performance liquid chromatography; % ID g^{-1}, percentage of the injected dose per gram tissue; $^{111}InCl_3$, ^{111}Indium chloride; ITLC, instant thin-layer chromatography; muMAbs, murine monoclonal antibodies; PBS, phosphate-buffered saline; Ph. Eur., European Pharmacopoeia; RPMI, Roswell Park Memorial Institute; SEC, size exclusion chromatography; s.e.m., standard error of the mean; TCA, trichloroacetic acid; UV, ultraviolet

Introduction

Trastuzumab, a recombinant humanised 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 tumour 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 (Slamon *et al*., 2001). Overexpression of the HER2 receptor is a predictor of poor prognosis, because it is associated with aggressive tumour growth and metastatic activity (Slamon *et al*., 2001). Several trials investigated the role of trastuzumab alone and in combination with chemotherapeutic drugs, especially in metastatic breast cancer (McKeage & Perry, 2002). 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 (Slamon *et al*., 2001).

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 (Crone *et al*., 2002). The HER2 receptor plays a critical role in cardiac development. Conditional *ErbB2* mutants developed severe dilated cardiomyopathy (Ozcelik *et al*., 2002). 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

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Advance online publication: 2 August 2004

compared to the levels in HER2-overexpressing breast cancer cells that are the intended targets of trastuzumab therapy (Schneider *et al*., 2001; Keefe, 2002).

The risk of developing cardiotoxicity makes patient selection for trastuzumab therapy of great importance. A reliable test to predict tumour response and the risk of developing cardiac failure is therefore needed. Currently, there is no noninvasive method to visualise and stage HER2-overexpressing tumour localisations. Radionuclide imaging using radiolabelled trastuzumab might be useful for the detection of tumour localisations, 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*. (2001) recently suggested that pretreatment scanning with a tracer dose of radiolabelled trastuzumab might predict therapy response and cardiotoxicity.

The aim of the present study was to develop adequate radiolabelling of trastuzumab and to characterise and validate ^{111}In radiolabelled trastuzumab as a new tracer intended for future clinical use. The optimisation of the labelling method, the *in vitro* characterisation and the biodistribution of ^{111}In-DTPA-trastuzumab in tumour-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 × *g*). The chelator diethylenetriamine pentaacetic 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 (Hnatowich *et al*., 1983a, b). Conjugation was performed at a 1 : 1 molar ratio. In brief, 20 μl 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 μl) were subsequently added and gently mixed at room temperature for 5 min. Unbound DTPA was then removed by ultrafiltration (twice 30 min at 2684 × *g*). The purified immunoconjugate DTPA-trastuzumab was either stored at −20°C or immediately used for radiolabelling.

Radiolabelling

$^{111}InCl_3$ (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 $^{111}InCl_3$ stock solution and carefully mixed, resulting in pH 5.5. The $^{111}InCl_3$ 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 ^{111}In. The resulting ^{111}In-DTPA was then removed by ultrafiltration. The product was diluted in normal saline and sterilised by filtration through a 0.2 μm Millex GV filter (Millipore).

The complete labelling procedure was optimised by subsequently varying reaction pH, incubation times, molar DTPA to trastuzumab ratios, amounts of $^{111}InCl_3$ added per mg conjugate and purification methods with a Sephadex G25 column (PD-10 column, Amersham Biosciences AB, Uppsala, Sweden) *versus* ultrafiltration.

The optimal labelling 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 labelling 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 sterilised (15 min at 121°C). Glassware, materials and solutions for the labelling procedure were sterilised, pyrogen-free and metal-free. Centricon filters were not sterilised.

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 (Rheodyne™ 7725i Injector, Milford, MA, U.S.A.), 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 analyser (Ortec, Nieuwegein, The Netherlands). Chromatograms were analysed using the Breeze software (Waters, Etten-Leur, The Netherlands). The size exclusion column used was a Bio Silect SEC 250-5, 300 × 7.8 mm^2 column from Bio-Rad Laboratories BV (Veenendaal, The Netherlands). The mobile phase was phosphate-buffered saline (PBS; NaCl 140 mmol l^{-1}, Na_2HPO_4 9.0 mmol l^{-1} and NaH_2PO_4 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-Compugamma system (LKB Wallac, Turku, Finland)).

ITLC was performed on silica-impregnated glass fibre sheets (ITLC-SG 2.5 × 10 cm^2, 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 radiolabelled compound

The stability of the labelled 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 labelling efficiency and radiochemical purity were determined.

Iodination of trastuzumab

Direct iodination of purified trastuzumab with ^{125}I (^{125}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 radiolabelled trastuzumab was essentially determined by cell-binding assays at infinite antigen excess, as described by Lindmo *et al*. (1984). 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 (McKeage & Perry, 2002). 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% foetal 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 × *g*) and resuspended in fresh medium to 40×10^6 cells ml^{-1}. A fixed amount of ^{111}In-DTPA-trastuzumab (50 ng, 10,000 c.p.m.) or ^{125}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 h. To determine uptake, cell suspensions were centrifuged (10 min, 167 × *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.

Internalisation assay

This assay was performed as described by Zalutsky *et al*. (1999) (Xu *et al*., 1997). About 5–10 ng ^{125}I-trastuzumab or ^{111}In-DTPA-trastuzumab was added to 1.4×10^6 SK-OV-3 cells and incubated at 4°C for 1 h. After washing twice with cold PBS, cells were incubated at 37°C in medium. After 1, 2, 4, 20 and 24 h 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, radioimmunoscintigraphy and immunohistochemistry in tumour-bearing mice

The *in vivo* behaviour 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 Matrigel™ (Becton Dickinson, Bedford, MA, U.S.A.). Animals were used for *in vivo* studies approximately 2 weeks after inoculation, when the tumour measured between 0.5 and 0.8 cm in maximal diameter.

^{111}In-DTPA-trastuzumab was injected intravenously (i.v.) in the penile vein (450 ± 25 kBq, 25 μg, 0.2 ml). At six time points (t = 5 h, 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}). Tumour-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 (s.e.m.) 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 tumours were stained with antibodies against HER2/neu (HercepTest™, 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).

Radioimmunoscintigraphy was performed with tumour-bearing mice to further demonstrate the tumour localisation of ^{111}In-DTPA-trastuzumab, as well as illustrate the potential use of the radioimmunoconjugate in nuclear medicine

applications. The mice were anaesthetised 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 h after administration of ^{111}In-DTPA-trastuzumab.

Statistical analysis

Data are presented as means ± s.e.m. Statistical analysis was performed using Student's *t*-test (SPSS version 10.0.7). A *P*-value of $\leqslant 0.05$ was considered significant.

Results

^{111}In-DTPA-trastuzumab radiolabelling

First, both the DTPA conjugation step and the final labelling step were optimised 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 labelling yields considerably. In the conjugation step, the optimal pH is 8. Incubation times longer than 5 min did not increase the labelling 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 labelling efficiency. The conjugated trastuzumab was radiolabelled with a specific activity of 18.5 MBq (0.5 mCi) per mg protein. Labelling with $^{111}InCl_3$ resulted in labelling yields of 92.3 ± 2.3% ($n = 4$). After purification of the final product by ultrafiltration, the radiochemical purity of the final product ^{111}In-DTPA-trastuzumab was 97.0 ± 1.5%, as determined by HPLC and ITLC. The only impurities detected were unbound ^{111}In-DTPA and some labelled aggregates (3.35 ± 0.26%). The recovery of the HPLC column was always > 85%. A typical HPLC chromatogram of ^{111}In-DTPA-trastuzumab is shown in Figure 1.

The stability of the conjugated DTPA-trastuzumab stored at −20°C was investigated over a period of 15 months. HPLC-UV analysis of the stored conjugated product showed no degradation products or other impurities. ^{111}In labelling of the

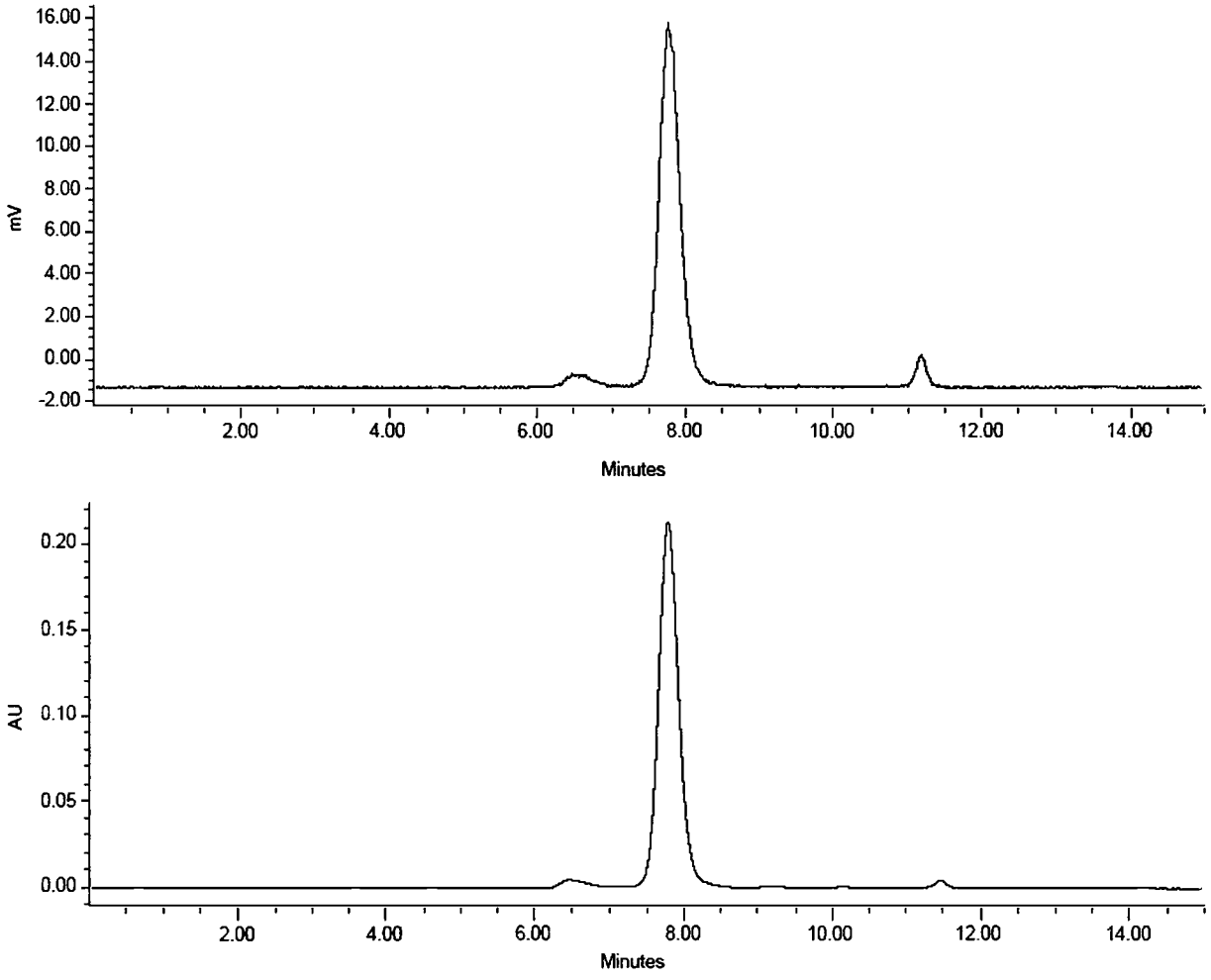

Figure 1 Typical SEC-HPLC-chromatogram of ^{111}In-DTPA-trastuzumab. Upper panel shows the radioactivity signal. Lower panel shows the UV signal ($\lambda = 280$ nm). Retention time for ^{111}In-DTPA-trastuzumab is 7.8 min and for ^{111}In-DTPA 11.7 min.

stored conjugated product resulted in a labelling efficiency of 89.4±6.3% (77–96%, $n=20$) and a radiochemical purity of 97.2±1.3%.

^{111}In-DTPA-trastuzumab in vitro *stability*

^{111}In-DTPA-trastuzumab stored at 4°C was highly stable in PBS over 14 days, with minimal decrease of protein-bound radioactivity (6%). In serum stored at 37°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.

^{111}In-DTPA-trastuzumab immunoreactivity

The immunoreactive fraction of the purified, radiolabelled product ^{111}In-DTPA-trastuzumab was 0.87 (±0.06, $n=3$), determined by an SK-OV-3 cell-binding assay. The same results were achieved using HER2-overexpressing SK-BR-3 tumour 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.

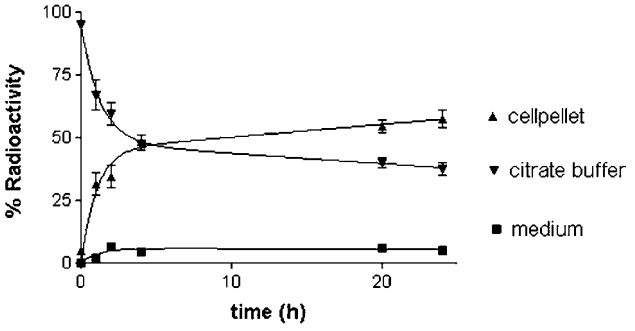

Figure 2 ^{111}In-DTPA-trastuzumab internalisation assay. The acid-soluble cell surface radioactivity (citrate buffer), intracellular acid-resistant radioactivity (cell pellet) and supernatant (medium) fractions, expressed as the percentage of the total activity±s.d. that was present in each of the fractions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

^{111}In-DTPA-trastuzumab internalisation

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

^{111}In-DTPA-trastuzumab biodistribution

Tables 1 and 2 show the biodistribution of ^{111}In-DTPA-trastuzumab in HER2-positive SK-OV-3 and HER2-negative GLC4 tumour-bearing mice, respectively.

Uptake in the SK-OV-3 tumour could be demonstrated already 5 h after injection (9.77±1.14% ID g^{-1}) and this uptake is even higher 2 days after injection (16.30±0.64% ID g^{-1}). The percentage of ^{111}In-DTPA-trastuzumab circulating in the blood meanwhile declined from 12.75±1.50 to 2.33±0.46% ID g^{-1}. In contrast with the marked uptake in the HER2-positive tumour, the uptake in the HER2-negative GLC4 tumour remained nearly constant around a mean of 4.3% ID g^{-1}. There is a higher tumour uptake in the SK-OV-3 *versus* GLC4 tumour at 24 h ($P<0.001$), 48 h ($P<0.001$) and 72 h ($P<0.02$) after injection (Figure 3). The 72 h gamma camera scintigram of the two mice also clearly shows tracer uptake in the HER2 receptor-positive tumour in contrast to the HER2-negative tumour (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 tumour-bearing mice. Uptake of labelled trastuzumab in these metastases was even more pronounced than the uptake in the primary tumour itself (15.4 *versus* 13.3% ID g^{-1} at 24 h and 11.2 *versus* 8.8% ID g^{-1} at 96 h). Whole body distribution in the SK-OV-3 tumour-bearing mice showed at $t=5$ h uptake in the liver (13.97±2.52% ID g^{-1}), spleen (8.40±1.67% ID g^{-1}), kidney (6.46±0.47% ID g^{-1}), lung (3.87±1.00% ID g^{-1}) and heart (3.83±0.15% ID g^{-1}), all well-circulated organs. At 5 h the blood contains 12.75±1.50% ID g^{-1}. With time the amount of the injected

Table 1 Biodistribution of ^{111}In-DTPA-trastuzumab in SK-OV-3 tumour-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)
Tumour	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 (s.e.m.).

Table 2 Biodistribution of ^{111}In-DTPA-trastuzumab in GLC4 tumour 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)
Tumour	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 (s.e.m.).

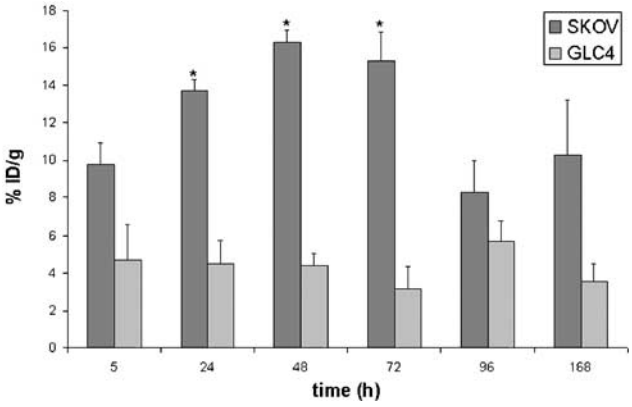

Figure 3 Uptake in the SK-OV-3 and GLC4 tumour at $t = 5$, 24, 48, 72, 96 and 168 h after injection of 450 kBq ^{111}In-DTPA-trastuzumab. Data are represented as %ID g^{-1} ± s.e.m. *$P < 0.05$.

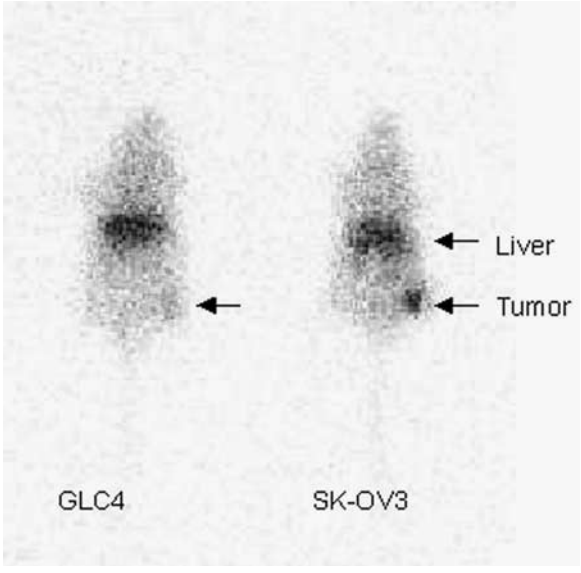

Figure 4 Typical scintigram of mice bearing HER2-positive human SK-OV-3 tumour (right, tumour weight 232 mg) and HER2-negative control GLC4 tumour (left, tumour weight 276 mg) in the right flank, imaged 72 h after injection of 450 kBq ^{111}In-DTPA-trastuzumab.

dose per gram tissue in nearly all organs decreases, while the amount in the SK-OV-3 tumour initially increases. Uptake in the organs is not significantly different in the SK-OV-3 *versus* GLC4 tumour-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 h (8.40 ± 1.67% ID g^{-1}) and still 4.26 ± 0.84% ID g^{-1} at $t = 72$ h. A minimal uptake of around 2% of the injected dose, remaining constant in time, is detected in bone, suggesting only limited leakage of ^{111}In from ^{111}In-DTPA-trastuzumab.

Discussion

In the present study, trastuzumab, a humanised monoclonal antibody against the HER2 receptor, was radiolabelled with ^{111}In, a residualising radiolabel that is trapped in the cell after internalisation of the antibody. We wanted to characterise this new tracer both *in vitro* and *in vivo* by performing a biodistribution study in mice bearing HER2-overexpressing tumour, before its use in the clinic.

A number of murine monoclonal antibodies (muMAbs) directed against the HER2 receptor have been developed and radiolabelled in the past (Goldenberg *et al*., 1989; De Santes *et al*., 1992; Rusckowski *et al*., 1997; Xu *et al*., 1997; Zalutsky *et al*., 1999; Tsai *et al*., 2000). Before 10 years, De Santes *et al*. (1992) pioneered the feasibility of targeting the HER2 receptor with radioiodinated muMAbs. 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 ^{111}In as radioisotope instead of shorter lived ^{99}mTc or ^{123}I, despite their better imaging qualities. Furthermore, trastuzumab is an antibody with internalising properties.

Residualising radionuclides (like ^{111}In) are preferred over the use of ^{131}I, which will not be trapped in the cell. Labelling of trastuzumab with PET radionuclides with middle long half-lives (^{89}Zr) 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.

^{111}In-DTPA-trastuzumab could be efficiently labelled 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 labelling 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 internalisation assay the prolonged cellular retention of ^{111}In as compared to ^{125}I is shown. Therefore, the use of ^{111}In-DTPA-trastuzumab is preferred over the use of iodinated trastuzumab. As also described by Lotti, internalisation of erbB-2/p185 is fast and resembles that of epidermal growth factor receptor 1 (EGFR1) (Lotti *et al*., 1992).

The biodistribution study in mice showed well the *in vivo* targeting potential of radiolabelled trastuzumab of a tumour overexpressing the human HER2 receptor. Since mice lack the human HER2 receptor and trastuzumab is a humanised monoclonal antibody, the biodistribution of ^{111}In-DTPA-trastuzumab over organs other than the tumour 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 tumour-to-tissue ratio, together with the absolute uptake in the tumour, is important to evaluate the potential future use of the labelled antibody for HER2 receptor visualisation. The tumour-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).

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

Less extensive biodistribution studies of radiolabelled trastuzumab in different animal models and using a different chelator have been described earlier (Garmestani *et al*., 2002; Kobayashi *et al*., 2002). The aim of Garmestani *et al*. was to develop a simple and rapid procedure for purification of cyclotron produced ^{86}Y. 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 radiolabelled trastuzumab (^{125}I and ^{111}In) for comparison. Our data are largely in accordance with the data described in these

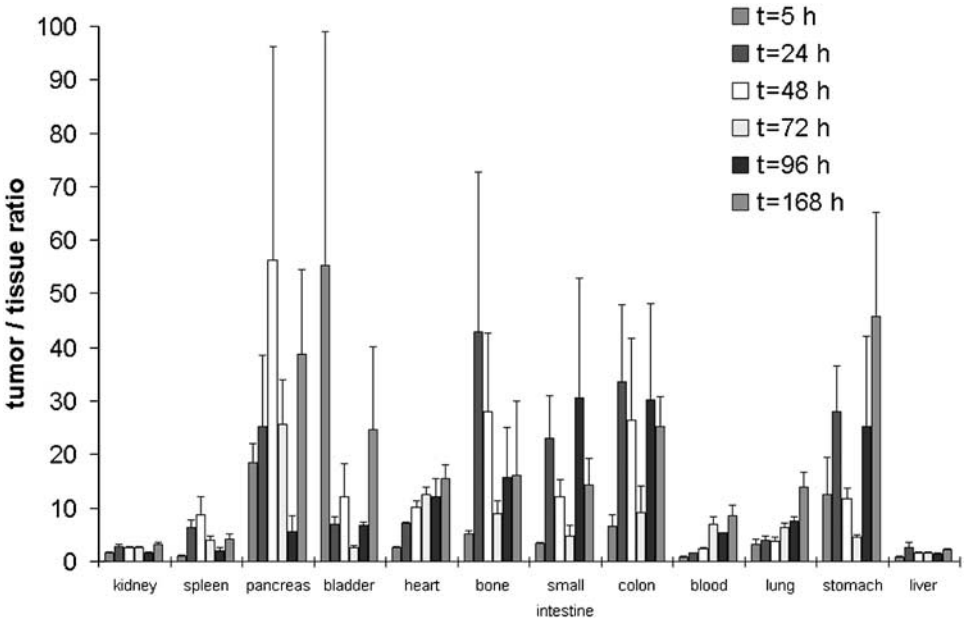

Figure 5 Tumour-to-tissue ratio (±s.e.m.) for several mice tissues at $t = 5$, 24, 48, 72, 96 and 168 h after injection. Data presented are for the SK-OV-3 tumour-bearing mice.

studies. The most distinct difference is their lower liver uptake and more prolonged blood circulation and our observation that metastases also take up labelled trastuzumab at even higher concentrations than the primary tumour. 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 localisation of radiolabelled muMAbs in human tumours growing as xenografts in nude mice (Pimm, 1995). Shedding of the extracellular domain (ECD) of the HER2 receptor is a well-described phenomenon and serum HER2 levels normally rise as tumours enlarge (Colomer *et al*., 2000). In our animal model, a correlation existed between tumour size and tumour uptake at 168 h. Larger tumours showed less uptake per gram tumour than smaller tumours. This is most likely the result of necrosis inside the tumour, although shedding is a possibility that cannot be ignored. Since trastuzumab is a humanised 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 labelled with ^{111}In using DTPA as chelator. ^{111}In-DTPA-trastuzumab is produced with high yield and great stability. The immunoreactivity and internalisation properties are largely preserved. Specific tumour targeting was proven in an animal model using human HER2 tumour-bearing mice. From the preclinical characterisation, it is therefore concluded that ^{111}In-DTPA-trastuzumab appears suitable for clinical use in humans to visualise HER2 receptor presence.

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(Received February 19, 2004
Revised June 15, 2004
Accepted June 17, 2004)