PET imaging of epidermal growth factor receptor expression in tumours using $^{89}$Zr-labelled ZEGFR:2377 affibody molecules

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Abstract. Epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor, which is overexpressed in many types of cancer. The use of EGFR-targeting monoclonal antibodies and tyrosine-kinase inhibitors improves significantly survival of patients with colorectal, non-small cell lung cancer and head and neck squamous cell carcinoma. Detection of EGFR overexpression provides important prognostic and predictive information influencing management of the patients. The use of radionuclide molecular imaging would enable non-invasive repeatable determination of EGFR expression in disseminated cancer. Moreover, positron emission tomography (PET) would provide superior sensitivity and quantitation accuracy in EGFR expression imaging. Affibody molecules are a new type of imaging probes, providing high contrast in molecular imaging. In the present study, an EGFR-binding affibody molecule (ZEGFR:2377) was site-specifically conjugated with a deferoxamine (DFO) chelator and labelled under mild conditions (room temperature and neutral pH) with a positron-emitting radionuclide $^{89}$Zr. The $^{89}$Zr-DFO-ZEGFR:2377 tracer demonstrated specific high affinity (160±60 pM) binding to EGFR-expressing A431 epidermoid carcinoma cell line. In mice bearing A431 xenografts, $^{89}$Zr-DFO-ZEGFR:2377 demonstrated specific uptake in tumours and EGFR-expressing tissues. The tracer provided tumour uptake of 2.6±0.5% ID/g and tumour-to-blood ratio of 3.7±0.6 at 24 h after injection. $^{89}$Zr-DFO-ZEGFR:2377 provided higher tumour-to-organ ratios than anti-EGFR antibody $^{89}$Zr-DFO-cetuximab at 48 h after injection. EGFR-expressing tumours were clearly visualized by microPET using $^{89}$Zr-DFO-ZEGFR:2377 at both 3 and 24 h after injection. In conclusion, $^{89}$Zr-DFO-ZEGFR:2377 is a potential probe for PET imaging of EGFR-expression in vivo.

Introduction

Targeting of cancer-associated abnormalities with specific drugs is a promising strategy in treatment of disseminated malignancies. The epidermal growth factor receptor (EGFR) is a transmembrane tyrosine kinase receptor that regulates cell proliferation and survival, but is abnormally expressed and/or activated in many epithelial tumours (1,2). EGFR is a target for several anticancer therapeutics, such as tyrosine kinase inhibitors gefitinib and erlotinib and monoclonal antibodies cetuximab and panitumumab (3).

High level of EGFR expression is associated with poor prognosis in a number of cancers, such as head and neck squamous cell carcinoma (HNSCC) (4), breast (5) and non-small-cell lung cancer (NSCLC) (6). High EGFR expression predicts resistance to neoadjuvant therapy with anthracyclines and taxanes in triple-negative breast cancer (7) and relapse of HNSCC after radiotherapy (8). Overexpression of EGFR can be used for stratification of patients with advanced NSCLC to gefitinib (9,10) and to first-line chemotherapy in combination with cetuximab (11). Patients with a high expression of EGFR in NSCLC might also benefit from the addition of cetuximab to chemoradiotherapy, but cetuximab might be detrimental for patients with low EGFR expression (12). Patients with high EGFR expression in HNSCC may benefit from hyperfractionated accelerated radiotherapy (13). Thus, detection of high EGFR expression levels in malignant tumours may provide important prognostic and predictive information influencing management of the patients.

Currently, EGFR expression level is determined using analyses of biopsy material. However, the biopsy-based methods are invasive and associated with morbidity. Therefore, a limited number of biopsy samples are usually taken. Furthermore, the EGFR expression might change over time due to genetic instability of the cancer and/or in response to therapy (14). An appreciable discrepancy in EGFR expression in primary tumours and corresponding metastases has been documented in colorectal cancer (15,16) and NSCLC (17,18).
There is an unmet clinical need to establish a methodology enabling non-invasive repeatable assessment of EGFR expression during course of disease.

Radionuclide molecular imaging with EGFR-specific agents might be a way to provide repetitive non-invasive assessment of target expression level. Goldenberg and co-workers (19) have demonstrated that the 111In-labeled anti-EGFR antibody 225 (murine predecessor of cetuximab) accumulates in human cancer xenografts in mice proportionally to the EGFR-expression. A clinical study confirmed that 111In-225 can visualize EGFR-expressing tumours in patients (20). The cited study also identified the major obstacle for EGFR visualization in vivo, which is the expression of the receptors in multiple normal tissues, foremost in the liver. However, the authors have shown that increasing the injected antibody dose saturates receptors in normal tissues but not in tumours making imaging possible. Still, there are general issues with antibody-based imaging agents, such as a long residence time in circulation and slow penetration into tumours. This results in low tumour-to-blood ratios, translating into low contrast and therefore, a low sensitivity of imaging. Positron-emission tomography (PET) provides better resolution and sensitivity than single photon emission computed tomography (SPECT). Therefore, labelling of anti-EGFR antibodies with long-lived positron emitting radionuclides 64Cu (T1/2=12.7 h) (21,22), 86Y (T1/2=14.7 h) (23,24), and 89Zr (T1/2=78.4 h) (25,26) has been evaluated. While 64Cu and 86Y-labelled antibodies have shown promising results in murine models at 24-48 h, the half-life of these nuclides is too short for clinical translation. The use of more long-lived 89Zr is probably the only viable option in clinical settings. Still, it would be desirable to have higher contrast than antibodies could provide even if PET is used for imaging. An alternative to antibodies is the use of a radionuclide labelled natural ligand of EGFR, epidermal growth factor (EGF) (27-30). This small (6 kDa) protein has much more rapid extravasation rate than bulky (150 kDa) antibodies. Small size results also in a rapid clearance of unbound imaging agent via the kidneys, which is a precondition for high contrast imaging. Rallly and co-workers (28) have demonstrated that 111In-EGF provides higher tumour-to-blood ratio than anti-EGFR antibody labelled with the same nuclide. The major issue with the use of EGF as an imaging agent is its strong agonistic action causing nausea, vomiting and hypotension at higher injected doses (27). An ideal agent for EGFR imaging should be as small as EGF but without agonistic properties upon binding to the receptor.

Affibody molecules constitute a rather new class of imaging agents that meet the requirements of small size and absence of agonistic action (31). Affibody molecules are engineered scaffold protein binders, which are originally based on a domain of protein A (32). The use of molecular display techniques enables selection of high-affinity affibody binders to variety of molecular targets (31,32). Due to the small size (7 kDa) and high affinity (low nanomolar or subnanomolar level), affibody molecules is a very promising format of targeting proteins for radionuclide molecular imaging (33,34). In the clinic, affibody molecules have demonstrated feasibility of imaging of HER2-expressing breast cancer metastases with high specificity and sensitivity (35). Earlier, we have reported development of high-affinity anti-EGFR affibody molecules for the use in radionuclide imaging (36-38). These studies resulted in development of the ZEGFR:2377 affibody molecule that has equal affinity to human and murine EGFR (38). This makes mouse models relevant for preclinical evaluations. In preclinical studies, 111In-DOTA-ZEGFR:2377 provided a tumour-to-blood ratio exceeding the tumour-to-blood ratios of any anti-EGFR monoclonal antibody. However, the use of the radionuclide 111In requires SPECT for imaging. As PET imaging provides better sensitivity of diagnostics, development of an affibody-based agent labelled with a positron emitting nuclide would be desirable.

Previous studies (38) have shown that 111In-DOTA-ZEGFR:2377 provides the highest tumour-to-blood ratio and therefore the best sensitivity at 24 h after injection. Hence, the use of short-lived positron emitting nuclides, such as 18F (T1/2=109.8 min) or 68Ga (T1/2=67.6 min), for labelling of ZEGFR:2377 would be suboptimal. The use of a more long-lived positron emitter would be desirable. Earlier studies have demonstrated that the use of radiometal labels for anti-EGFR affibody provide better contrast than the use of radiohalogens (37), which excludes the positron-emitting halogen 124I (T1/2=109.8 min) as a label. Therefore, a long-lived positron emitter, such as 89Zr (T1/2=78.4 h) would be a better choice for labelling of ZEGFR:2377.

The goal of the present study was to evaluate a 89Zr-labelled anti-EGFR ZEGFR:2377 affibody molecule for imaging of EGFR expression in human xenografts in mice and to compare imaging properties of 89Zr ZEGFR:2377 with properties of anti-EGFR antibody 89Zr-cetuximab.

**Materials and methods**

**Material.** Zirconium-89 (solution in 1 M oxalic acid) was purchased from Perkin-Elmer (Waltham, MA, USA).

**Statistics.** Data on cellular uptake and biodistribution were analyzed by unpaired, two-tailed t-test using GraphPad Prism (version 4.00 for Windows GraphPad Software) in order to determine significant differences (P<0.05).

**Preparation of targeting conjugates.** Anti-EGFR ZEGFR:2377 affibody molecule having a single C-terminal cysteine was produced as previously described (38).

For labelling with 89Zr, ZEGFR:2377 was conjugated to a maleimido derivative of deferoxamine (DFO) chelator (Macrocyclics, Dallas, TX, USA). To reduce spontaneously formed intermolecular disulphide bonds, affibody molecules were treated with dithiothreitol (DTT; E. Merck, Darmstadt, Germany). Affibody molecules (2 ml, 2.3 mg/ml in PBS) were mixed with 100 μl 1 M Tris-HCl buffer, pH 8.0, and 63 μl DTT solution (0.5 M in water). The mixture was incubated at 40°C for 30 min. The reduced affibody molecules were then purified and the buffer was changed using a disposable PD-10 column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with 0.2 M ammonium acetate, pH 5.5. The DFO-to-ZEGFR:2377 ratio was optimized to obtain conjugate in high yield. To do this, a 2-, 3-, 5- and 8-fold molar excess of DFO (0.0337 μmol/μl DMSO) was added to the ZEGFR:2377 (0.5 mg in 0.72 ml 0.1 M ammonium acetate buffer, pH 5.5) under gentle shaking,
and incubated for 30 min at 40°C. Unconjugated molecules and excess chelators were separated from the DFO-conjugated affibody molecules on a semi-preparative RP-HPLC column (Zorbas 300SB-C18 9.4x250 mm, 5 µm particle size; Agilent Technologies, Palo Alto, CA, USA) using a gradient from 35-60% B for 18 min at a flow rate of 0.5 ml/min (A: 0.1% trifluoroacetic acid in water, B: 0.1% trifluoroacetic acid in acetonitrile). The analysis was performed using high-performance liquid chromatography and on-line mass spectrometry (HPLC-MS) using an Agilent 1100 LC/MSD system equipped with electrospray ionization and single quadrupole (Agilent Technologies). The analysis was performed using a Zorbas 300SB-C18 2.1x150 mm, 3.5 µm column. Agilent ChemStation Rev. B.02.01 software (Agilent Technologies) was used for analysis and evaluation of HPLC data. The purified conjugate (further designated as DFO-ZEGFR:2377) was freeze-dried.

Cetuximab was conjugated with p-isothiocyanatobenzyl-desferrioxamine (DF-Bz-NCS) as previously described (39). The conjugate was purified using a NAP-5 size-exclusion column equilibrated with PBS.

Radiolabeling. DFO-ZEGFR:2377 was labelled with 89Zr using a modified method published by Vosjan and co-workers (39). A solution of 89Zr (67 µl, 30-40 MBq) was mixed with 30.2 µl 2 M Na2CO3, and the mixture was incubated for 3 min. Thereafter, 100 µl 0.5 M HEPES buffer, pH 7.1 was added followed by DFO-ZEGFR:2377 (50 µg dissolved in 240 µl ammonium acetate, pH 5.5) and 234.5 µl 0.5 M HEPES, pH 7.1. The mixture was incubated for 60 min at room temperature. The final purification of 89Zr-DFO-ZEGFR:2377 was performed using a NAP-5 size-exclusion column equilibrated with PBS.

For labelling of DFO-cetuximab, a solution of 89Zr (7 µl, 4 MBq) was mixed with 3 µl 2 M Na2CO3, the mixture was incubated for 3 min and 10 µl 0.5 M HEPES buffer, pH 7.1 was added. DFO-cetuximab (100 µg dissolved in 60 µl ammonium acetate, pH 5.5) and 60 µl 0.5 M HEPES, pH 7.1, were added and the mixture was incubated for 90 min.

Radio-instant thin layer chromatography (radio-ITLC) was used to measure yield, purity and stability of radiolabelled conjugates. ITLC strips (150-771 Dark Green Tec-Control Chromatography strips; Biodex Medical Systems, Shirley, NY, USA) were eluted with 0.2 M citric acid, pH 2.0. An SDS-PAGE analysis was performed [NuPAGE 4-12% Bis-Tris Gel in MES buffer (both from Invitrogen AB Foster City, CA, USA), 200 V constant] to cross-validate the ITLC.

Stability of 89Zr-DFO-ZEGFR:2377 was measured in PBS and murine blood plasma up to 24 h. For blood stability studies, freshly labelled 89Zr-DFO-ZEGFR:2377 (10 µl) was diluted in a serum sample (240 µl) to a concentration similar to the concentration in blood at the time of injection.

In vitro studies. In vitro binding and cellular processing studies were performed using EGFR-expressing A431 epidermoid carcinoma cell line (ATCC; purchased via LGC Promochem, Borås, Sweden). Binding specificity and cellular processing of 89Zr-DFO-ZEGFR:2377 were evaluated according to methods previously described (40). To determine binding specificity, A431 cells (3 cell culture dishes) were incubated for 1 h at 37°C with 10 nM 89Zr-DFO-ZEGFR:2377. Two sets of control dishes were pre-treated with 100-fold molar excess of either non-labelled ZEGFR:2377 or cetuximab 5 min before adding 10 nM 89Zr-DFO-ZEGFR:2377 and incubated at the same conditions. After 1-h incubation, the incubation media were collected, the cells were detached using trypsin and collected. Radioactivity in cells and incubation media was measured, and percentage of cell-bound radioactivity was measured.

To determine internalization rate, A431 cells were incubated with 10 nM 89Zr-DFO-ZEGFR:2377 at 37°C in a humidified incubator. At 1, 2, 4, 8 and 24 h after incubation start, internalized and membrane-bound radioactivity in a set of three dishes was determined by the acid wash method, as previously described (40). Briefly, the incubation medium was collected, cells were washed by an ice-cold medium and treated with 4 M urea solution in a 0.1 M glycine buffer, pH 2.5, for 5 min on ice. The buffer was collected, the cells were additionally washed with the buffer and the acidic fractions were pooled. Thereafter, the cells were lysed by a treatment with 1 M sodium hydroxide solution (0.5 h at 37°C) for at least 0.5 h. The basic solution containing cell debris with internalized radioactivity was collected. Dishes were additionally washed with sodium hydroxide and alkaline fractions were pooled. Radioactivity of the fractions was measured. Radioactivity in acidic fractions represented membrane-bound tracer, and radioactivity of alkaline fraction presented internalized tracer.

Animal studies. The animal experiments were planned and performed in accordance with the national regulation on laboratory animals’ protection and were approved by the Ethics Committee for Animal Research in Uppsala. Euthanasia was performed under Ropumpan/Ketalar anaesthesia, and all efforts were made to minimize suffering. Female outbred BALB/c nu/nu mice were purchased from Taconic M&B a/S (Ry, Denmark). At the time of the experiment, the average animal weight was 19±1 g. EGFR-expressing xenografts were established by subcutaneous injection of 107 A431 cells in the right hind leg. The tumours were grown for 12-14 days before the experiment. The animals were randomized into groups of four.

For biodistribution measurements, three group of mice were intravenously injected with 89Zr-DFO-ZEGFR:2377 (20 kBq in 100 µl PBS per mouse). The injected protein dose was adjusted to 40 µg per mouse by non-labelled affibody molecule. One group was euthanized at 3 and another at 24 h after injection, and distribution of radioactivity was measured. To confirm the EGFR specificity of in vivo targeting, the receptors in one group of mice were pre-saturated by injection of 400 µg of non-labelled ZEGFR:2377 40 min before injection of 89Zr-DFO-ZEGFR:2377. Biodistribution in this group of mice was measured at 3 h after injection. For comparison, one group of mice was injected with 89Zr-DFO-cetuximab (30 kBq/50 µg in 100 µl PBS per mouse) and the biodistribu-
tion was measured at 48 h after injected. After euthanasia, blood and organ samples were collected and weighed, and their radioactivity was measured. Tissue uptake (decay corrected) was calculated as percent of injected dose per gram (% ID/g).

Whole body positron emission tomography (PET)/computed tomography (CT) scans of the mice injected with $^{89}$Zr-DFO-Z2377 (45 µg, 4.6 MBq) were performed in Triumph™ tri-modality system (TriFoil Imaging, Inc., Northridge, CA, USA) at 3 and 24 h p.i. The animals were sacrificed by CO$_2$ asphyxiation immediately before being placed in the camera. The urinary bladder was excised post-mortem due to its proximity to tumour xenografts and kidneys. The PET scans were conducted for 30 min followed by CT examination at the following parameters: field of view (FOV), 8 cm; magnification, 1.48; one frame and 512 projections for 2.13 min. CT raw files were reconstructed by filter back projection (FBP). PET data were reconstructed into a static image using OSEM-3D (20 iterations). The scatter and attenuation correction were performed using their respective CT data. PET and CT files were analyzed using PMOD v3.508 (PMOD Technologies Ltd., Zurich, Switzerland). Coronal PET-CT images are presented as maximum intensity projections (MIP) in RGB color scale.

**Results**

**Preparation of targeting conjugates.** Increase of chelator-to-protein molar ratio from 3:1 to 5:1 improved the conjugation yield from 81 to 93%. Further increase of the chelator-to-protein molar ratio to 8:1 did not improve the conjugation (yield of 93%). Semipreparative HPLC enabled efficient separation of DFO-ZEGFR:2377 from unconjugated ZEGFR:2377. HPLC analysis demonstrated that the purity of DFO-ZEGFR:2377 was >95%. The mass spectrometry analysis showed an excellent agreement between the molecular mass of DFO-ZEGFR:2377 with the theoretical value (expected 8102.8 Da, observed 8103 Da).

**Radiolabelling.** Labelling of DFO-ZEGFR:2377 with $^{89}$Zr provided yield of 99±1%. Purification using a disposable NAP-5 size-exclusion column provided purity of 100%. The identity of conjugates was confirmed by radio-SDS-PAGE. There was no measurable release of $^{89}$Zr from $^{89}$Zr-DFO-ZEGFR:2377 during incubation in PBS (Table I). A minimum release (<3%) was observed in murine blood plasma after incubation for 24 h.

**Table I. Stability of $^{89}$Zr-DFO-ZEGFR:2377 in PBS and murine blood plasma.**

| Protein-associated radioactivity, % | 1 h   | 2 h   | 4 h   | 24 h  |
|-----------------------------------|-------|-------|-------|-------|
| PBS                               | 100±0 | 100±0 | 100±0 | 100±0 |
| Plasma                            | 100±0 | 100±0 | 100±0 | 98.5±1.5 |

Data are presented as an average from two measurement ± maximum error.

**In vitro studies.** Data concerning specificity of $^{89}$Zr-DFO-ZEGFR:2377 binding to EGFR-expressing A431 cells are presented in Fig. 1A. Pre-saturation of EGFR on A431 cells with an excess of both non-labeled ZEGFR:2377 and cetuximab caused a highly significant ($P<1x10^{-5}$) reduction of $^{89}$Zr-DFO-ZEGFR:2377 binding to the cells. This demonstrates saturability of binding and proves that the binding of the conjugate to EGFR-expressing cells is receptor-mediated. Similarly, specificity of $^{89}$Zr-DFO-cetuximab binding to A431 cells was confirmed (data not shown).

Data concerning cellular processing of $^{89}$Zr-DFO-ZEGFR:2377 are presented in Fig. 1B. The internalization of $^{89}$Zr-DFO-ZEGFR:2377 by A431 cells was relatively slow. The internalized fraction was ~20% after 4-h incubation and ~40% after 24 h.

A representative LigandTracer sensorgram of $^{89}$Zr-DFO-ZEGFR:2377 binding to and dissociation from living A431 cells is presented in Fig. 2. The interaction of $^{89}$Zr-DFO-ZEGFR:2377 was characterized by rapid binding (association rate of 1.95±0.45x10$^5$ 1/M x s) and slow dissociation (dissociation rate of 3.3±1.2x10$^{-5}$ 1/s). This provided a very high affinity. The dissociation constant (KD) of $^{89}$Zr-labelled DFO-ZEGFR:2377 interaction with A431 cells was 160±60 pM.

**Animal studies.** The results of an in vivo specificity test are presented in Fig. 3. Pre-saturation of EGFR with a large excess of non-labelled affibody molecule caused a significant ($P<0.0005$) reduction in tumour uptake. Radioactivity uptake was also significantly ($P<0.05$) reduced in EGFR-expressing tissues, such as liver, salivary gland and lung. There was
also a significant reduction of uptake in blood and muscle. Radioactivity uptake in kidneys was increased in the blocked group.

Comparison of $^{89}$Zr-DFO-ZEGFR:2377 biodistribution at 3 and 24 h and $^{89}$Zr-DFO-cetuximab biodistribution in nude mice bearing EGFR-expressing xenografts is presented in Fig. 4. $^{89}$Zr-DFO-ZEGFR:2377 demonstrated relatively rapid blood clearance. Already at 3 h after injection, the blood concentration was 2.0±0.4% ID/g. Low radioactivity uptake in gastrointestinal tract (2.5±0.4% ID pre-whole sample, including content) suggested that hepatobiliary excretion played a minor role in clearance of $^{89}$Zr-DFO-ZEGFR:2377. On the contrary, the high kidney uptake suggested that $^{89}$Zr-DFO-ZEGFR:2377 underwent a rapid glomerular filtration followed by renal re-absorption, which is typical for affibody molecules.

The tumour uptake (4.3±0.6% ID/g) exceeded the uptake in other organs and tissues except for liver (4.1±0.9% ID/g) and kidneys. At 24 h after injection, the blood concentration of $^{89}$Zr-DFO-ZEGFR:2377 decreased nearly 3-fold, from 2.0±0.3% ID/g to 0.7±0.07% ID/g. There was no significant decrease of uptake in salivary gland, spleen, colon, and kidney between 3 and 24 h after injection. There was significant ($p<0.05$) decrease of $^{89}$Zr uptake in the tumour, or in lung, liver and muscle. The $^{89}$Zr uptake in bone increased at 24 h.

Biodistribution of $^{89}$Zr-DFO-cetuximab was measured at 48 h after injection, i.e. an optimal time-point for imaging using radiolabelled monoclonal antibodies. At this time-point, the tumour uptake of $^{89}$Zr-DFO-cetuximab was at the same level as uptake of $^{89}$Zr-DFO-ZEGFR:2377 at 24 h after injection. However, uptake of $^{89}$Zr-DFO-cetuximab was significantly higher in blood, salivary gland, lung, liver, spleen and muscles. The use of monoclonal antibody provided significantly lower uptake in kidneys.

Tumour-to-organ ratios for $^{89}$Zr-labelled tracers, which determine contrast and therefore sensitivity of imaging, are presented in Fig. 5. At both time-points, $^{89}$Zr-DFO-ZEGFR:2377 provided significantly higher tumour-to-organ ratios than $^{89}$Zr-DFO-cetuximab except from the tumour-to-kidney ratio and, at 4 h after injection, the tumour-to-salivary gland ratio. At 24 h after injection, the tumour-to-blood ratio for $^{89}$Zr-DFO-ZEGFR:2377 (3.7±0.6) was 1.7-fold higher than at 3 h after injection. At the same time, tumour-to-muscle ratio was reduced 1.3-fold, tumour-to-bone ratio 2.8-fold and tumour-to-spleen ratio 1.4-fold. There was no significant difference in tumour-to-organ ratios for other tissues.
PET-CT images are presented as maximum intensity projections (MIP) in RGB colour scale.

MicroPET imaging of EGFR expression in mice bearing A431 xenografts using $^{89}$Zr-DFO-ZEGFR:2377 is presented in Fig. 6. The images were in a good agreement with the biodistribution data. The highest concentration of radioactivity was in kidneys. Liver showed also a prominent uptake of radioactivity. Tumours on right hind legs were clearly visualized. The tumour uptake was at the same level as in the liver (somewhat higher at 3 h after injection) and noticeably higher than uptake in any other tissue. The image at 3 h provided somewhat better contrast towards bones.

**Discussion**

PET as an imaging modality has traditionally been associated with applications using radio labelled small molecules as imaging agents and protein-based imaging agents were typically applied in SPECT imaging. However, the intrinsic advantages of PET, such as higher sensitivity, better resolution and superior quantification accuracy caused an increased interest in the labelling of proteinaceous imaging probes with positron-emitting nuclides and their use for PET imaging (42). The short-lived positron-emitting nuclides, such as $^{18}$F ($T_{1/2}$=109.8 min) or $^{68}$Ga ($T_{1/2}$=67.6 min), are perfect labels for small peptides providing high contrast already a few hours after injection (42). These nuclides have been successfully used for labelling of e.g. anti-HER2 and anti-PDGFR affibody molecules (43-45). However, anti-EGFR affibody molecules have unusually slow blood clearance. This might be associated with their slow internalisation after binding to EGFR in normal tissues (38). Clearance from blood causes dissociation of the reversibly bound tracer from EGFR-expressing tissues and its release back to blood circulation. Thus, the tumour-to-blood ratio for $^{18}$F-labelled anti-EGFR ZEGFR:1907 affibody molecules was around 2 within the time window for this short-lived nuclide, although three different labelling approaches were tested (46,47). The low tumour-to-blood ratio translates into low overall contrast and compromises sensitivity of imaging diagnostics. Previous studies have shown that the tumour-to-blood ratio for $^{111}$In-DOTA-ZEGFR:2377 affibody molecule is significantly higher at 24 than at 4 h after injection. However, next-day-imaging requires a more long-lived positron-emitting label.

Radionuclide $^{89}$Zr seems suitable for labelling of affibody molecules for PET imaging on the next day after injection. This nuclide has been successfully used for labelling of several antibodies for preclinical (25,26,48,49) and clinical (50,51) PET imaging. Derivatives of deferoxamine (DFO) chelator provide attachment of $^{89}$Zr to targeting proteins under mild conditions (room temperature, neutral pH) with adequate stability (52). $^{89}$Zr is currently commercially available. In this study, a maleimido derivative of DFO was site-specifically conjugated to a unique cysteine at the C-terminus of ZEGFR:2377 affibody molecules, providing a homogeneous conjugate. DFO-ZEGFR:2377 was efficiently labelled with $^{89}$Zr. The conjugate was stable both in PBS and in murine plasma during incubation up to 24 h (Table I). The $^{89}$Zr-DFO-ZEGFR:2377 affibody tracer demonstrated specific binding to EGFR-expressing cells (Fig. 1) and high affinity (160±60 pM, as measured by LigandTracer). The slow internalization of $^{89}$Zr-DFO-ZEGFR:2377 by A431 cells (Fig. 1) was consistent with earlier findings for $^{111}$In-DOTA-ZEGFR:2377 (38). In vivo, $^{89}$Zr-DFO-ZEGFR:2377 bound specifically to EGFR-expressing xenografts and EGFR-expressing murine organs at 3 h after injection (Fig. 3). Saturation of uptake in normal tissues resulted in lower release of the conjugate back to circulation, which resulted in lower concentration of radioactivity in blood and muscles (Fig. 3). In addition, non-bound $^{89}$Zr-DFO-ZEGFR:2377 was cleared from blood and re-absorbed in the kidneys, which resulted in higher kidney uptake after blocking (Fig. 3). The increased uptake in the kidneys upon blocking suggests that the renal uptake of $^{89}$Zr-DFO-ZEGFR:2377 is not EGFR-mediated. Most likely, the renal re-absorptions of $^{89}$Zr-DFO-ZEGFR:2377 is mediated by one of the ‘scavenger receptor’ systems that is responsible for recovery of proteins from primary urine (53). Although the high renal re-absorption is a common feature of affibody molecules (34), its exact mechanism is still not known. It has to be noted that the high renal uptake does not prevent visualization of tumours in close vicinity of the kidneys. For example, a HER2-expressing adrenal metastasis has been clearly visualized by the anti-HER2 $^{111}$In-ABY-025 affibody molecule during a clinical study (35), although the renal uptake of this tracer was as high as the uptake of $^{89}$Zr-DFO-ZEGFR:2377 in preclinical studies (54).

At 24 h after injection, the uptake of $^{89}$Zr-DFO-ZEGFR:2377 in tumours and majority of tissues was reduced compared to uptake at 3 h (Fig. 4). The only exception was bone, where the uptake of $^{89}$Zr was higher at 24 h after injection. This is consistent with the data for $^{89}$Zr-labelled antibodies, which also demonstrated increase of the bone uptake at later time-points (48,52). This might be an indication of release of the radionuclide from the conjugate in vivo (52). As $^{89}$Zr-DFO-ZEGFR:2377 was stable in the blood plasma ex vivo (Table I), we can suppose that the release takes place during catabolism in excretory organs and EGFR-expressing tissues. As the decrease of tumour uptake was slower than blood clearance, the tumour-to-blood ratio significantly (P<0.05) increased from 2.2±0.3 to 3.7±0.6, leading to increased overall imaging.
contrast. The EGFR-expressing xenografts were clearly visualized at both 3 and 24 h after injection (Fig. 6).

For comparison, we investigated targeting of EGFR-expressing xenografts using ⁸⁹Zr-labelled anti-EGFR antibody cetuximab. The biodistribution of ⁸⁹Zr-DFO-cetuximab was measured at 48 h after injection because the literature data (25) suggest that this time-point is optimal for imaging of A431 xenografts, as there was neither significant increase of tumour uptake nor decrease of blood concentration at later time-points. Our data were in a good agreement with the data published by Aerts and co-workers (25) as the difference was within the measurement accuracy. At the optimal time-point, the tumour uptake of ⁸⁹Zr-DFO-cetuximab (2.6±0.4% ID/g) was at the same level as uptake of ⁸⁹Zr-DFO-ZEGFR:2377 at 24 h after injection (2.6±0.5% ID/g) and was significantly lower than uptake at 3 h after injection (4.3±0.6% ID/g). The normal organ uptake of ⁸⁹Zr-DFO-cetuximab at 48 h was at the same level or higher than uptake of ⁸⁹Zr-DFO-ZEGFR:2377 at 3 h after injection (Fig. 4). As a result, ⁸⁹Zr-DFO-ZEGFR:2377 provided at both time-points significantly higher tumour-to-organ ratios than ⁸⁹Zr-DFO-cetuximab for all organs except from kidneys (Fig. 5). Thus, PET imaging using ⁸⁹Zr-DFO-ZEGFR:2377 should provide better contrast and, therefore, sensitivity than using ⁸⁹Zr-DFO-cetuximab. The imaging could be performed earlier after injection, which is an additional clinical advantage.

In conclusion, labelling of anti-EGFR ZEGFR:2377 antibody molecule with the long-lived positron emitting radio-nuclide ⁸⁹Zr provides a probe capable of specific PET imaging of EGFR expression in malignant tumours. ⁸⁹Zr-DFO-ZEGFR:2377 provides higher tumour-to-organ ratios than the anti-EGFR cetuximab, cetuximab. This should ensure higher sensitivity in clinical imaging.

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