In vivo and in vitro studies on renal uptake of radiolabeled affibody molecules for imaging of HER2 expression in tumors

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ABSTRACT

Affibody molecules (6-7 kDa) are a new class of small robust three-helical scaffold proteins. Radiolabeled subnanomolar anti-HER2 affibody $Z_{HER2:342}$ was developed for imaging of HER2 expression in tumors and a clinical study has demonstrated that the ^{111}In- and ^{68}Ga-labeled affibody molecules can efficiently detect HER2 expressing metastases in breast cancer patients. However, significant renal accumulation of radioactivity after systemic injection of radiolabeled anti-HER2 affibody conjugate is observed. The aim of this study was to investigate the mechanism of renal reabsorption of anti-HER2 affibody at the molecular level. Renal accumulation of radiolabeled anti-HER2 affibody molecules was studied in murine model and in vitro using opossum derived proximal tubule cells (OK). It was found that kidney reabsorption of affibody was not driven by megalin/cubilin. Amino acids in the target-binding side of affibody were involved in binding to OK cells. On OK cells two types of receptors for anti-HER2 affibody were found: K_{D1}=0.8 nM, B_{max1}=71,500 and K_{D2}=9.2 nM, B_{max2}=367,000. The results of the present study indicate affibody and other scaffold-based targeting proteins with relatively low kidney uptake can be selected using in vitro studies with tubular kidney cells.

Key words: affibody molecules, HER2, OK cells, megalin, renal reabsorption

INTRODUCTION

Radionuclide molecular targeting is a promising method for detection, characterization and treatment of both localized and metastasized tumors. Several classes of radiolabeled proteins have been used for the delivery of radionuclides to tumors in vivo, utilizing the specific affinity of these agents for phenotypic alterations in malignant cells. In fact, tumor targeting with radiolabeled antibodies for detection of malignancy or for therapeutic purposes has been reported as early as the 1950's. [1] During the last decades, there was a general trend towards using smaller targeting proteins like antibody fragments, peptide hormones and scaffold proteins in order to overcome slow clearance of bulky antibodies. This strategy enables improved imaging with high contrast and reduction of the radiation dose to normal tissues during therapy. Nowadays, peptide-based radionuclide imaging and therapy plays a well established role in the management of patients with neuroendocrine tumors. [2] The use of somatostatin analogues for imaging and therapy of neuroendocrine tumors [3] and RGD (Arg-Gly-Asp) peptides to visualize tumor-associated angiogenesis [4] are some well known examples. Selectivity of these radionuclide loaded peptides to cancer cells, rapid penetration to the tumor tissue in combination with rapid clearance from normal tissues, designates them as very promising targeting agents.

Unfortunately, high kidney reabsorption after glomerular filtration was often a stumbling block for further development of many peptide-based agents as efficient radiolabeled targeting probes. This tubular re-absorption of tracers smaller than the cut-off for glomerular filtration (~60 kDa) might potentially compromise reliable diagnostic accuracy in the retroperitoneal, periaortic and epigastric regions, especially with residualizing labels such as radiometals. [5] Moreover during radionuclide therapy, high accumulation of the radiotracer in the kidneys can be a limiting factor

for successful delivery of appropriate radiation doses to tumors. For example, detoriation of renal functions is the dose limiting toxicity for treatment of neuroendocrine tumors with ^{90}Y-DOTATOC despite a number of kidney-protecting precautions (dose fractionation, co-injection of positively charged amino acids and pre-estimation of patient's risk factors). [6]

It is known that megalin and cubilin, two endocytic receptors, expressed in proximal tubule, play a major role in normal reabsorption of proteins in kidneys. [7] It has been demonstrated that the scavenger receptor megalin, is the main mediator of renal accumulation of somatostatin analogues and other small peptides. [8,9] Melis et al. showed that the reabsorbed somatostatin analogue, ^{111}In-DTPA-octreotide localized in the renal cortex. [10] Moreover, a profound decrease in radioactivity accumulation in the kidneys of megalin-deficient mice was observed for several radiolabeled small peptides, thus clearly indicating direct involvement of the megalin/cubilin system in the reabsorption process of these agents. [11] Accumulation of the radiotracer in the kidney by the megalin/cubilin system is a complex process. Megalin is a large multi-ligand receptor (517 kDa) with several domains, each of which might interact with different parts of the ligand. Different binding sites might be involved in the accumulation of different peptides. [12] Moreover, it was proposed that the differences in renal accumulation of radiopeptides may be related to the number of charged amino acids of a molecule. [13] These data indicate that the kidney reabsorption mechanism for each peptide-based radioactive probe could be individual and the optimal strategy for decreasing renal reabsorption could differ from one protein to another.

Affibody molecules (6-7 kDa) are a new class of small robust three-helical scaffold proteins, based on a modified B-domain of staphylococcal protein A. [14] Randomization of 13 amino acids on helices 1 and 2 provided a large library which enables molecular-display selection of high-affinity (ranging from low nanomolar to picomolar) binders to different proteins. [14,15]

Radiolabeled anti-HER2 affibody molecule $Z_{HER2:342}$ and its derivatives have been evaluated pre-clinically for imaging of HER2 expression in tumors. [16] A clinical study has demonstrated that the ^{111}In- and ^{68}Ga-labeled affibody molecules can efficiently detect HER2 expressing metastases in breast cancer patients. [17] However, similar to other small proteins and peptides, efficient tumor targeting was associated with significant renal accumulation of the radioactivity. Fusion of $Z_{HER2:342}$ with an albumin-binding domain (ABD) allows non-covalent binding of the conjugate to albumin in the circulation resulted in a dramatic decrease of renal uptake, [18] suggesting that the kidney uptake was associated with tubular re-absorption. At the same time the use of labeling methods yielding non-residualizing radiocatabolites caused a significant reduction of renal retention. [19-25]

Better understanding of the fate of affibody molecules in the kidneys would open the way for the rational design of new affibody conjugates and/or could facilitate the development of substances that can effectively block kidney reabsorption. Therefore, here we studied the mechanism of kidney reabsorption of affibody molecules in detail both in vitro and in vivo. The opossum derived proximal tubule cell line (OK cells) was selected as an in vitro model for this study. In these cells the expression of scavenger receptors, transporters and kidney ion channels are preserved. [26-28] $Z_{HER2:2395}$-Cys, a cysteine-containing derivative of $Z_{HER2:342}$ anti-HER2 affibody molecule was selected as a model molecule. This affibody molecule with K_D = 27 pM [22] was labeled with indium-111 via site-specifically conjugated maleimido derivative of DOTA and with technetium-99m using a peptide based natural chelating system at the C-terminus. [22,23] Earlier it was shown that both labeling methods yielded residualising radiocatabolites and similar biodistribution profiles (kidney radioactivity uptake was 191±15%ID/g for ^{99m}Tc and 168±24%ID/g for ^{111}In at 4 h p.i.). [22,23]

The aim of this study is to investigate the mechanism of renal reabsorption of anti-HER2 affibody molecules at the molecular level, and to determine the role of the megalin/cubilin receptor complex system in this process.

MATERIALS AND METHODS

^{99m}Tc-pertechnetate solution was acquired by elution of an Ultra-TechneKow generator (Covidien, Hazelwood, US) eluted with sterile 0.9 % sodium chloride (Covidien). ^{111}In-indium chloride was purchased from Covidien. All affibody molecules ($Z_{HER2:2395}$-Cys, $Z_{taq:1154}$, $Z_{insulin:810}$, and $Z_{EGFR:2377}$) were kindly provided by Affibody AB (Stockholm, Sweden). Radioactivity of samples was measured in an automated gamma counter with a 3 inch NaI(Tl) well detector (1480 WIZARD^{TM}, Wallac Oy, Turku, Finland). Cells in in vitro experiments were counted manually using a cell counter (Scepter, Millipore, Billerica, US). Labeling of affibody molecules with indium-111 was performed as described previously. [22] Labeling with technetium-99m was performed using a two-vial freeze-dried kit method as described previously. [29] After purification on the NAP-5 size-exclusion column (Amersham Biosciences, Uppsala, Sweden) purity of ^{99m}Tc-$Z_{HER2:2395}$-C was >95 %, whereas the content of hydrolyzed ^{99m}Tc did not exceed 1 %.

Animal experiments

All animal experiments were planned and performed in accordance with national legislation on laboratory animals' protection. The studies had been approved by the Local Ethics Committee for Animal Research. In all in vivo experiments indium-labeled $Z_{HER2:2395}$-Cys was used.

To evaluate a gender influence of renal re-absorption of radiolabeled affibody molecules, four NMRI male and four NMRI female mice were iv injected with 1 μg of ^{111}In-labeled anti-HER2 affibody molecule (30 kBq). Biodistribution was determined at 4 h pi. Mice were exsanguinated by heart puncture under anesthesia, organs and tissue samples were collected, weighed and the radioactivity content was determined.

For SPECT imaging, five kidney-specific megalin deficient mice (30) and five wild-type mice were injected iv with 10 μg of ^{111}In-labeled anti-HER2 affibody molecule (20 MBq). SPECT images were acquired at 4 h pi, using the USPECT II (MILabs, Utrecht, The Netherlands). Mice were scanned in prone position under general anesthesia (isoflurane/N_2O/O_2) for 45 min using the 1.0-mm diameter pinhole collimator tube. After completion of the acquisition of the images mice were euthanized and the concentration of the radioactivity in dissected tissues was determined as described above. Scans were reconstructed with MILabs reconstruction software, using an ordered-expectation maximization algorithm, with a voxel size of 0.375 mm.

Cell Experiments

Opossum kidney cell line (OK) and ovarian cancer cells (SKOV-3) were purchased from ATCC, via LGC Standards AB, Boras, Sweden. OK cells were cultured in MEM Earle's and SKOV-3 cells in RPMI medium complemented with 10% fetal calf serum (FCS), 2mM L-glutamine and PEST (penicillin 100 IU/ml, streptomycin 100 μg/ml), all from Biochrom AG, Berlin, Germany. These media are referred to as complete media in the text. In all experiments with OK cells, cells were incubated in 1% FCS medium. Cells were detached using trypsin-ethylenediaminetetraacetic acid (EDTA) solution (0.25% trypsin, 0.02% EDTA in buffer; Biochrom AG, Berlin, Germany). In vitro studies were performed in triplicate. In all cell experiments, ^{99m}Tc-labeled affibody molecule $Z_{HER2:2395}$-Cys was used. Cells were seeded two days before the experiment in Petri dishes (ø 3.5 cm) in amount $2 \cdot 10^6$ cell/dish.

Binding kinetics: cell-associated radioactivity as a function of the incubation time

Cultured OK cells were washed once with serum-free medium and incubated with 1 mL of 1 nM ^{99m}Tc –$Z_{HER2:2395}$-Cys in 1% FCS medium at 4°C up to 4 h. At pre-determined time points, the cells were washed two times with serum free-medium and treated with 0.5 ml/dish trypsin-EDTA

at room temperature. When the cells were detached, 0.5 ml/dish serum-free medium was added and the cells were re-suspended and collected. The radioactivity was measured and percentage of cell-associated radioactivity at different time points was calculated. Data were normalized to maximum cell associated radioactivity.

Binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys to OK cells at different conditions

The goals of this experiment were to investigate if the binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys to OK cells is saturable by non-labeled $Z_{HER2:342}$, whether the presence of FCS interferes with binding and whether internalization contributes to uptake of ^{99m}Tc –$Z_{HER2:2395}$-Cys by OK cells. In total, four sets of cells (12-well plate, Nunclone Surface, Nunc, Denmark) were prepared. Radiolabeled conjugate was added to cells in concentration of 1 nM and incubated at 37°C. One set of cells was pre-incubated for 1 h at 37°C in medium without FBS and to another set a 10,000-fold excess of unlabeled affibody molecule $Z_{HER2:342}$ was added 5 min before adding the labeled conjugate. One set of cells was incubated for 1 h at 4°C. After incubation supernatants were collected and cells were treated as described above. Radioactivity of samples was measured and percentage of cell-bound radioactivity was calculated.

Competitive binding assay, IC_{50} determination

The ability of different affibody molecules and human albumin to inhibit binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys to OK cells was studied in vitro. The half maximum inhibitory concentration (IC_{50}) of HER2- ($Z_{HER2:342}$) and non-HER2- ($Z_{taq:1154}$, $Z_{insulin:810}$ and $Z_{EGFR:2377}$) affibody molecules and of albumin was determined. Cell monolayers were washed once with serum-free medium and incubated for 2 h at 4°C with ^{99m}Tc –$Z_{HER2:2395}$-Cys (1 nM) in the presence of increasing concentrations (range 0 - 9.7 10^{-4} M) of competitors. After incubation, cells were

treated as described above. In addition, the half maximum inhibitory concentration (IC_{50}) of $Z_{HER2:342}$ for binding of ^{99m}Tc-Z_{2395}-C to SKOV-3 cells (high HER2 expression) was determined.

Receptor expression and affinity, Scatchard analysis

OK cells were incubated for 2 h at 4°C with ^{99m}Tc –$Z_{HER2:2395}$-Cys (concentration range of 0.5 to 300 nM). For each data point, four dishes were used, including 1 pre-saturated with 30 μM unlabeled $Z_{HER2:342}$ to determine un-specific binding. After incubation, medium was aspirated and the cells were treated as described above. From the cell suspension 0.1 ml was used for cell counting and the rest - for radioactivity measurements. Finally data were corrected on un-specific binding.

Cellular processing of ^{99m}Tc –$Z_{HER2:2395}$-Cys by OK cells

OK cells were incubated with 3 or 30 nM ^{99m}Tc –$Z_{HER2:2395}$-Cys in 1% FCS medium at 37°C **(**5% CO_2). At various time points, the medium was collected and the cells were washed once with serum-free medium. The membrane-bound and internalized radioactivity was estimated essentially as described by Wållberg. [31] Briefly, cells were treated with 0.5 ml 4 M urea solution in a 0.2 M glycine buffer, pH 2.5, for 5 min on ice. The acid supernatant was collected and the cells were washed twice with an additional 0.5 ml of acid buffer, which were also collected. The radioactivity in the acid fractions was considered as membrane bound radioactivity. For lysis, 0.5 ml of 1 M sodium hydroxide solution was added and the cells were incubated at 37˚C for at least 0.5 h. The alkaline solution containing cell debris was collected. Dishes were washed with an additional 0.5 ml basic solution and the basic fractions were pooled. The radioactivity in alkaline fractions was considered to contain the internalized activity.

Data analysis and statistics

Data for cell analysis were investigated by unpaired, two-tailed *t* test using GraphPad Prism (version 4.00 for Windows GraphPad Software, San Diego, California, USA) to find significant statistical difference ($p<0.05$). Binding data were analyzed using the GraphPad Prism software package.

RESULTS

Animal experiments

The biodistribution data of ^{111}In-labeled anti-HER2 affibody molecules are presented in Tables 1 and 2. No apparent difference in radioactivity uptake in kidneys related to mice gender was found. Remarkably, uptake of ^{111}In-DOTA-$Z_{HER2:2395}$-Cys in the kidney of the megalin-deficient mice was identical to the uptake in the kidneys of the wild-type mice. Micro-SPECT images of kidney in megalin-deficient mice and wild type mice of ^{111}In-DOTA-$Z_{HER2:2395}$-Cys confirmed the identical accumulation of radioactivity in kidneys (Fig. 1), indicating that megalin expression in the kidney is not essential for renal uptake of ^{111}In-labeled anti-HER2 affibody molecules.

In Vitro Characterization

The in vitro assay showed that ^{99m}Tc –$Z_{HER2:2395}$-Cys at 4°C showed rapid binding to OK cells that reached a plateau almost in 1.5 h with cell associated radioactivity 3.6% from added (Fig. 2). It was also found that incubation of cells at 4°C should not exceed four hours, since cells start to detach. Pre-incubation of OK cells in serum-free medium to stimulate protein transport did not influence binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys to OK cells (Fig. 3). Incubation at 4°C or addition of an excess of non-labeled affibody molecule significantly decreased binding.

The IC_{50} values of the anti-HER2 affibody molecule $Z_{HER2:342}$, the non-HER2 affibody molecules Z_{EGFR}, $Z_{insuline}$, Z_{taq} and albumin to OK cells were determined (see Fig. 4A and Table 3). The IC_{50}

value of $Z_{HER2:342}$ was in low nanomolar range (1.46 nM), and was five orders of magnitude lower than that of the other affibody molecules and albumin. Comparison of binding of ^{99m}Tc-Z_{2395}-C to kidney cells (OK) and cancer cells with high HER2 expression (SKOV-3) showed that inhibition concentration, IC_{50}, for binding of anti-HER2 affibody molecule $Z_{HER2:342}$ to OK cells was more than 30 times lower than for binding to HER2-expressing cells (1.5 nM for OK and 49 nM for SKOV-3 cells). Interestingly, the inhibition curve for OK cells showed a rather flat slope (slope = -0.7), which could be due to the presence of more than one binding site with different affinities for ^{99m}Tc –$Z_{HER2:2395}$-Cys on OK cells.

The expression of receptors on OK cells capable to bind to ^{99m}Tc –$Z_{HER2:2395}$-Cys was quantified in a saturation experiment (Fig. 5). In good agreement with inhibition data the resulting binding curve fitted to two sites (receptors) binding model with two different affinities for ^{99m}Tc –$Z_{HER2:2395}$-Cys, K_{D1} = 0.8 nM (B_{max1} = 71,500 receptors/cell) and K_{D2} = 9.2 nM (B_{max2} = 367,000 receptors/cell). Total amount of receptors capable to bind to the anti-HER2 affibody molecule on OK cells was 440,000 receptors/cell.

To study the role of the two different receptor types in anti-HER2 affibody binding and cellular processing, OK cells were incubated with two different concentrations ^{99m}Tc –$Z_{HER2:2395}$-Cys (Fig. 6). The binding and internalization patterns at two different concentrations demonstrated different cellular processing of ligand-receptor complexes. At concentration of 3 nM (same amount of ^{99m}Tc –$Z_{HER2:2395}$-Cys bound to each receptor type), the cellular binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys reached a maximum at 2 h. At later time points cell binding decreased and dropped to 50% of the maximum after 8 h. In contrast, when OK cells were incubated with 30 nM concentration of ^{99m}Tc –$Z_{HER2:2395}$-Cys (assuming ^{99m}Tc –$Z_{HER2:2395}$-Cys more abundantly bound to the receptor with lower affinity), the cell-associated and internalized radioactivity

increased continuously during the experiment (about 80% bound within first hour).

DISCUSSION

The mechanism of renal reabsorption and retention of radiolabelled peptides and small proteins has been studied by several groups during the last decade. Possible renal toxicity and tentative mechanisms of its prevention utilizing various blockers were the main driving forces for these studies. The majority of these studies was performed using in vivo models. In particular the molecular mechanism of kidney reabsorption of radiolabeled peptides and proteins mediated by megalin/cubilin co-receptors has attracted substantial attention during last years. Megalin and cubilin are synergistically acting endocytic receptors located mainly on the brush border of the apical parts of proximal tubules. [32] Megalin is a large transmembrane receptor; cubilin is a somewhat smaller receptor with no extracellular domain, which directs the trafficking of its ligands towards lysosomes through dimerization with megalin. Both receptors are important for the recovery of proteins such as albumin, vitamin binding proteins, hormones and apolipoproteins from the glomerular filtrate. Involvement of megalin in renal reabsorption of radiolabeled somatostatin analogues has been demonstrated. [8,26] Later, it has been confirmed that the same receptor pair is partly responsible for renal reuptake of other peptides and proteins such as exendin, minigastrin and neurotensin. [11] Interestingly, although the level of expression of megalin/cubilin co-receptors in mice does not differ between the sexes, the same radiolabeled peptide exhibits higher renal uptake in female than in male mice. [11] Co-infusion of substances that are ligands of megalin/cubilin, such as positively charged amino acids and albumin fragments, reduce the kidney uptake of radiolabeled small protein. [9,33,34] Recently, involvement of megalin in renal reabsorption of radiolabeled anti-EGFR nanobodies (15 kDa) has been demonstrated, [35] despite EGFR expression in proximal tubule. [36] In summary, the studies so far

suggest that the most important mechanism responsible for renal reabsorption of small radiolabeled proteins and peptides is megalin-mediated tubular reabsorption.

Affibody molecules appeared as promising imaging probes for in vivo detection of several tumor-associated molecular abnormalities. [16] Their prompt extravasation, high uptake by tumors, low uptake in normal tissues and rapid renal clearance provided high contrast images within a few hours after injection. [17] However, affibody molecules labeled with radiometals consistently show high radioactivity accumulation in the kidneys. Although the kidneys are a well-defined structure, high uptake in the kidney might obscure imaging of lesions in the kidneys or in their vicinity and it might cause high radiation doses during therapeutic application. It is known that HER2 is expressed only in developing proximal tubules. [36] Moreover, it was found that high renal reabsorption occurs for anti-HER2 affibody molecules independent of the labeling method used, but in the case of non-residualizing labels (radiohalogens and some technetium-99m and rhenium-188) renal retention is low. [25,37] At the same time tumor uptake and retention of radioactivity is stable for both residualizing radiometals and non-residualizing radiohalogens (see Fig. 7). Long retention of radioactivity in tumors correlates well with slow internalization rates of anti-HER2 affibody molecules in HER2-expressing tumor cells. [31,38] This mismatch indicates that different processes are involved in cellular processing of radiolabeled affibody molecules in tumor cells and in kidneys. Knowledge about different chelators and the properties they confer to the targeting agent and optimization of labeling chemistry enabled reduction of renal retention of radioactivity. [19,21,24,39,40] However, the molecular mechanism of reabsorption of affibody molecules presumably by tubular cells is not fully understood.

In the present study, ^{111}In-labeled anti-HER2 affibody molecules were injected in NMRI mice: the same level of the injected activity was retained in the kidneys in both male and female mice.

This observation was the first indication that megalin/cubilin system is not involved in kidney reabsorption of affibody molecules, considering the pronounced difference of megalin activity in male and female mice. Additional in vivo experiments did not show any difference in renal uptake of ^{111}In-labeleed $Z_{HER2:2395}$-Cys between megalin deficient and wild type mice. This observation indicated that a megalin-mediated mechanism for kidney retention could be excluded.

Further experiments were carried out in cultured immortal tubular opossum kidney OK cells, which retained the molecular properties of kidney tubular cells. The time course of ^{99m}Tc – $Z_{HER2:2395}$-Cys binding to OK cells showed that equilibrium was reached fast and binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys to OK cells was significantly reduced by pre-incubation with an excess of unlabeled conjugate, which was an indication that binding of the radiolabeled anti-HER2 affibody molecule to tubular cells is mediated by a saturable mechanism. Pre-incubation of OK cells in a serum-free medium before the incubation with radiolabeled proteins had been used to remove serum albumine from megalin/cubilin to examine megalin/cubilin driven uptake of albumine. [27] Because pre-fasting had no effect on binding of radiolabeled affibody ^{99m}Tc – $Z_{HER2:2395}$-Cys to OK cells, megalin/cubilin is not involved.

Incubation of cells with ^{99m}Tc –$Z_{HER2:2395}$-Cys at 37^{o}C provided a significantly higher cell uptake than incubation at 4^{o}C, suggesting internalization of radiolabeled tracer. To exclude the influence of internalization, the IC_{50} was determined at 4^{o}C. In the competition experiments, five orders of magnitude higher concentration of anti-EGFR, -insulin and –taq-polymerase affibody molecules was required to inhibit the binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys to OK-cells by 50%, compared to the unlabeled parent affibody molecule ($Z_{HER2:342}$). Affibody molecules consist of a scaffold part, which is common for all tested affibody molecules, and a target-binding area determined by 13

amino acids on surface of helices 1 and 2. The target-binding region is unique for $Z_{HER2:2395}$-Cys and $Z_{HER2:342}$. The results of the inhibition experiments clearly showed that amino acids in the target-binding parts of $Z_{HER2:2395}$-Cys and $Z_{HER2:342}$ were involved in the binding to OK cells. Furthermore, albumin was as inefficient in blocking of ^{99m}Tc –$Z_{HER2:2395}$-Cys to OK cells as non-HER2-binding affibody molecules. As albumin is a ligand of megalin, this is another strong indication that megalin/cubilin system is not involved in binding of anti-HER2 affibody molecule that correlates with in vivo findings.

To evaluate a possibility that opossum ErbB2 (homologue of human HER2) is directly involved in the binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys by OK cells, an inhibition profile of ^{99m}Tc –$Z_{HER2:2395}$-Cys was measured on HER2-expressing human ovarian carcinoma SKOV-3 cell line. The half inhibition concentration on OK cells was 30-fold lower than that on SKOV-3 cells, where uptake is HER-2 mediated. These data suggest that another receptor, different from the targeted antigen HER2, is responsible for this extensive uptake of ^{99m}Tc –$Z_{HER2:2395}$-Cys by tubular OK cells.

Further determination of the equilibrium dissociation constant K_D indicated the presence of two types of receptors on the OK cells that bind the anti-HER2 affibody molecule ^{99m}Tc –$Z_{HER2:2395}$-Cys: one with a high affinity of 0.8 nM and one with a lower affinity of about 9.2 nM. Cellular processing of ^{99m}Tc –$Z_{HER2:2395}$-Cys by OK cells was studied by continuous incubation at physiological conditions at two different concentrations of radiolabeled affibody molecule. The lower concentration (3 nM) was chosen at such level that a contribution of a receptor with K_{D1} would be substantial (about 50% of bound radioactivity). At this concentration, $4 \cdot K_{D1}$ or $0.3 \cdot K_{D2}$, approximately 80% of high affinity receptors and 25% of low affinity receptors will be occupied. At higher concentration (30 nM), the more abundant low affinity receptor should predominantly contribute to binding and cellular processing pattern of ^{99m}Tc –$Z_{HER2:2395}$-Cys. We found that

binding kinetics were very rapid for both receptors and internalization was slow. After 2 h incubation at the lower concentration, the cell-associated activity started to decline, and decreased to 60% of maximum value at 8 h of incubation. It should be noted that no depletion of radiolabeled affibody molecules occurred. Such a pattern points to internalization of the receptor-ligand complex without subsequent repopulation of the receptors. This pattern has not been observed for anti-HER2 affibody molecules bound to HER2 expressing cells. [21] In HER2 expressing tumor cells, monomeric affibody molecules do not trigger intracellular signaling, [41] which is reflected in slow internalization of a ligand-receptor complex. [21] The binding and processing pattern after binding to the low affinity receptor was different: after rapid binding during the first two hours, the cell-associated radioactivity increased slowly during the 8 h period. Taking into account that the high affinity receptor bound activity decreased, it can be assumed that the low affinity receptor is constantly repopulated. After 8 h internalized radioactivity was at a level that was 2-fold higher than that observed in HER2 expressing tumor cells. [21]

We started this project with the aim to identify molecular mechanism of renal reabsorption of anti-HER2 affibody molecule $Z_{HER2:2395}$-Cys in particular and affibody molecules as scaffold protein in general. Development of efficient predictive in vitro models can help to reduce a number laboratory animals used for development of new imaging agents. We hypothesized that the mechanism of renal reabsorption for affibody molecules was similar to that of other small proteins, e.g. megalin/cubilin mediated. Unexpectedly, we have found that this co-receptor system is not involved in renal reabsorption of anti-HER2 affibody molecules. Moreover, in our in vitro model anti-HER2 affibody molecule binds to two types (or sub-types) of receptors with

high affinity in low nanomolar range. The nature of these receptors on OK cells still needs to be identified.

In conclusion, we have shown that megalin/cubilin co-receptors do not play a significant role in renal uptake of radiolabeled anti-HER2 affibody molecules in mice. Experiments with opossum tubular OK cells indicated that binding of anti-HER2 affibody molecule ^{99m}Tc –$Z_{HER2:2395}$-Cys is mediated by scavenger receptors cross-reacting with the binding site of the anti-HER2 affibody molecules, but it is unlikely that HER2 is one of them. We are considering approaches to further identify these receptor types. The results of this study indicate that affibody molecules and other scaffold-based targeting proteins with relatively low kidney uptake can be selected using in vitro studies with tubular kidney cells.

ACKNOWLEDGEMENT

This work was supported by a grant from Swedish Research Council (Vetenskapsrådet). The authors express their gratitude to Affibody AB for providing affibody molecules for this study. Affibody AB holds intellectual property rights and trademarks for affibody molecules. A.Orlova is a member of Scientific Advisory Board of Affibody AB.

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Table 1. Biodistribution of ^{111}In in NMRI male vs. female mice at 4 h after injection of ^{111}In-DOTA- $Z_{HER2:2395}$-Cys. Mice were injected iv (tail vein) with 1 µg of protein per animal. Data are presented as average % ID per organ ± SD (n =4).

	male	female
Blood	0.12±0.03	0.12±0.03
Liver	1.28±0.07	1.0±0.2
Spleen	0.05±0.01	0.035±0.008
Kidney	79±2	70±10
GI tract	0.9±0.1*	1.4±0.1
Carcass	3.7±0.08	2.8±1.0

* significant difference ($p < 0.05$) in a un-paired t-test.

Table 2. Biodistribution of ^{111}In in megalin deficient mice vs. wild type mice 4 h pi of ^{111}In-DOTA- $Z_{HER2:2395}$-Cys. Mice were injected iv (tail vein) with 10 μg of protein per animal. Data are presented as average % ID per organ ± SD (n =5).

	megalin deficient mice	wild type mice
Blood	0.080±0.010	0.08±0.02
Muscle	0.09±0.02	0.08±0.02
Lung	0.32±0.05	0.35±0.10
Pancreas	0.15±0.02	0.12±0.03
Spleen	0.51±0.06	0.51±0.08
Kidney	202±16	214±26
Liver	1.6±0.2	1.5±0.3
Stomach	0.25±0.02	0.23±0.05
Duodenum	0.33±0.04	0.29±0.06

Table 3. Inhibition concentrations (IC_{50}) for different Affibody molecules and albumin for binding of binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys to OK cells.

Inhibitor	**IC_{50} concentration, M**
$Z_{HER:342}$	1.46×10^{-9}
$Z_{EGFR:2377}$	$1.5\text{-}2 \times 10^{-4}$
$Z_{insuline:810}$	$2.5\text{-}3 \times 10^{-4}$
$Z_{taq:1154}$	$3.5\text{-}4 \times 10^{-4}$
Albumine	5×10^{-4}

Figure 1. micro-SPECT images of megalin deficient mice (A) vs. wild type mice (B) 4 h pi of ^{111}In-DOTA-$Z_{HER2:2395}$-Cys.

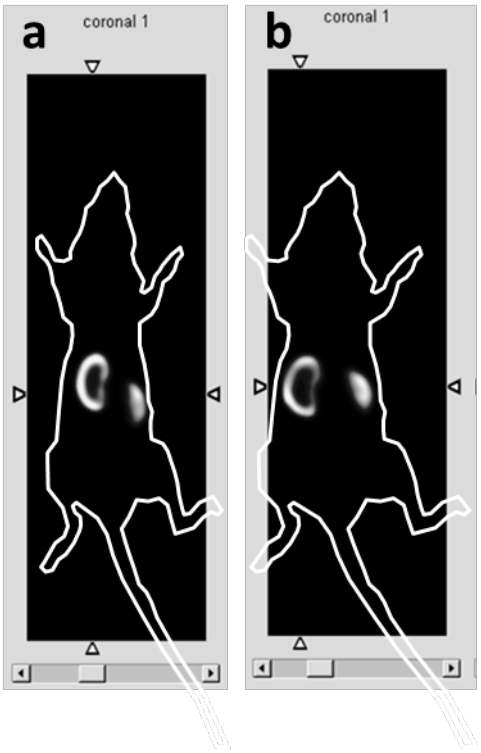

Figure 2. Binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys to OK cells at 4°C as a function of time. Data presented as an average of three samples with standard deviations, error bars might be not seen because they are smaller than point symbols.

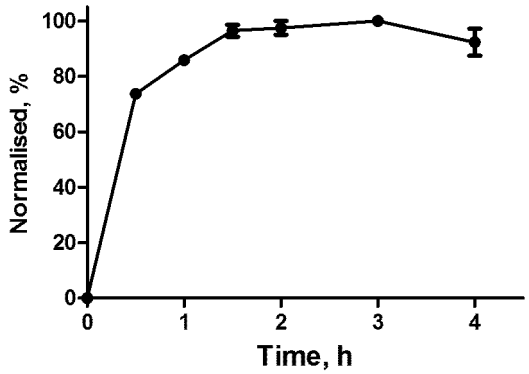

Figure 3. Binding of ^{99m}Tc –$Z_{HER2:2395}$-Cys (1nM) to OK cells in different conditions (block – pre-incubation for 15 min with 10 μM $Z_{HER2:342}$, pre-fasting – pre-incubation for 1 h with FSB-free media).

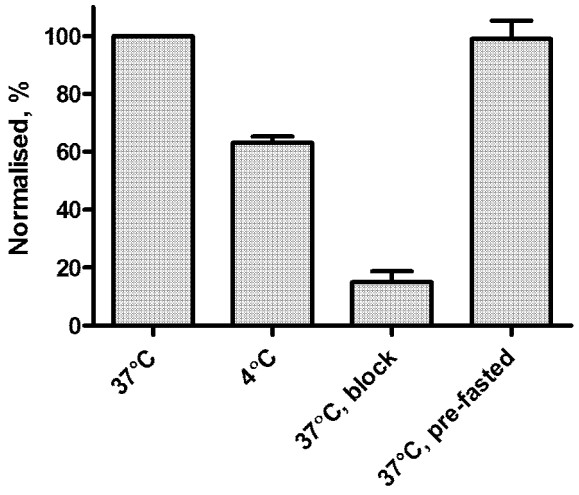

Figure 4. A. Inhibition of ^{99m}Tc-Z_{2395}-C binding to OK cells with different Affibody molecules. B. Inhibition of ^{99m}Tc –$Z_{HER2:2395}$-Cys binding to OK and SKOV-3 (ovarian cancer with high HER2-expression) cells. Data presented as an average of three samples with standard deviations, error bars might be not seen because they are smaller than point symbols.

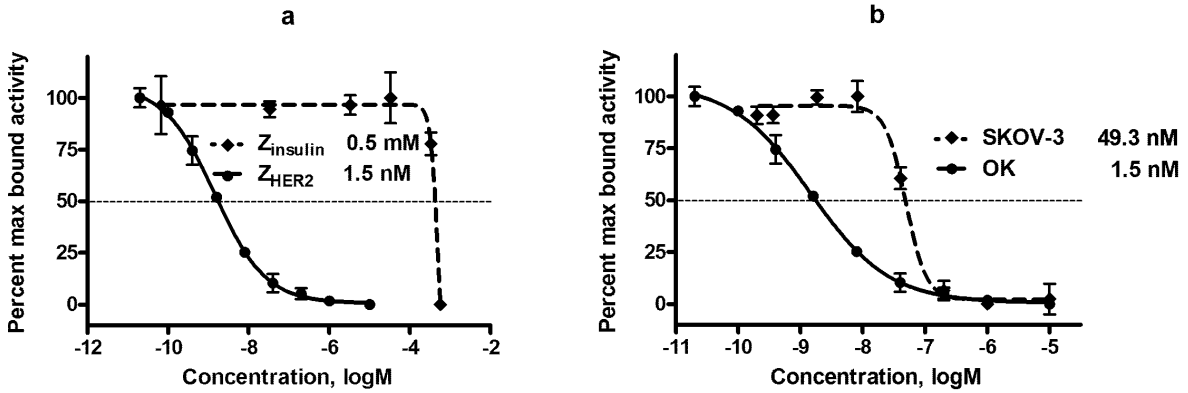

Figure 5. Non-linear regression analysis for two sites binding of data from binding saturation experiment. Increasing concentrations of ^{99m}Tc –$Z_{HER2:2395}$-Cys (x-axis) were added to dishes with OK cells.

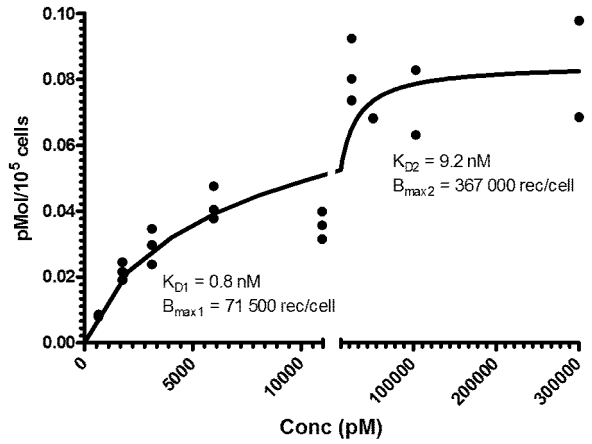

Figure 6. Binding and cellular processing of ^{99m}Tc –$Z_{HER2:2395}$-Cys by OK cells at continuous incubation with two different concentrations at 37ºC. Data presented as an average of three samples with standard deviations, error bars might be not seen because they are smaller than point symbols.

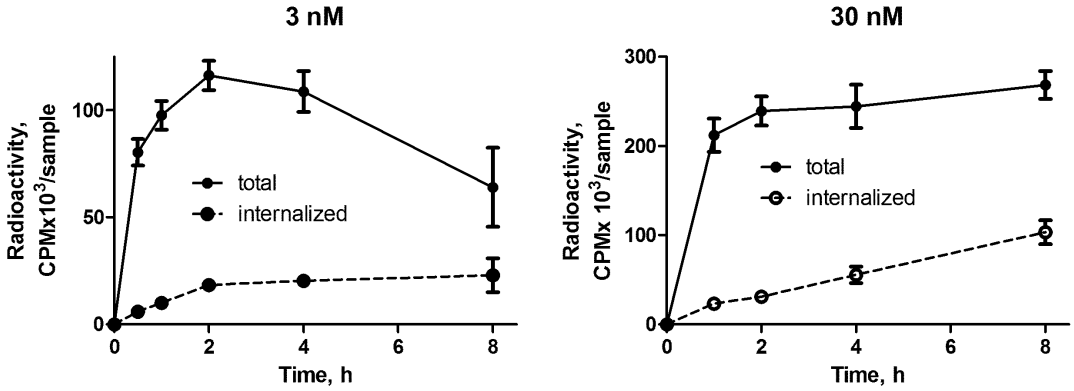

Figure 7. Biodistribution over time in SKOV-3 tumor bearing BALB/c nu/nu mice after administration of ^{111}In-DOTA-$Z_{HER2:342}$ [37] and ^{125}I-PIB- $Z_{HER2:342}$. [25]