In vivo and *in vitro* uptake of ^{111}In, delivered with the affibody molecule $(Z_{EGFR:955})_2$, in EGFR expressing tumour cells

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Abstract. The epidermal growth factor receptor, EGFR, is overexpressed in many carcinomas. Targeting this receptor with radionuclides is important for imaging and therapy applications in nuclear medicine. We investigated the *in vitro* and *in vivo* properties of a new high affinity EGFR binding affibody molecule, $(Z_{EGFR:955})_2$, when conjugated with CHX-A"-DTPA and labelled with ^{111}In. The binding time patterns and retention studies were performed using cultured squamous carcinoma A431 cells that overexpress EGFR. In the *in vivo* studies, female BALB/c nu/nu mice carrying tumours from xenografted A431 cells were used. The *in vitro* studies showed EGFR specific binding, high uptake and good retention of ^{111}In when delivered as $[^{111}In](Z_{EGFR:955})_2$. The retention after 72 h of incubation was 38.0±1.15% of the initial level. The biodistribution study showed a tumour specific ^{111}In uptake of 3.8±1.4% of injected dose per gram tumour tissue 4 h post-injection. The tumour to blood ratio was 9.1 and the tumours could easily be visualized with a gamma camera at this time-point. ^{111}In delivered with $[^{111}In](Z_{EGFR:955})_2$ gave an EGFR specific uptake and the results indicated that the $(Z_{EGFR:955})_2$ affibody molecule is a candidate for radionuclide-based tumour imaging. Potential therapy applications are discussed.

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Introduction

Overexpression of epidermal growth factor receptors, EGFR, is common in squamous carcinomas from the cervix, lungs and head and neck region. Some adenocarcinomas, such as colon and ovarian tumours, also express high levels of EGFR as well as gliomas and urinary bladder cancers (1,2). Thus, different types of tumours can have high expression of EGFR. It is therefore important to find targeting agents suitable for imaging applications with the purpose to select suitable treatments for the individual patient.

EGFR belongs to a transmembrane tyrosine kinase receptor family. This family which consists of four receptors, EGFR, HER2, HER3 and HER4, regulates many important cellular processes such as proliferation, apoptosis and migration (3-5). Receptor activation is dependent on receptor dimerisation and activation triggers autophosphorylation of the kinase domain which starts a signalling cascade, resulting in increased proliferation and decreased apoptosis (3,6).

There are presently at least five marketed products designed to target receptors in the EGFR family. These are the monoclonal antibodies trastuzumab, that influence HER2 mediated signal transduction, cetuximab and panitumumab, which blocks EGF from binding to the EGFR receptor (7-9), and the tyrosine kinase inhibitors gefitinib and erlotinib, which inhibit the activation of EGFR (10,11).

A new category of targeting agents that are interesting for imaging applications is affibody molecules (Affibody®) (12). These molecules are based on 58 amino acid monomers and are derived from the IgG-binding domains of staphylococcal protein A. They are small, 7 or 15 kDa (depending on monomeric or dimeric state), resulting in good tumour penetration and fast blood clearance compared to larger molecules, e.g. antibodies (13,14). An anti-HER2 affibody molecule, $Z_{HER2:342}$, has been characterized and tested in a few patients (15,16). This molecule has been labelled with ^{111}In for SPECT or ^{68}Ga for PET and has potential to be used for detection of HER2 expression in primary breast cancers and related metastases.

The new dimeric EGFR binding affibody molecule $(Z_{EGFR:955})_2$ presented in this study may, in a similar way, be a candidate for imaging of EGFR expression in primary tumours and metastases. The design and biochemical characterization of $(Z_{EGFR:955})_2$ has been described by Friedman *et al* (17), where the K_D-value was established. It was found that the radioiodinated $(Z_{EGFR:955})_2$ has an apparent affinity (avidity) of ~1 nM in binding to EGFR-expressing cells.

The cellular binding and processing of $(Z_{EGFR:955})_2$ when labelled with ^{125}I have previously been analyzed in our laboratory (18). Competitive binding studies showed that $[^{125}I](Z_{EGFR:955})_2$, [^{125}I]EGF and [^{125}I]cetuximab, which all bind EGFR, competed for an overlapping binding site. The binding study suggested that $[^{125}I](Z_{EGFR:955})_2$ binds to domain III of EGFR. Furthermore, $(Z_{EGFR:955})_2$ was internalized as demonstrated with confocal microscopy (18). For nuclear medicine applications, it is well known that ^{111}In, for gamma camera-based imaging, and ^{68}Ga, for positron emission tomography-based imaging, are important candidates. The use of chelator-conjugated proteins enables kit formulation, which simplifies labelling in a hospital radiopharmacy. Development of an ^{111}In-labelled $(Z_{EGFR:955})_2$ affibody molecule facilitates its introduction in clinical practice.

In this study we investigated the *in vitro* and *in vivo* properties of the new affibody molecule $[^{111}In](Z_{EGFR:955})_2$ with the intention to evaluate the capacity of visualising EGFR expression in tumours using a gamma camera.

Materials and methods

Nomenclature. In this article $(Z_{EGFR:955})_2$ coupled to CHX-A''-DTPA and labelled with ^{111}In is referred to as $[^{111}In](Z_{EGFR:955})_2$.

Cell culture. The squamous carcinoma cell line A431 (CRL 1555, LGC-promochem, Borås, Sweden) with high EGFR-expression, was used in all studies. The cells were cultured in Ham's F-10 medium, supplemented with 2 mM L-glutamine, PEST (penicillin 100 IU/ml and streptomycin 100 μg/ml) and 10% fetal calf serum (Biochrom Kg, Berlin, Germany). Such culture medium is from now and on called complete medium. The cells were cultured in 75 cm^2 culture bottles (Nunclon™ surface, Roskilde, Denmark). The cells were grown at 37˚C in an incubator with humidified air equilibrated with 5% CO_2.

Radiolabelling. The $(Z_{EGFR:955})_2$ and the irrelevant $(Z_{abeta3\text{-}C28S})_2$ affibody molecules were conjugated to CHX-A''-DTPA in borate buffer (0.07 M pH 9.2) at 37˚C overnight, using initial chelator with a protein molar ratio of 1:1. The conjugated CHX-A''-DTPA-$(Z_{EGFR:955})_2$ was purified on a NAP-5 size exclusion column (Sephadex G-25, Amersham Biosciences, Uppsala, Sweden), using 0.2 M ammonium acetate buffer pH 5.5 as eluent, and then split into aliquots (which were frozen in -20˚C and later used for chelation). The ^{111}In was chelated for 30 min at room temperature and the purity of the ^{111}In-CHX-A''-DTPA-$(Z_{EGFR:955})_2$, denoted in this article as $[^{111}In](Z_{EGFR:955})_2$, was controlled with instant thin-layer chromatography (ITLC) SG plates (German Sciences, Ann Arbour, MI, USA) eluted with citric acid (0.2 M). The plates were measured on a Cyclone Phosphorimager (Packard, IL, USA) and analysed using OptiQuant image analysis software (Hach Co., Loveland, USA). A CHX-A''-DTPA chelator was selected because it provides both rapid labelling with ^{111}In at room temperature and high stability *in vivo*. Our experience (19) showed that of CHX-A''-DTPA suites well for labelling of affibody molecules.

Cellular uptake of $[^{111}In](Z_{EGFR:955})_2$. Approximately 9x10^4 cells/well were seeded and cultured for ~2 days in 2 ml complete medium. $[^{111}In](Z_{EGFR:955})_2$ (1.5 nM, 0.25 MBq/μg) were diluted in complete medium and added to pre-washed cells (washed with serum-free medium) to a total volume of 1 ml/well. The cells were incubated at 37˚C for 0.5, 2, 8, 12 or 24 h. In some wells a 1,000-fold excess of non-radioactive $(Z_{EGFR:955})_2$ was added together with the labelled variant to test if the binding was specific. After incubation the cells were washed, trypsinised and counted, and the cell-associated radioactivity was measured with the gamma counter (1480 Wizard, Wallac Oy, Turku, Finland). Triplicate samples were prepared.

Retention of $[^{111}In](Z_{EGFR:955})_2$. Approximately 9x10^4 cells were seeded and incubated as already described. $[^{111}In](Z_{EGFR:955})_2$ (1.5 nM, 0.25 MBq/μg), were diluted in complete medium and added to pre-washed cells to a total volume of 1 ml/well. In some wells a 1,000-fold excess of non-radioactive $(Z_{EGFR:955})_2$ was added together with the labelled variant, to test if the binding was specific. $[^{111}In](Z_{EGFR:955})_2$ was preincubated at 37˚C for 11 h and the cells was washed six times in cold serum-free medium and further incubated with 1 ml fresh complete medium for further 0, 2, 4, 8, 12, 24, 48 and 72 h. After the last incubation the medium was collected and the cells were washed, trypsinised and counted, and the radioactivity was measured as described earlier. Triplicate samples were prepared.

Biodistibution. The local ethics committee for animal research approved the animal studies. Female BALB/c nu/nu mice were injected with ~7x10^6 A431 cells (in 100 μl complete medium) in the right fore leg. Xenografts were allowed to establish during eight days. The mice were then randomly selected into groups each containing four mice. Two groups were injected with 100 μl (1 μg, 0.25 MBq) of $[^{111}In](Z_{EGFR:955})_2$ into the tail vein. The third group was injected with 100 μl (1 μg, 0.25 MBq) irrelevant $[^{111}In](Z_{abeta3\text{-}C28S})_2$ control affibody molecules. The fourth group was preinjected subcutaneously with 500 μl (500 μg) of unlabelled $(Z_{EGFR:955})_2$ 45 min before the i.v. injection of 100 μl (1 μg, 0.25 MBq) of $[^{111}In](Z_{EGFR:955})_2$. The mice were anaesthetized with a mixture of ketamine and xylazine and euthanized by heart puncture 4 or 8 h after the injection of the radiolabelled affibody. Organs and tissue samples were dissected and weighed and their radioactivity content was measured in the gamma counter. The percent of injected dose per gram tissue (% ID/g) was calculated by dividing the radioactivity in an organ by the amount of injected radioactivity, and dividing the quotient by the organ weight.

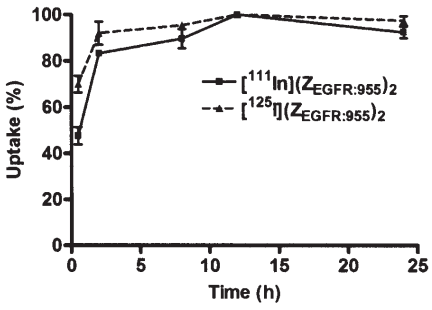

Figure 1. Time-dependent uptake of ^{111}In when delivered as $[^{111}In](Z_{955:EGFR})_2$. The highest uptake of ^{111}In was obtained after ~12 h and the remaining cell-associated radioactivity was ~90% after 24 h of incubation. The ^{111}In uptake data were compared to the ^{125}I uptake, after administration of $[^{125}I]$-$(Z_{955:EGFR})_2$, as reported in a previous study by Nordberg *et al* (18). Mean values and standard deviations are shown.

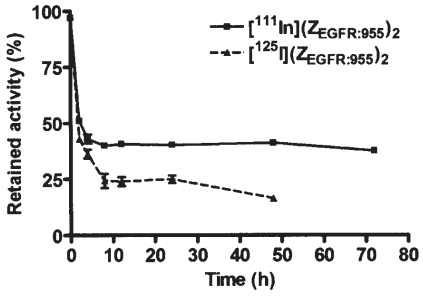

Figure 2. Cellular retention of ^{111}In delivered as $[^{111}In](Z_{955:EGFR})_2$. The remaining cell associated radioactivity after 72 h of incubation was ~38%. The ^{111}In retention data were compared to the data on ^{125}I retention reported by Nordberg *et al* (18). Mean values and standard deviations are shown.

Gamma camera imaging. A431 cells were injected as described above and xenografts were established after two weeks. Each of the three mice was injected with 100 μl (5 μg, 5 MBq) $[^{111}In](Z_{EGFR:955})_2$ into the tail vein. One mouse was preinjected s.c. with 500 μl (500 μg) of unlabelled $(Z_{EGFR:955})_2$ 45 min before the i.v. injection. After 4 h the mice were anaesthetized with a mixture of ketamine and xylazine i.p. The mice were then imaged using a e.CAM (Siemens, Erlangen, Germany) gamma camera equipped with a medium-energy, general-purpose collimator. The images were stored in a 256x256 bit matrix.

Results

Time-dependent cellular uptake. $[^{111}In](Z_{EGFR:955})_2$ gave the highest uptake (3x10^5 CPM/10^5 cells) of ^{111}In after 12 h of incubation, and ~90% of the cell-associated ^{111}In remained after 24 h (Fig. 1). The uptake pattern of ^{111}In was similar to the ^{125}I uptake, when delivered as $[^{125}I](Z_{EGFR:955})_2$, as described in an earlier study (18). The cellular binding of the ^{111}In-labeled substance could effectively be inhibited by an excess of the corresponding non-labelled substance, confirming specific uptake (data not shown).

Cellular retention. The cellular retention of ^{111}In delivered as $[^{111}In](Z_{EGFR:955})_2$ was studied at various times (Fig. 2). The remaining cell associated ^{111}In activity after 72 h of incubation delivered as $[^{111}In](Z_{EGFR:955})_2$ was 38.0±1.2%, which was more than the retention of ^{125}I delivered in the form of $[^{125}I](Z_{EGFR:955})_2$, as studied earlier (18). The cellular binding of $[^{111}In](Z_{EGFR:955})_2$ could effectively be inhibited by an excess of the corresponding non-labelled substance (data not shown).

Biodistribution in tumour-bearing mice. The radioactivity distribution of ^{111}In delivered as $[^{111}In](Z_{EGFR:955})_2$, was followed up to 8 h in Balb/mice carrying xenografted A431 tumours (Fig. 3). Four hours p.i. the tumour uptake was 3.8±1.4% ID/g and after 8 h it had decreased to 2.0±0.5% ID/g. The radiotracer had a rapid blood clearance, with a concentration <0.4±0.1% ID/g in the circulation after 4 h at which time the tumour to blood ratio was 9.1. The binding of $[^{111}In](Z_{EGFR:955})_2$ in the tumours was EGFR-specific, which was tested with the irrelevant affibody molecule $[^{111}In](Z_{abeta3-C28S})_2$. The uptake of this control affibody molecule in the tumours was low (Fig. 3).

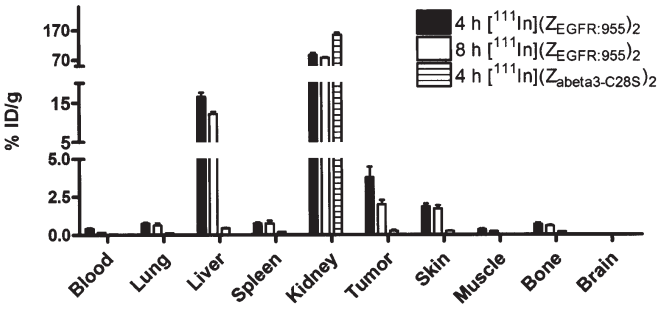

Figure 3. Biodistribution of ^{111}In, delivered as $[^{111}In](Z_{955:EGFR})_2$, expressed as percent injected dose per gram tissue in tumour (A431 cells) bearing nude mice, 4 and 8 h after injection. The control mice injected with the irrelevant affibody molecule $[^{111}In](Z_{abeta3-C28S})_2$ were analyzed 4 h after injection. Mean values and standard deviations are shown.

Table I. Uptake in liver and kidneys, 4 h post-injection, of ^{111}In delivered as $[^{111}In](Z_{955:EGFR})_2$ without or with blocking amounts of non-labelled $(Z_{955:EGFR})_2$. Data also given for the control affibody $[^{111}In](Z_{abeta3-C28S})_2$.

Substance	Liver	Kidneys
$[^{111}In](Z_{955:EGFR})_2$	16.5±2.2	86.4±14.4
$[^{111}In](Z_{955:EGFR})_2$ + excess $(Z_{955:EGFR})_2$	1.8±0.1	165.2±28.3
$[^{111}In](Z_{abeta3-C28S})_2$	0.5±0.0	153.9±15.2

Numbers are percentage injected dose per gram tissue (% ID/g). Mean values from four mice ± standard deviations are given.

There were high ^{111}In uptake in the kidneys and liver when delivered with $[^{111}In](Z_{EGFR:955})_2$. The liver uptake could be blocked by administration of an excess of unlabelled $(Z_{EGFR:955})_2$ while this was not the case for the kidney uptake. The kidney uptake of ^{111}In was high also for the irrelevant affibody molecule (Table I).

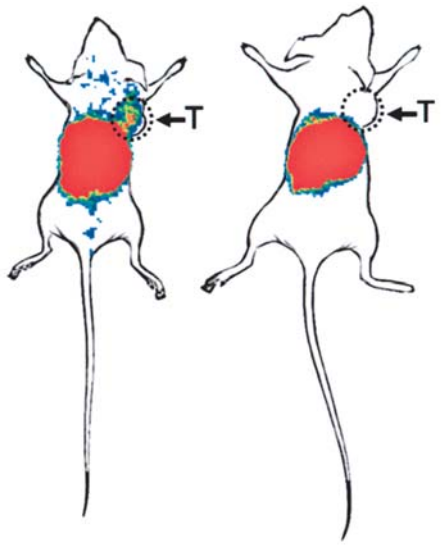

Figure 4. Gamma camera images after injection of [^{111}In]($Z_{EGFR:955}$)$_2$ in tumour-bearing mice. The images were taken 4 h after injection. The tumour areas are marked T. The receptors in the mouse to the right were blocked with an excess of non-labelled ($Z_{EGFR:955}$)$_2$.

Imaging. The localization of ^{111}In, delivered as [^{111}In]($Z_{EGFR:955}$)$_2$, was detectable using a gamma camera 4 h after injection (Fig. 4). The tumour on the right fore leg was visible while no radioactivity could be seen on the contralateral side or in the tumour of the control mouse that in addition to [^{111}In]-($Z_{EGFR:955}$)$_2$ was pre-injected with an excess of non-radio-labelled ($Z_{EGFR:955}$)$_2$, confirming antigen specific tumour uptake.

Discussion

The affibody molecule [^{111}In]($Z_{EGFR:955}$)$_2$ was developed and studied with the aim that it could be a candidate for imaging of EGFR expression in tumours and metastases. Before investigating the binding *in vivo* it was necessary to characterize the binding *in vitro*.

The [^{111}In]($Z_{EGFR:955}$)$_2$ showed a rapid cellular binding of [^{111}In] (Fig. 1) and initially also a fast release as seen in the retention analysis (Fig. 2). The quick ligand dissociation during the first 4 h was probably due to [^{111}In]($Z_{EGFR:955}$)$_2$ molecules detaching from the receptors at the cell surface without being internalised. After 4 h, it appeared that another fraction, ~38%, of the [^{111}In], was still associated to the cells for at least 72 h. This fraction was probably internalised. Similar retention patterns were also detected in our earlier study (18) performed with the same affibody molecule but labelled with ^{125}I (Fig. 2). However, the retention after several hours of incubation was lower in the ^{125}I case. Radiometals are known to be trapped in lysosomes, resulting in good cellular retention (20,21), and this is a reasonable explanation for the higher level of retention of [^{111}In] in comparison to ^{125}I.

To analyze the uptake *in vivo*, a biodistribution study was done (Fig. 3). The tumour uptake of ^{111}In mediated by [^{111}In]($Z_{EGFR:955}$)$_2$ was ~3.8% ID/g 4 h after injection. This uptake was better than the ^{99m}Tc uptake delivered with the clinically used substance [^{99m}Tc]-depreotide (~1.5% ID/g), targeting the somatostatin receptor, when analysed 4 h after injection (22). The tumour to blood ratio was also compared with the same study and was found to be in good agreement (tumour to blood ratios of 9.1 as compared to 12.1). The tumour to blood ratio were further compared to studies using radiometal-labelled anti-EGFR antibodies or EGF-ligand. When analyzed within 24 h after injection, the affibody showed better tumour-to-blood ratios (9.1) compared to the antibody (1.5) or the natural EGF-ligand (3.0) (23,24).

The kidneys and liver obtained the highest uptake of [^{111}In]. A high kidney uptake is probably a consequence of kidney clearance and tubular reabsorption that is common for small proteins. As radioactive metals are residualizing, the [^{111}In] will be trapped in the kidneys following tubular resorbtion of the labelled affibody molecule (25). The high liver uptake is most likely due to the normal expression of EGFR in liver tissue (26) and cross-reactivity of [^{111}In]($Z_{EGFR:955}$)$_2$ with murine EGFR. This is supported by the fact that the liver uptake could be blocked by a factor of ten by administration of an excess of unlabelled ($Z_{EGFR:955}$)$_2$, and that the non-specific affibody molecule was not accumulating in the liver. The uptake in the kidneys could not be inhibited this way. Note that the kidney uptake of [^{111}In] increased when the uptake in the liver was blocked (Table I).

The tumours in the right fore leg of the mice were clearly visualized in gamma camera, even though they were quite close to the kidneys and liver, that also gave a strong signal in the gamma camera. Thus, tumour tissue reasonable close to the liver and kidneys in mice can be visualised. An excess amount of unlabeled ($Z_{EGFR:955}$)$_2$ could block the [^{111}In]-($Z_{EGFR:955}$)$_2$ mediated gamma camera signal of the tumour, which supported the biodistribution results regarding receptor specificity (Fig. 4).

Small peptides like somatostatin analogues labelled with ^{177}Lu have been shown to give therapy effects (27,28) and it is possible that ($Z_{EGFR:955}$)$_2$ labelled with ^{177}Lu also could be of therapeutic interest. However, due to the high liver and kidney uptake the molecule used in this study might only be used through locoregional administration, e.g. for treatment of the urinary bladder carcinoma (29) or gliomas (30). High molecular weight substances are most likely favourable for therapeutic applications since high renal uptake of radio-nuclides is avoided, e.g. [^{111}In] and ^{177}Lu, as demonstrated with radiolabelled cetuximab (24), pertuzumab (31) and albumin-ABD-conjugated affibody molecules (32). Furthermore, unlabelled ($Z_{EGFR:955}$)$_2$ might be used therapeutically by blocking EGF-binding to EGFR, in analogy with cetuximab or panitumumab (9).

The results presented in this article shows that [^{111}In]-($Z_{EGFR:955}$)$_2$ is a promising candidate for EGFR targeted imaging. An interesting aspect is that the biodistribution of [^{111}In] delivered with [^{111}In]($Z_{EGFR:955}$)$_2$ turned out to be comparable to ^{99m}Tc delivered with [^{99m}Tc]-depreotide (NeoSpect™ or NeoTect™) (22). This substance is clinically used for imaging of somatostatin receptors in lung cancers. Thus, it is possible that [^{111}In]($Z_{EGFR:955}$)$_2$ could be used for clinical imaging of EGFR expression. If so, it could be used for early detection of EGFR expressing primary tumours and corresponding metastases at least outside the liver. It could

potentially also be used for identification of patients with tumours that are suitable for treatment with EGFR targeting agents, e.g. treatment of non-small cell lung cancer with gefitinib (33), and for follow-up of such therapy to evaluate if receptors are up- or down-regulated.

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