Affibody Molecules for HER3-targeted Theranostics of Malignant Tumours

MARIA ROSESTEDT
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjölds väg 20, Uppsala, Friday, 9 November 2018 at 13:00 for the degree of Doctor of Philosophy (Faculty of Pharmacy). The examination will be conducted in English. Faculty examiner: Professor Sten Nilsson (Karolinska Institutet).

Abstract

The HER3 receptor plays a strong role in disease progression and resistance to therapies in several cancer types. Due to its endogenous expression and low overexpression in malignant tumours, it is a particularly challenging target. The primary aim of this thesis project was to develop, evaluate and characterize affibody molecules for theranostic applications in HER3-expressing malignant tumours.

Paper I investigated the in vivo targeting properties and therapeutic efficacy of a bivalent affibody construct fused with an albumin binding domain, Z\(_{\text{HER3}}\)-ABD-Z\(_{\text{HER3}}\). This construct could slow down the growth of HER3-expressing tumour xenografts without causing health problems or side effects in mice.

Paper II compared the in vitro and in vivo properties of two HER3-targeting affibody molecules (Z\(_{08698}\) and Z\(_{08699}\)) to select an imaging probe for HER3 diagnostics. While the two constructs had similar properties, Z\(_{08698}\) demonstrated better blood clearance and better radioactivity retention in tumours.

Paper III and IV present the development of a HER3 imaging probe for PET using gallium and cobalt isotopes. We demonstrated that imaging of HER3 expression could be obtained as soon as 3 h pi using gallium-68. Additionally, we demonstrated that affibody molecules labelled with a neutral cobalt-NOTA complex had a lower radioactivity uptake in the liver than molecules radiolabelled with a positive gallium-NOTA complex. Imaging contrast increased over time. As the dose of the injected protein increased, the activity uptake in normal organs decreased, whereas the tumour uptake remained the same, which improved the imaging contrast and allowed discrimination between xenografts with high and low HER3 expression. This modification did not influence tumour activity uptake.

Paper V presents the HER3-targeting affibody molecule trimer as a tool to block hepatic uptake in order to increase the imaging contrast in the liver. The trimer demonstrated its ability to bind to endogenous receptors in the liver, which decreased the hepatic uptake of the radiolabelled monomer. This phenomenon enabled the monomer to pass the liver barrier, which increased tumour radioactivity uptake and improved imaging contrast.

Keywords: affibody molecules, theranostics, HER3, molecular imaging

Maria Rosestedt, Department of Medicinal Chemistry, Theranostics, Dag Hammarskjöldsv 14C, Uppsala University, SE-751 83 Uppsala, Sweden.

© Maria Rosestedt 2018

ISSN 1651-6192

urn:nbn:se:uu:diva-360973 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-360973)
Nog finns det mål och mening i vår färd – men det är vägen, som är mödan värd.

*Karin Boye*

*To my family –*

Мама, Папа, Бася, Вова.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


*Equal contribution.*

Reprints were made with permission from the respective publishers.
Published papers not included in this thesis:


*Equal contribution.*
Contents

Introduction ............................................................................................................. 11
Cancer ..................................................................................................................... 11
Cancer diagnostics ............................................................................................... 13
  Cancer staging .................................................................................................... 13
  Tissue sampling .................................................................................................. 13
  Molecular profiling ............................................................................................. 14
Treatment ............................................................................................................... 15
  Personalised therapy .......................................................................................... 15
  Targets for therapy ............................................................................................. 16
  Treatment response monitoring ......................................................................... 16
  Theraonics .......................................................................................................... 17
Human epidermal growth factor receptor family (HER) ................................... 18
  HER1 .................................................................................................................. 18
  HER2 .................................................................................................................. 18
  HER3 .................................................................................................................. 19
  HER4 .................................................................................................................. 20
HER3-targeted therapy .......................................................................................... 21
Radionuclide imaging ........................................................................................... 22
  Single-photon emission computed tomography (SPECT) .................................. 22
  Positron emission tomography (PET) ................................................................. 22
  Imaging agents ................................................................................................... 23
  Size and affinity in tumour targeting ................................................................... 24
  Sensitivity and specificity of imaging ................................................................. 25
Affibody molecules ............................................................................................... 26
  HER3-targeting Affibody molecules .................................................................. 26

Aim of thesis .......................................................................................................... 27

Methodology ......................................................................................................... 28

Results of present investigations ........................................................................ 31
  Paper I ............................................................................................................... 31
  Paper II .............................................................................................................. 35
  Paper III ............................................................................................................. 38
  Paper IV ............................................................................................................. 42
  Paper V .............................................................................................................. 46
Concluding remarks .................................................................51
Future studies ...........................................................................53
Acknowledgements ....................................................................55
References ..................................................................................58
Introduction

Cancer
Sustaining proliferative signalling, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumour-promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death and deregulating cellular energetics. These hallmarks describe the differences between normal cells and cells with uncontrolled growth, thereby forming the malignant tumours that cause cancer (1,2). However, what is cancer? Briefly, it could be described as an accumulation of genetic alterations in a specific cell or group of cells for which the regulatory functions of the human body have lost control. Furthermore, cancer will cause the formation of a primary, sometimes malignant tumour, which could spread to other sites in the body (Figure 1).

Even though many cancers have common features, it is important to remember that it is not possible to describe cancer as a single disease, as it constitutes a group of several hundred malignancies (2). The malignant transformation includes changes in the gene coding system, which creates different types of cells, each having their own phenotype. Thus, they express different kinds of molecular entities that could be targeted for therapies for specific tumour types. The challenges with these molecular targets are that they might exhibit variations 1) in their phenotypic expression within the same tumour (intratumoural heterogeneity), 2) between the primary tumour and the metastases (intertumoural heterogeneity) and 3) between different metastases. Additionally, as outlined above, there may be differences in the phenotypes of identical cancer types between patients (3).

Because of the high diversity among different tumours, theoretically, the most effective treatment should ideally be tailored to the cancer subtype in each individual patient, creating a personalised medicine approach.
Figure 1. Basic illustration describing the formation of primary and secondary tumours.
Cancer diagnostics
The chances of patient recovery increase dramatically if the cancer is detected, diagnosed and treated as early as possible. However, most patients receiving a cancer diagnosis already have a macroscopic tumour. Furthermore, to select appropriate treatment, the tumour should be classified based on its origin, malignancy, and invasiveness. This classification can be made using different techniques, such as palpation, blood tests, and anatomical imaging.

Cancer staging
Cancer staging is the process by which the growth and spread of the disease are assessed and serves as an important guide for prognosis and future therapy. Currently, the tumour staging system TNM is the most widely used system describing the presence and size of the tumour (T), local and/or distant lymph nodes affected (N), and presence of distant metastases (M). There is also a less detailed description of the cancer divided into five stages: stage 0 indicates the presence of abnormal cells that are not yet cancer, stages 1-3 indicate the presence of cancer, and higher numbers indicate larger tumours and spread to surrounding tissues, and stage 4 indicates metastases to distant parts of the body (4).

While staging is essential for the prognostication and treatment of cancer, the system is not 100% accurate due to the variable nature of the disease. A tumour that is 1 cm in diameter might be very difficult to detect by palpation, and it is impossible to predict the behaviour of a particular cancer based on only its gross anatomy. Additionally, no information about cancer-related molecular expression changes is provided using the staging system.

Tissue sampling
Generally, when a cancer is suspected, tissue sampling is performed. This sample is often referred to as a biopsy sample. Taking a biopsy sample permits detection of cancer-associated changes in cell morphology. Tissue sampling, which can enable the detection of mutations in multiple genes simultaneously, serves as an invasive method that can potentially cause severe tissue damage and is not easily repeatable. Furthermore, there are risks of collecting non-representative samples, such as missing the tumour tissue during the biopsy procedure, as well as due to heterogeneities within the tumour tissue. Additionally, a clonal selection of malignant cells during the disease and as a response to applied treatment might occur.

When the biopsy sample is taken, a molecular analysis is performed on the tissue material using techniques such as histochemistry, immunohistochemistry (IHC) and molecular pathology (e.g., fluorescent in situ hybridization (FISH)).
Biopsy sampling, although it is an important standard technique, can provide only limited information. For example, it does not provide information on tumours forming distant metastatic lesions. Therefore, diagnostic tools enabling visualization of the tumour together with the possible metastatic sites within the body would provide a valuable complement and a more accurate cancer diagnosis.

**Molecular profiling**

Historically, IHC has been the most commonly used technique for molecular profiling of tumours, mainly due to its relatively fast, standardized and uncomplicated performance as well as its low cost and wide availability. The method is used to determine and evaluate the protein expression levels in a tissue sample, and it provides information about the tumour origin and type, tumour classification and therapy prediction. However, the method is observer-dependent, requires analysis by a trained pathologist and is inherently qualitative.

FISH is a different method for tumour profiling and molecular pathology. It provides with the genetic information (such as deletions or translocations) present in the tumour sample. FISH is a more sensitive technique, which is associated with high costs and requires extended laboratory procedures and higher qualifications.

Nevertheless, due to the dynamic changes in the molecular characteristics of the tumour over time, the molecular profile determined at one time point might not reflect the actual tumour status at later time points.
Treatment

The ideal goal of cancer therapy is to completely eradicate the tumour without affecting or damaging healthy tissue in the rest of the body. Depending on the type and location of the tumour and possibly of its metastases, treatments such as surgery, chemotherapy, radiation therapy and immunotherapy can be applied as a monotherapy or in combination. Surgical procedures are sometimes not practically possible due to the location of the tumour or to the possibly of severe damage to vital organs that might occur as a side effect. Additionally, an issue with both chemotherapy and radiation therapy is that neither targets cancer cells exclusively. Thus, they will, in turn, affect all growing cells and damage the surrounding healthy tissue, which gives rise to unwanted side effects.

A possible route towards increasing the effectiveness of the currently available therapies is to detect and characterize the cancer on a molecular level, which would identify a specific target expressed on the surface of the cancer cell. This target could serve as a bioindicator of ongoing processes (normal and pathogenic) in the body and reflect responses to therapeutic interventions. The expression levels of a protein or receptors can serve as predictors and could also be linked to patient survival and treatment outcomes (5).

Personalised therapy

As mentioned previously, cancer is often comprised of several different diseases, which means that the cancer must be treated based on its individual characteristics. Identifying targets representative of the specific disease in every patient could make determining the specific molecular pathways that are targetable by therapeutic molecules possible. In this way, patients could be stratified based on the protein expression profiles of their cancer, side effects associated with overtreatment could be avoided and the therapy could be tailored. This therapy strategy should be applied together with strong diagnostic tools to select patients who would benefit from and respond to a specific therapy (Figure 2).

As of today, the concept of personalised therapy is still in early development. It is not yet an established treatment approach for any indication, mostly due to the relatively high costs, lack of experience and companion diagnostics (6). However, the high cost of diagnostic procedures associated with personalised medicine (e.g., imaging) are expected to be off-set by the overall decreased healthcare cost due to early identification of the correct treatment regimen.
Figure 2. The strategy for conventional and personalised therapy for patients with the same cancer diagnosis. By using molecular diagnostics, patient are matched with a suitable treatment, which will hopefully lead to the best therapeutic outcome.

**Targets for therapy**

The standard approach to eradicate rapidly dividing cancer cells is the use of chemotherapy. Unfortunately, chemotherapy drugs affect all cells in the body that divide rapidly (cells in bone marrow, GI-tract, skin, hair, etc.), which impairs normal function of the body and causes serious side effects. Targeted therapy selects one or several phenotypical features (molecular targets) of the cancer cell that make it distinct from normal cells. The Food and Drug Administration (FDA) has approved several targeted cancer therapies, including *hormone therapies* (affects the growth of hormone-dependent tumours, preventing the production/action of the hormones), *signal transduction inhibitors* (blocks the series of biochemical responses required to generate growth signals), *gene expression modulators* (affects the mutated gene involved in cancer growth), *apoptosis inducers*, *angiogenesis inhibitors* and *immunotherapies* (programming the immune system to kill cancer cells). *Cancer vaccines*, such as the preventive human papillomavirus (HPV) vaccine (7), can be considered a targeted therapy due to their interference with the growth of the potential cancer.

**Treatment response monitoring**

It is important to evaluate the response to a certain treatment, and biopsy sampling is not an optimal method due to the invasiveness of the procedure and tumour heterogeneity. Instead, a method relying on the measurement of tumour progression in response to treatment is used. Accompanied by ana-
tomical imaging (CT or MRI), response evaluation criteria in solid tumours (RECIST) are used to determine the growth of a solid tumour (8). By using a CT image, the tumour lesions are measured, and the longest diameter of a targeted lesion is defined. Furthermore, to evaluate the treatment outcome, the longest diameters of all targeted lesions are summed, and a decrease in the sum by at least 30% would indicate partial response to therapy (9).

However, clinical response can be underestimated by evaluation of tumour size measurements. Morphological changes (e.g., necrosis) affecting the treatment outcome and patient survival can occur in response to therapy, and they are not distinguished from active cancer tissue by CT.

A useful addition to the currently available methods for treatment response monitoring would be an alternative enabling visualization of biochemical changes in all lesions at a functional rather than anatomical level. For example, fluorine-18 (^{18}F)-labelled fluorodeoxyglucose (^{18}F-FDG) is used to evaluate the metabolic changes that occur in cancer. However, ^{18}F-FDG is a general marker of glucose metabolism, and strong uptake may indicate many different processes in the tumour microenvironment apart from an actual increase in tumour glycolysis. These processes include infiltration of immune cells, which is actually a marker of tumour response to immuno-oncological treatments rather than progression. Thus, quantitative imaging of overexpression of specific receptors in cancer would be highly desirable and could contribute to an improved personalisation of treatment.

**Theranostics**

Theranostics is a nascent field of medicine that combines targeted therapy and diagnostic imaging. The key focus is to move away from the one-fits-all therapy approach and identify the most suitable and effective therapy for each patient. Diagnostic and therapeutic applications are united in a manner combining diagnosis, drug delivery and treatment response monitoring (10).
Human epidermal growth factor receptor family (HER)

This family of receptors is aberrantly expressed in a variety of cancer types. It consists of HER1/EGFR, HER2, HER3 and HER4, all transmembrane receptor tyrosine kinases (Figure 3). The major signalling routes for the HER family are the Ras-Raf-MAPK and PI-3K/Akt pathways, both of which are strongly connected to apoptosis reduction and cell proliferation promotion. Because of the implications of the HER family of receptors in the development and progression of cancer, it is considered a group of potential biomarkers for diagnosis, therapy and therapeutic monitoring (11).

**HER1**

The overexpression of HER1/EGFR is present in many malignancies, such as colorectal cancer, neck squamous cell carcinoma (HNSCC) and glioma (12). HER1/EGFR is activated by specific ligands, e.g., EGF and transforming growth factor α (TGFα). Its intrinsic intracellular protein-tyrosine kinase activity will be stimulated, triggering downstream signalling pathways (MAPK, Akt) and leading to increased DNA synthesis, cell survival and proliferation (13). Due to its involvement in cancer, HER1/EGFR has been identified as an oncogene, leading to the aim of developing therapeutic approaches, such as monoclonal antibodies (e.g., cetuximab, which blocks the extracellular ligand binding domain) and small-molecule kinase inhibitors (e.g., gefitinib, which inhibits the HER1 tyrosine kinase and prevents self-activation).

**HER2**

HER2 is expressed at a very low level in adult normal tissue, and it lacks a natural ligand for activation. However, it exists in a constantly active conformation, which allows for dimerization with other activated receptors, preferably other HER family members (11,14). When activated, HER2 is
one of the main activators of the MAPK and Akt cascades, which are important for cancer development, making it a proto-oncogene. As overexpression of HER2 is specific to tumours, targeting agents (e.g., antibodies, such as trastuzumab) and tyrosine kinase inhibitors have been developed for therapeutic use. Since only tumours specifically expressing HER2 can respond to HER2-targeted therapies, it is important to estimate the level of HER2 overexpression in each patient being considered for this expensive treatment, in line with the emerging personalised medicine paradigm described herein.

**HER3**

HER3 has an inactive intracellular tyrosine kinase domain that requires heterodimerization with HER1/EGFR, HER2 or HER4 for signalling. The preferable heterodimerizing partners for HER3 are HER1/EGFR and HER2, and the HER2/HER3 dimer is considered the most potent oncogenic unit (15). HER3 has two known extracellular ligands, heregulin (HRG) 1 and 2, and neu differentiation factor (16).

In the last decade, HER3 has received increasing attention in the context of cancer. Today, it is considered one of the main players in breast, prostate, melanoma, colon and lung cancer as well as in many cancers overexpressing HER2 or HER1 (17,18). Importantly, HER3 may become upregulated and cause therapy resistance. This resistance could happen with HER2 inhibition as a response to targeted therapy involving trastuzumab in HER2-overexpressing breast cancer (17,19). Additionally, there is evidence that the HER1/HER3 and HER2/HER3 dimers promote the invasiveness of prostate cancer by affecting its progression towards androgen independence (20,21).

HER3 has strong potential as a therapeutic molecular target. This targeting could be used in anticancer therapy, and several monoclonal antibodies targeting HER3 are currently being evaluated in clinical trials (22,23). To successfully fulfil the aim of anti-HER3 therapy, patients with HER3-overexpressing tumours have to be stratified from patients without HER3 overexpression (potential non-responders). As mentioned above, HER3 expression can change in response to therapy, and a biopsy sample taken from the primary tumour would therefore not be conclusive. It would require repeated samplings, which is not desirable and is questionable in clinics.

Molecular imaging potentially enables stratification of patients with HER3-overexpressing tumours, and this methodology will be described in detail in the upcoming sections of this thesis. The challenges of this approach to targeting HER3 in cancer lesions is the low receptor expression, usually below 50 000 receptors/cell. Additionally, only a subset (17-60%) of cells in individual tumours, depending on the cancer origin, actually overexpress HER3 (24,25).
**HER4**

HER4 is less explored than the other HER family members, and the mechanism of action and detailed signalling of the receptor are still poorly understood. Structurally, HER4 is similar to HER1 and exists in homo- and heterodimeric forms, which can be activated by a variety of ligands, leading to increased mitogenesis, differentiation and cell survival (26).
HER3-targeted therapy

The function and role of HER3 in a variety of cancers as well as its crucial involvement in drug resistance has produced an increasing need for HER3-targeting therapeutic agents. HER3 protein expression is present in many normal organs in the body. Hence, the development of suitable therapeutics is critical to avoid on/off-target toxicity in normal tissues. However, there are no approved protein-based drugs that target HER3.

The HER3-targeting therapeutic proteins are designed to interfere with HER3 cellular mechanisms to inhibit tumour cell growth. Therapeutic targeting of HER3 can include the following (18):

- blockage of ligand-induced HER3 activation by outcompeting HRG 1/2 binding,
- inhibition of receptor dimerization by trapping HER3 in its closed conformation,
- inhibition of dimerization by allowing the binding of several receptors at the dimerization region, and
- induction of antibody-dependent cytotoxicity (ADCC) by recruitment of an effector cell.

Currently, several HER3-targeting monoclonal antibodies are under clinical investigation. The monoclonal antibody patritumab (U3 Pharma, Amgen, Daiichi Sankyo) (23) has reached phase 3 clinical studies and is being investigated for its therapeutic and imaging applications in non-small cell lung cancer and head and neck cancer. Serinbantumab (Merrimack, Sanofi-Aventis) is being investigated in clinical phase 2 trials in patients with breast, lung, and ovarian cancer (27). Lumretuzumab (RG7116, Roche) has shown an ability to prevent HRG binding to HER3, which prevents heterodimerization and phosphorylation, and has been shown to be well-tolerated in phase 1 clinical trials (28). Elgemtumab (LJM716, Novartis) is another monoclonal antibody currently in clinical testing, and it binds to HER3 in a way such that one binding arm remains inaccessible (29). This process traps HER3 in its inactive conformation, preventing it from heterodimerization with HER2 or EGFR/HER1 (30).
Radionuclide imaging

For the application of personalised therapy approaches for patients diagnosed with cancer, identifying, localizing and classifying the tumour as well as the molecular target of interest must be possible. As the goal of personalised therapy is to obtain knowledge about the ongoing molecular processes of a certain disease, imaging of specific molecules in their native state is performed in vivo.

As mentioned previously, biopsies are usually performed to determine the phenotype of a tumour. One problem with this approach is that the tumour phenotype might change during the progression of the disease. These events would make it unrealistic to stay updated about the actual cancer phenotype and require repeated biopsy sampling from tumour and metastatic sites, which is invasive, impractical and sometimes impossible.

Radionuclide molecular imaging is a noninvasive, phenotypic and repeatable method that potentially enables monitoring of cellular functions and molecular processes in a living object, mostly in cancer, but has also been widely implemented in the diagnosis of cardiovascular and neurological diseases.

Single-photon emission computed tomography (SPECT)

SPECT is the most commonly used imaging modality in clinics today. Single or multiple scintillation cameras are rotating around the patient recording the emitted photons from a radionuclide accumulated in different locations within the body. Depending on the radionuclide, photons with different energies are emitted and further registered as the radionuclide decays. Images are acquired by rotating detectors that record a number of projections obtained at defined time points during the rotation, and by applying specific reconstruction programmes, a 3D distribution of activity can be obtained. In combination with computed tomography (CT) or magnetic resonance imaging (MRI), SPECT allows for localization of activity accumulation with regard to other anatomical structures, such as bone.

Radionuclides being used in SPECT are most commonly long-lived (hours to days) (Table 1), which simplifies the production process, contributing to lower costs and making this technique more available to clinics.

However, the accuracy of detecting the radioactivity concentration using SPECT is reduced by its lowered sensitivity and poor spatial and temporal resolution. Additionally, SPECT is not normally quantitative unless a cross-calibrated reference is included in the examination.

Positron emission tomography (PET)

PET uses positron-emitting radionuclides with half-lives varying from a few minutes to several days (Table 1). As the radionuclide decays, positrons
annihilate with electrons in the medium and emit photons travelling in opposite directions with energy of 511 keV. The PET scanner is constructed as a ring of detectors (creating opposite detector pairs) that encircle the patient. The technique relays the coincidence detection of photons moving 180 degrees apart, and photons not arriving in pairs are ignored by the scanning device. This process results in lower background noise, increased spatial resolution and a more accurate quantification of activity concentrations.

Because of the short half-life of many positron-emitting radioisotopes, a cyclotron and a shielded radiopharmaceutical laboratory must be located in close proximity to the PET imaging facility, which is associated with high costs. However, the most commonly used PET radiopharmaceutical, $^{18}$F-FDG, can be transported to a remote site lacking a cyclotron.

Table 1. Overview and properties of selected radionuclides used in PET/SPECT-based radionuclide molecular imaging (31).

<table>
<thead>
<tr>
<th>Nuclide</th>
<th>Production</th>
<th>Imaging modality</th>
<th>Emission</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{82}$Rb</td>
<td>Generator</td>
<td>PET</td>
<td>$\beta^+$ (95%)</td>
<td>1.27 min</td>
</tr>
<tr>
<td>$^{11}$C</td>
<td>Cyclotron</td>
<td>PET</td>
<td>$\beta^+$ (98%)</td>
<td>20 min</td>
</tr>
<tr>
<td>$^{68}$Ga</td>
<td>Generator</td>
<td>PET</td>
<td>$\beta^+$ (89%)</td>
<td>68 min</td>
</tr>
<tr>
<td>$^{18}$F</td>
<td>Cyclotron</td>
<td>PET</td>
<td>$\beta^+$ (97%)</td>
<td>110 min</td>
</tr>
<tr>
<td>$^{64}$Cu</td>
<td>Cyclotron</td>
<td>PET</td>
<td>$\beta^+$ (18%)</td>
<td>12.7 h</td>
</tr>
<tr>
<td>$^{86}$Y</td>
<td>Cyclotron</td>
<td>PET</td>
<td>$\beta^+$ (33%)</td>
<td>14.7 h</td>
</tr>
<tr>
<td>$^{55}$Co</td>
<td>Cyclotron</td>
<td>PET</td>
<td>$\beta^+$ (76%)</td>
<td>17.6 h</td>
</tr>
<tr>
<td>$^{89}$Zr</td>
<td>Cyclotron</td>
<td>PET</td>
<td>$\beta^+$ (23%)</td>
<td>78.4 h</td>
</tr>
<tr>
<td>$^{124}$I</td>
<td>Cyclotron</td>
<td>PET</td>
<td>$\beta^+$ (23%)</td>
<td>4.2 d</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>Generator</td>
<td>SPECT</td>
<td>$\gamma$</td>
<td>6 h</td>
</tr>
<tr>
<td>$^{123}$I</td>
<td>Cyclotron</td>
<td>SPECT</td>
<td>$\gamma$</td>
<td>13.2 h</td>
</tr>
<tr>
<td>$^{111}$In</td>
<td>Cyclotron</td>
<td>SPECT</td>
<td>$\gamma$</td>
<td>2.8 d</td>
</tr>
</tbody>
</table>

**Imaging agents**

To enable molecular imaging, targeting molecules, also known as imaging agents, tracers or radiopharmaceuticals, must be used. The tracers are designed to bind specifically to disease-associated targets, such as receptors. The ideal situation for achieving successful tumour imaging is the exclusive expression of targets within the tumour and their absence in normal tissues. The tracers are labelled with a suitable radionuclide with a half-life corresponding to the circulatory half-life of the tracer (32).

The specific binding of the radiolabelled targeting molecule makes it possible to obtain whole-body information about target expression by measurements of radioactivity accumulation in specific areas using different imaging technologies.

Examples of imaging agents include the following:
• **Antibodies** (~ 150 kDa) have exceptional targeting ability and have long been considered very potent imaging agents when bearing a radiolabel. The limiting factor of antibodies is their large size, which causes non-specific accumulation in target-negative tumours due to an enhanced permeability and retention effect (EPR), and clearance from healthy tissues is slow. The antibody fragments (Fab) are able to overcome these size-related issues, but they are often unstable and have low affinities. Additionally, their slow biological half-life requires radionuclides with radioactive half-lives of days, which entails considerable absorbed radioactive doses. Taken together, these factors complicate the ability to obtain high-contrast images (33).

• **Natural peptide ligands** have the following valuable properties that could be used in the development of an imaging agent: low immunogenicity, low nanomolar affinities and small size (34). The targeting possibilities using natural peptide ligands are complicated (strong agonistic action, enzymatic degradation in circulation) and sometimes impossible if a target is missing or has no known ligand, such as HER2.

• **Alternative affinity scaffold proteins** are binders produced from a robust structure of amino acids. They are structurally able to withstand surface alterations in order to produce a large library of binders (35). These scaffold proteins could be referred to as antibody alternatives, and affibody molecules and DARPinS are successful examples in the field of imaging. This thesis will further focus on the role of affibody molecules in therapy and theranostics.

**Size and affinity in tumour targeting**

Depending on the intended use of the tracer, whether for imaging or therapy, several aspects must be considered. For imaging, the size and affinity of the tracer are the most important factors influencing its specificity and sensitivity (24,36). By using small tracers, rapid extravasation and fast clearance from the blood is possible. To enable sufficient accumulation in the tumour, it is of crucial importance that the tracer has a high affinity for its target, which, in turn, will provide a good imaging signal (37). However, due to its size, the small tracer will circulate in the blood for a shorter period, which is not optimal for therapy.

The EPR effect is another reason why smaller molecules should preferentially have a high affinity for their target (38). Initially, the effect describes the process in which macromolecules (larger than 40-50 kDa) accumulate in the tumour irrespective of their target affinity. This process is due to the nature of the tumoural vessels that are generally more permeable than normal vessels together with reduced lymphatic drainage. Molecules that are smaller than 40-50 kDa penetrate the tumour more efficiently than large molecules. However, because of the abovementioned reasons of the high
permeability of tumoural blood vessels, the unbound small molecules are released back into the circulation and further cleared by the kidneys (33,38). This process is why only the bound tracer would remain in the targeted tissue.

**Sensitivity and specificity of imaging**

The accuracy of tumour detection using imaging techniques relies on the specificity and sensitivity of the tracer to its target. The off-target interactions of the tracer, i.e., binding in nontargeted tissues, regulate the specificity. The sensitivity is dependent on the imaging contrast, which, in turn, relies on a high tumour-to-blood ratio, tumour-to-surrounding tissue ratio and the type of imaging modality being used (39,40).
Affibody molecules

Affibody molecules are small and robust high-affinity scaffold proteins that have been demonstrated to be good candidates for molecular imaging.

The affibody molecule is derived from the B-domain of the immunoglobulin binding region of staphylococcal protein A. The protein consists of a three-helical structure containing 58 amino acids (6-7 kDa), and randomization of the positions of the amino acids on helices 1 and 2 can generate multiple variants to select high-affinity binders (41, 42). The excellent ability of the affibody molecule to refold along with a very high pH tolerance allows for chelation to radiometals under harsh conditions, which are not well tolerated by many other protein-based probes.

Twenty years have passed since the first publication of the affibody molecule (43), and affibody molecules against more than 40 different targets have since been reported (44). The molecules included cancer-related EGFR, (45), HER2 (40), HER3 (46), PDGFRβ (47) and IGF-1R (48).

The potential of the affibody molecule to provide high-contrast images shortly after administration has been demonstrated in both preclinical and clinical studies (39, 49–51).

HER3-targeting Affibody molecules

This thesis project involves affibody molecules that were selected based on their high affinity towards the HER3 receptor. Three different forms of affibody-based agents were used for different applications. The monomers Z08698 and Z08699 (~8 kDa) have picomolar affinities (50 and 21 pM, respectively), and their amino acid sequences differ at only two positions.

Due to the small size of the monomeric form of the affibody molecule, it is rapidly cleared from blood circulation. A therapeutic agent benefits from having an extended half-life, and one of the main aims of this thesis project was to facilitate the therapeutic application of HER3-targeting affibody molecules. Therefore, two monomeric affibody molecules, Z08699, were linked by an albumin binding domain (ABD) to prolong the biological half-life (52). This dimeric form is referred to as 3A3 (Z08699-ABD-Z08699, 22.5 kDa).

The anti-HER3 affibody trimer consists of three linked monomeric variants ((Z08699)3, 24.3 kDa). The affinity of the trimer is high, but its size limits the tissue penetration. This trimer, in turn, saturates endogenous HER3 expression and should hypothetically enable undisturbed transport of targeting smaller monomeric molecule to the tumour.
Aim of thesis

The primary aim of this thesis project was to develop, evaluate and characterize affibody molecules for theranostic applications in HER3-expressing malignant tumours. To accomplish this aim, the following steps were performed.

- Investigations of the potential of the new affibody-based bivalent construct ZHER3-ABD-ZHER3 (3A3) as a HER3-targeting therapeutic agent (paper I)
- Selection of the superior HER3-targeting affibody molecule for PET/SPECT imaging by a comparison of two anti-HER3 affibody variants (paper II)
- Optimization of the radiolabel ($^{68}$Ga and $^{57}$Co) for HER3-targeting affibody molecule towards improved biodistribution and imaging properties (papers III and IV)
- Optimization of administration of HER3-targeting affibody molecule to improve imaging properties (paper V)
Methodology

Cells
This thesis included a panel of cell lines with various HER3 expression levels (Table 2). The different cell lines were characterized on the level of HER3 expression (52,63) and used to evaluate the receptor-mediated binding of the HER3-targeting affibody molecules (HER3, 3A3, (HER3)3). Additionally, they were implanted into mice for in vivo studies.

Table 2. Properties of the cell line panel used in this thesis (53).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HER3 expression</th>
<th>Cancer origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT474</td>
<td>25×10³</td>
<td>Breast</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>12×10³</td>
<td>Pancreas</td>
</tr>
<tr>
<td>LS174T</td>
<td>8×10³</td>
<td>Colon</td>
</tr>
<tr>
<td>DU-145</td>
<td>7×10³</td>
<td>Prostate</td>
</tr>
<tr>
<td>A431</td>
<td>4×10³</td>
<td>Squamous cells</td>
</tr>
</tbody>
</table>

Affibody production
The affibody molecule selection, maturation, derivatization and characterization (identity, purity, Tm, refolding capacity and affinity) were performed by our expert close collaborators at the Department of Biotechnology at the Royal Institute of Technology in Stockholm. This process is described in details in the PhD theses of Malm in 2013, Bass and Andersson in 2017 (54–56).

Radiolabelling
The conjugates used in this thesis were labelled with different radionuclides – indium-111, gallium-68 and cobalt-57. The labelling conditions, including the incubation temperature, incubation time, and buffer pH, were optimised to find a stable radionuclide attachment to the anti-HER3 affibody molecules.

Stability and identity test
The labelled conjugates were analysed by using radio-ITLC (instant thin layer chromatography) eluted with citric acid where the free radionuclide would migrate with the solvent front and the radiolabelled protein would
remain at the point of application. The ITLC analysis was also cross-validated by SDS-PAGE (gel electrophoresis) analysis to obtain more detailed information about the identity of the conjugate. Additionally, before injecting the radiolabelled conjugate in vivo, its stability was tested and analysed by in vitro incubation in mouse serum and/or in a large molar excess of EDTA dissolved in PBS.

**Binding specificity assay**

Modifications induced by labelling (both attachment of a chelator for radio-labelling and the reaction conditions, e.g., temperature or pH) could influence the targeting properties of the tracer. Therefore, the specificity of the radiolabelled affibody molecule to its intended target molecule must be validated. This validation is a qualitative assay in which only the targeting ability of the labelled conjugate is analysed. This is done by comparing the radioactivity associated with cells in pre-saturated and non-saturated samples.

**Cellular processing including internalization assay**

This type of in vitro assay is commonly the beginning of the identification of lead compounds for further pre-clinical investigations. When identifying new targeting molecules, it is particularly important to evaluate their targeting potential before proceeding to in vivo studies. The assay measures the receptor-mediated radioactivity uptake in cells and can provide insight into the cellular uptake and retention of a radiopharmaceutical, which is especially important for diagnostic and therapeutic applications. The different patterns of the target molecule internalisation in cells can depend on several aspects, among which are the characteristics of the radiolabel (e.g., residualizing/non-residualizing properties, radiometal/halogen). If a high percentage of the radiopharmaceutical is quickly internalised in the cell, it could indicate that labels trapped inside the cell after catabolic degradation (residualizing labels) should be used. In contrast, slow internalisation points to the possible utility of non-residualizing labels. The internalisation assay for affibody molecules was previously validated by Wållberg and Orlova in 2008 (57) and was applied in the present thesis project.

**Affinity measurements**

The affinity of a targeting molecule to its target was previously mentioned as an essential aspect. An important feature of imaging and therapeutic agents is to bind to their target, interact and remain attached for a desired amount of time. Initial affinity measurements for the newly selected affibody molecules were previously performed using surface plasmon resonance. However, many ongoing processes within the cell can interfere with the targeting agent and influence chemical modification during radiolabelling, and it might therefore be valuable to study the binding process on living cells using a radiolabelled conjugate. Ligand Tracer is an instrument that is used for real-time studies on the interaction between a ligand and its target on cells (e.g.,
drug interaction with cell surface receptors). This method provides information about the kinetic properties of the interaction from which the affinity of the ligand to its target and the influence of labelling on the affinity could be determined.

**In vivo studies**
Biodistribution of radiolabelled conjugates and their *in vivo* targeting can be studied in small animals, such as mice in our case. These experiments require approval from the local Ethics Committee for Animal Research. The activity distribution in normal organs and tumour uptake can be visualized using available imaging devices for small animals (PET/CT, PET/MR, SPECT/CT).

**Dual isotope technique**
Paper V includes use of the dual isotope technique to study the biodistribution profile of the anti-HER3 monomer and trimer simultaneously. When using this approach *in vivo*, the number of animals needed is reduced, and the statistical power is improved. To briefly describe the procedure, the HER3 monomer and corresponding trimer were labelled separately with $^{57}$Co- and $^{111}$In, respectively, and injected into the same mouse. The dissected organs were separately measured on activity content in the energy window for $^{111}$In and for $^{57}$Co.

**Therapy**
This thesis project includes a therapy study with two groups (treated/non-treated control) of mice bearing subcutaneously implanted xenografts. Both groups were intraperitoneally injected with an affibody-based pharmaceutical or vehicle three times per week over 4 weeks. Tumours in the treated and control groups were measured continuously. The well-being of the mice during treatment was controlled in accordance with the Guidelines for Pain and Distress in Laboratory Animals established by the National Institute of Cancer (NIH, USA) and adopted by Uppsala University. The controlled parameters were general condition, behaviour, stress, pain, ataxia, appetite, sores and blistering, skin colour, inflammation of eyes, porphyria, function of urinary and gastrointestinal systems, respiration, body scoring and body weight. The therapy study was terminated on day 30, as the ethical permit was limited in terms of the number of injections and duration of therapy due to an unknown toxicity profile.
Results of present investigations

**Paper I**

*In vivo evaluation of a novel format of a bivalent HER3-targeting and albumin-binding therapeutic affibody construct*

**Background and aim**

Over the last decade, several monoclonal antibodies have been developed to target HER3 and are under evaluation in clinical trials. Additionally, non-immunoglobulin scaffold proteins have been developed for therapy purposes. However, to our knowledge, there is only one reported alternative scaffold protein aimed at our target of interest, HER3. Based on affinity maturation of an anti-HER3 affibody molecule, Z\textsubscript{08699} was selected for its low picomolar affinity (21 pM) to HER3 (51).

*In vitro* work performed by Gostring and colleagues in 2012 demonstrated that the anti-HER3 affibody molecule has the ability to completely inhibit heregulin-induced cancer growth, which encouraged further studies on HER3-targeted cancer therapy (58). Efficient therapeutic strategies require long blood residence times for the agent, which can be achieved for affibody molecules by a genetic fusion to an albumin binding domain (ABD). Using (S\textsubscript{4}G\textsubscript{4})-linkers, two Z\textsubscript{08699} molecules were connected to an ABD-domain, creating the fusion construct Z\textsubscript{08699}-(S\textsubscript{4}G\textsubscript{4})\textsubscript{4}-ABD-(S\textsubscript{4}G\textsubscript{4})\textsubscript{4}-Z\textsubscript{08699} (further referred to as 3A3).

To study the biodistribution of the new construct, its radiolabelled version was used. By introducing a C-terminal cysteine residue, a DOTA-chelator was conjugated to 3A3, which further enabled radiolabelling with radiomets. Indium-111 (\textsuperscript{111}In) was selected as the radiolabel, and the biodistribution was studied 1) in tumour-free mice to analyse different routes of administration of the construct and the influence of the administered protein dose, and 2) in immunodeficient mice bearing HER3-expressing BxPC3 xenografts to study the targeting ability of the construct as well as its biodistribution in the tumour and normal organs. As a control, a structurally identical fusion construct binding to the Taq-polymerase (Z\textsubscript{Taq}-(S\textsubscript{4}G\textsubscript{4})\textsubscript{4}-ABD-(S\textsubscript{4}G\textsubscript{4})\textsubscript{4}-Z\textsubscript{Taq}, further denoted as TAT) was included in this study. Additionally, a pilot therapy study was performed using 3A3 to evaluate the treatment effect on HER3-expressing xenografts.
This study aimed to evaluate the new bivalent construct 3A3 and its *in vivo* targeting properties, biodistribution and possibility for use as a therapeutic agent.

**Results**

Both 3A3 and TAT were labelled with $^{111}$In ($^{111}$In-DOTA-3A3 and $^{111}$In-DOTA-TAT) with a high yield (>95%) and purity (>99%). The stability of the labelling was tested in 500-fold molar excess EDTA, and after 90 minutes of incubation, a high association of activity with proteins was observed for both $^{111}$In-DOTA-3A3 and $^{111}$In-DOTA-TAT. The specific characteristics of 3A3 binding to HER3-expressing cells was demonstrated by pre-incubation of cells with a nonlabelled construct, which significantly reduced the associated activity.

Tumour-free NMRI mice were used for biodistribution studies to determine the optimal administration route, protein dose and frequency of injections for the *in vivo* therapy study. Different administration routes (intravenous, subcutaneous, and intraperitoneal injections) of 40 µg of conjugate were compared, and measurements were done at 24 h pi (Figure 4). The blood activity uptake was at the same level (approximately 1 %ID/g) for all administration routes, whereas the liver uptake was the highest for the intravenous injection at 8.04±1.07 %ID/g. Intraperitoneal injection was chosen for further experiments due to the multiple injections that this therapy study would require.

The biodistribution of $^{111}$In-3A3 was studied at 6, 24, 48, 72 and 168 h following intraperitoneal injections of 40 µg of $^{111}$In-3A3. The conjugate was efficiently taken up by blood, and already 6 h pi, the activity concentration in blood was at the level of 7.9±0.7 %ID/g. The results presented by Andersson and colleagues in paper II demonstrated that $^{111}$In-Z08699 nonfused with ABD had a blood uptake of 0.42±0.02 %ID/g at 8 h pi, which was appreciably lower than that of the 3A3 variant. This difference demonstrates that it is possible to prolong the circulation time of anti-HER3 affibody molecules when fused with ABD.

The therapy pilot study was performed in immunodeficient mice bearing HER3-expressing (BxPC-3) xenografts. They were injected with either 80 µg of 3A3 in PBS or PBS three times per week during 4 weeks. To assess the health of the mice after the injections, parameters such as behaviour, stress, appetite, pain, body weight and inflammation were controlled, and we did not detect any differences between the treated group and the control group (Figure 5). This finding indicated that the multiple injections of the anti-HER3 affibody-based construct caused no visible side effects. It also demonstrated that the HER3-expressing xenografts responded to 3A3 therapy. The average tumour size in the treated group became significantly smaller than that in the control group on day 26, suggesting that 3A3 therapy is well tolerated in mice and delays the growth of HER3-expressing xenografts *in vivo* (Figure 5).
Discussion
Upon initiating this therapy study, it was known that the residence time of the affibody-based therapeutic construct in blood circulation had to be prolonged. This prolongation was achieved by fusing two scaffold proteins comprising Z08699 to an ABD-domain. As the study required multiple injections over a long period of time, the most optimal route of injection had to be selected. Comparing the biodistribution of $^{111}$In-3A3 injected intravenously, subcutaneously and intraperitoneally, it was demonstrated that the biodistribution of the labelled compound was similar for all tested routes. However, since the mouse tail vein is fragile, it might not tolerate injections several times per week. Therefore, we considered the subcutaneous or intraperitoneal injections to be more suitable.

![Figure 4. Biodistribution of $^{111}$In-3A3 in NMRI mice at 24 h pi. Injections of the conjugate were made intravenously (iv), intraperitoneally (ip) and subcutaneously (sc). The results are presented as an average of the percentage of injected activity per gram of tissue ($\%IA/g$) for 4 animals ± SD. *The uptake in the gastrointestinal tract (GI tract) and carcass are presented as the %ID in the whole sample.](image)

The biodistribution pattern after intraperitoneal injection was most similar to that of the intravenous route, although the blood concentration and radioactivity retention in the gastrointestinal tract and carcass were significantly ($p < 0.05$) higher for the intraperitoneal route. The subcutaneous injection demonstrated significantly lower uptake of the construct in the lung, liver, spleen and bone. The intraperitoneal route resulted in the highest pancreatic and abdominal fat uptake, however, we believe it is due to the lipophilic character of the conjugate and not receptor mediated. For this reason, the intraperitoneal injection route was selected for the following experiments, including in vivo therapy (Figure 4).
Figure 5. (a) tumour volumes and (b) mice weights. Mice bearing BxPC-3 xenografts were intraperitoneally injected 3 times per week with 100 μl PBS with 80 μg of 3A3 (treated) or without it (control), n = 6. Mice were implanted on day -7 and treatment was started on day 0 and discontinued on day 30. At time points marked with *tumour volumes in control group were significantly bigger than in treated group (p < 0.05). Results are presented as average for 6 animals ± SD.

From the pilot therapy study, we concluded that HER3-expressing tumours responded to the treatment, and we were able to demonstrate a delay in tumour growth.

In conclusion, the novel ABD-fused affibody molecule 3A3 demonstrated specific binding to HER3-expressing human tumour xenografts, as well as to mErbB3-expressing tissues. Also, the high-affinity ABD fusion protein was able to slow down the growth of HER3-expressing tumour xenografts in vivo without affecting the health statuses of the mice or having any visible side effects.

In this study, we demonstrated the ability of the affibody-based construct 3A3 to delay HER3-expressing tumour growth. We also demonstrated that the residence time of 3A3 in the blood was extended when fused to ABD. However, before applying therapy, proper selection of therapeutic agents must be performed, which could be done by imaging studies.
Paper II
Comparative evaluation of $^{111}$In-labeled NOTA-conjugated affibody molecules for visualization of HER3 expression in malignant tumors

Background and aim
The feasibility of using a technetium-99m labelled, (HE)$_3$-tagged anti-HER3 affibody molecule as an imaging agent for HER3-expression in vivo was previously demonstrated by our group (46). A general requirement for targeting therapy is proper selection of the patients who have tumours expressing the targeting molecule. The selection might be performed by imaging studies using an imaging agent with high affinity towards its intended target.

Two HER3-targeting affibody molecules were selected as possible imaging probes based on their high affinities to HER3, $Z_{08698}$ (50 pM) and $Z_{08699}$ (21 pM). Our group previously showed that small changes in the amino acid composition of the affibody molecules affected important parameters and pharmacokinetics, such as hepatic uptake and blood clearance (24).

We were interested in the development of an imaging agent for radionuclide labelling for both PET and SPECT; therefore, a NOTA chelator was site-specifically conjugated to the affibody molecules.

As this study is the first to present the universal PET/SPECT design of the anti-HER3 affibody molecule, we were interested in the possibility to study the biodistribution over a longer period of time. Additionally, previous findings from our group demonstrated that imaging contrast for affibody molecules targeting tyrosine kinases with endogenous expression in normal tissues (e.g., IGF1R, HER1) could be improved at later time points, such as at 24 h pi (59,60). Indium-111, a trivalent radiometal suitable for SPECT with a half-life of 2.6 days, was thus selected as a label.

This study aimed to 1) compare the previously used $Z_{08699}$ with the new variant $Z_{08698}$ to select the molecule with the best biodistribution and imaging properties and 2) develop a HER3-targeting affibody molecule that is suitable for labelling with PET and SPECT nuclides by introducing a NOTA chelator which provides a stable complex with trivalent metals, such as $^{111}$In for SPECT and $^{68}$Ga for PET.

Results
The conjugates were labelled with $^{111}$In with a high yield (>95%), purity (>98%) and stability and preserved binding specificity.

The conjugates were injected into mice bearing human breast cancer xenografts, BT474 cells, and both conjugates demonstrated specific binding to HER3 as well as to the murine counterpart (mErbB3). The pattern of activity accumulation was similar for both conjugates (Table 3). However, $^{111}$In-HEHEHE-$Z_{08698}$-NOTA demonstrated faster blood clearance, better activity...
retention in tumours and a higher tumour-to-blood ratio at 24 h pi (Table 3). The liver uptake was higher for Z_{08698} than for the Z_{08699} conjugate at 24 h pi (4.9±0.2 %ID/g and 3.0±0.1 %ID/g, respectively). The activity uptake in tumours exceeded the uptake in several organs (salivary glands, lungs and stomach) with murine ErbB3 expression. However, the activity uptake in the liver and small intestine were higher than the uptake in tumours at both time points.

Using a gamma camera, it was possible to visualise the HER3-expressing tumours for both conjugates already at 4 h pi (Figure 6).

Table 3. Tumor-to-organ ratios of $^{111}$In-HEHEHE-Z_{08698}-NOTA and $^{111}$In-HEHEHE-Z_{08699}-NOTA in BT474 tumor bearing mice. Results are presented as an average of 4 ± SD.

<table>
<thead>
<tr>
<th>Organ</th>
<th>$^{111}$In-HEHEHE-Z_{08698}-NOTA</th>
<th>$^{111}$In-HEHEHE-Z_{08699}-NOTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>4 h</td>
</tr>
<tr>
<td>Blood</td>
<td>5.3±0.4</td>
<td>12±3</td>
</tr>
<tr>
<td>Lung</td>
<td>2.5±0.6</td>
<td>3.0±0.4</td>
</tr>
<tr>
<td>Liver</td>
<td>0.66±0.06</td>
<td>0.59±0.06</td>
</tr>
<tr>
<td>Muscle</td>
<td>1.4±2</td>
<td>16±4</td>
</tr>
<tr>
<td>Bone</td>
<td>8±1</td>
<td>8.9±0.5</td>
</tr>
</tbody>
</table>

Discussion

We previously demonstrated the specific in vivo accumulation of $^{99m}$Tc-labelled Z_{08699} in HER3-expressing xenografts (46). To enable the use of both PET and SPECT imaging modalities, a NOTA chelator was site-specifically conjugated to the C-terminal cysteine of two anti-HER3 affibody molecules with a picomolar affinity to the target, which created a new format for the targeting protein (HEHEHE-Z-NOTA). It is important to note that the low picomolar affinity to HER3 was maintained after this conjugation. A significant feature of the anti-HER3 affibody molecules is their cross-reactivity to mErbB3, thus making the murine model appropriate for evaluation of the imaging agent. Considering the imaging contrast, $^{111}$In-HEHEHE-Z_{08698}-NOTA, was able to provide a reasonable one as early as 4 h pi, which points on the possibility of using short-lived nuclides, such as gallium-68, for PET imaging.
We concluded that the HEHEHE-containing NOTA-conjugated anti-HER3 affibody molecule should be considered as superior for development in future studies for imaging using PET and SPECT modalities.

Figure 6. Gamma-camera image of mice bearing BT474 xenografts, at 4 h pi of 1 µg of both conjugates. The arrow points at the tumour (T).

Although the difference in the amino acid sequences of the two compared variants was small, the biodistribution was influenced, resulting in a higher tumour-to-blood ratio for $^{111}$In-HEHEHE-Z08698-NOTA, which was considered as superior for imaging. Good imaging contrast was achieved already at 4 h pi. This enables the use of short-lived radionuclides for PET imaging, which further optimize molecular targeted imaging.
Paper III

Affibody-mediated PET imaging of HER3 expression in malignant tumours

Background and aim
Based on studies performed by our group, including paper II in this project, it was possible 1) to demonstrate the feasibility of using a radiolabelled anti-HER3 affibody molecule as an imaging agent in HER3-expressing tumours, 2) to develop a molecular format enabling labelling with radionuclides for both PET and SPECT by a C-terminal NOTA conjugation, 3) to select the anti-HER3 affibody molecule with the best imaging properties and 4) to obtain an image in a murine model with good contrast already at 4 h pi. Based on these findings, we continued with the short-lived positron-emitting radionuclide $^{68}$Ga for labelling of the HEHEHE-Z$_{08698}$-NOTA affibody molecule for PET imaging.

The aim of this study was to investigate whether $^{68}$Ga-HEHEHE-Z$_{08698}$-NOTA could enable PET imaging in tumour xenografts as well as to evaluate its ability to discriminate between tumours with high and low HER3 expression. A panel of cell lines with different levels of HER3 expression was hence used, including BT474 (breast cancer), BxPC-3 (pancreatic cancer), LS174T (colorectal cancer) and A431 (epidermoid cancer) cells.

Results
The anti-HER3 affibody molecule Z$_{08698}$ that was selected as the superior imaging agent in paper II was used in the current paper. HEHEHE-Z$_{08698}$-NOTA was labelled with $^{68}$Ga with a high yield and stability. After challenging the conjugate with 500-fold excess of EDTA, no release of activity was detected as compared to the control that was analysed in PBS. Cell studies showed that the internalisation pattern of activity was similar in all the tested cell lines. However, the internalisation pattern differed. Cell lines with higher HER3 expression (BT474 and BxPC-3) demonstrated more rapid internalisation than the cell line with low expression (LS174T) (Figure 7).

![Figure 7](image_url)

*Figure 7. Internalisation of $^{68}$Ga-HEHEHE-Z$_{08698}$-NOTA in LS174T (left), BxPC-3 (center) and BT474 (right) cell lines. Data is presented as mean values from 3 cell dishes±SD. Error bars might not be seen because they are smaller than the symbols.*
Mice with LS174T xenografts were injected with 1 and 2 µg of \(^{68}\)Ga-HEHEHE-Z08698-NOTA. We observed that the different protein doses did not affect the activity uptake in the tumours, however, the uptake in normal murine tissues expressing mErbB3 was significantly decreased when 2 µg of the protein was injected, and the tumour-to-organ ratios were significantly improved for liver, intestines and salivary glands, which are organs that express mErbB3 (Figure 8). Also, the activity uptake in mErbB3-expressing tissues was significantly reduced when 70 µg of the affibody molecule was injected compared to that resulting from injections of 1 and 2 µg (Figure 8).

Xenografts with higher HER3 expression demonstrated significantly higher activity uptake than xenografts from low expressing cell lines. Therefore, a correlation between radioactivity uptake and receptor expression in cell lines was shown (Figure 9). MicroPET was used to visualize the HER3-expressing tumour at 3 h pi (Figure 10).

**Discussion**

Characterization of the anti-HER3 affibody molecules showed that the blood clearance was faster than expected for the tracer accumulated in normal organs endogenous expression of the targeted receptor and that the internalised fraction of cell-associated activity at 4 h pi was as high as that observed for other affibody molecules at 24 h (46, 53, 57, 61). This finding suggests that \(^{68}\)Ga can be used for PET imaging, an imaging modality that can provide with a higher accuracy, improved quantitative information, higher resolution and better quantification of activity distribution *in vivo* than other imaging modalities. Additionally, using \(^{68}\)Ga is favourable due to its low production cost and short half-life, which reduce the radiation dose received by patients.

Considering the challenge with endogenous HER3 expression in the liver, we hypothesized that it would be possible to partially saturate the receptors in the liver by optimizing the injected protein dose, making it easier for the probe to reach the tumour. To investigate this hypothesis, we injected two different protein doses into tumour-bearing mice: 1 and 2 µg. We were able to demonstrate that by increasing the injected protein dose from 1 to 2 µg, the activity uptake decreased almost two-fold in normal organs with mErbB3 expression, such as the liver, whereas the radioactivity accumulation was remained the same in tumours. This finding points the importance of dosage optimization in clinics, as it could lead to considerable changes in imaging contrast.

Additionally, by using affibody molecules, it could be possible to make a receptor quantification in the tumours, which could allow for monitoring of receptor expression before, during and after onset of therapy. This monitoring would provide information about the response to the given therapy.

In conclusion, \(^{68}\)Ga-HEHEHE-Z\_08698\_NOTA has proven to be feasible as an imaging agent for PET. We also demonstrated the ability of the conjugate to discriminate between high and low HER3 expression in tumours.
Figure 8. Biodistribution of 1 and 2 µg of $^{68}$Ga-HEHEHE-Z08698-NOTA at 1 h pi in tumour bearing mice with LS174T xenografts. The graph includes the blocking dose of 70 µg of $^{68}$Ga-HEHEHE-Z08698-NOTA at 3 h pi. The injected doses are 1 and 2 µg, and the values are presented as average of 4 animals.

Figure 9. Correlation of tumor uptake with HER3 expression level in respective cell line at 3 h pi of $^{68}$Ga-HEHEHE-Z08698-NOTA. Dotted lines represent 95% confidence interval, deviation from linearity was nonsignificant (p=0.3).
Figure 10. microPET/CT images of mice bearing BT474 (left), BxPC-3 (center) and LS174T (right) xenografts at 3 h pi of 2 µg of $^{68}$Ga-HEHEHE-Z08698-NOTA. Upper row demonstrates the transaxial view, low row demonstrates coronal view. White arrow indicates tumours.

In this study, we demonstrated the specific binding of $^{68}$Ga-Z08698 to HER3-expressing cell lines. The conjugate was also able to saturate the uptake in HER3 xenografts and mErBb3 tissues, indicating its specificity in vivo. Dosage optimization improved HER3 imaging contrast in malignant tumours, and the activity uptake in tumours was proportional to the HER3 expression level in the panel of cell lines.
Paper IV
*Evaluation of a radiocobalt-labelled affibody molecule for imaging of human epidermal growth factor receptor 3 expression*

**Background and aim**
As mentioned earlier, HER3 has an endogenous expression in several organs, one of which is the liver, which complicates the imaging of HER3 expression in liver metastases. In previously performed studies involving $^{99m}$Tc-Z08698 (46), $^{111}$In-Z08698 (61) and $^{68}$Ga-Z08698 (53), it was not possible to achieve a complete reduction in hepatic uptake when high protein doses were injected *in vivo*. Based on this finding, we hypothesized that the hepatic uptake of the anti-HER3 affibody molecule depends on two mechanisms: one that is receptor mediated and one that is dependent on the structural characteristics of the protein, such as lipophilic patches and charge distribution.

Another member of the HER-family, HER1/EGFR, reminds of the HER3 in endogenous expression. Studies performed by our group using an affibody molecule targeting EGFR demonstrated that a decrease in the positive charge of the metal-chelator complex resulted in a lower radioactivity hepatic uptake *in vivo*. Based on these results, we aimed to use bivalent radiocobalt as a label instead of trivalent indium or gallium. Cobalt-55 ($^{55}$Co, $t_{1/2}=17.5$ h, charge of 2+) is a positron-emitting radionuclide that has been proposed for imaging a variety of diseases, such as multiple sclerosis and ischaemic stroke. Because of its suitable half-life, one production of $^{55}$Co can be used for imaging of several patients by using only one production, which is cost efficient. Additionally, it enables next-day imaging, which might provide with better contrast. A surrogate nuclide, $^{57}$Co ($t_{1/2}=271.6$ days), was used for convenience. Mitran B and colleagues (62) demonstrated that *in vitro* and *in vivo* data obtained using $^{57}$Co and $^{55}$Co were in good agreement.

The aim of this study was to evaluate $^{57}$Co-HEHEHE-Z08698-NOTA in mice bearing HER3-expressing xenografts by means of labelling chemistry, cellular processing and targeting ability analyses *in vivo*.

**Results**
HEHEHE-Z08698-NOTA was labelled with $^{57}$Co with a high yield and stability. After challenging the conjugate with 500-fold excess of EDTA, no release of radioactivity was detected as compared to the control that was analysed in PBS. The radioactivity uptake in the studied HER3-expressing cell lines, DU145 and LS174T, was significantly decreased after the receptors were presaturated with nonlabelled affibody molecule, indicating the specificity of the conjugate to the HER3 receptors.
The conjugate was injected into Balb/c nu/nu mice bearing DU145 or LS174T xenografts (Figure 11), and the HER3-mediated uptake of the $^{57}$Co-HEHEHE-Z08698-NOTA conjugate was demonstrated in both tumour models. The binding specificity of $^{57}$Co-HEHEHE-Z08698-NOTA in vivo was studied by injecting a high protein dose of 70 µg. A significant decrease in activity uptake in the blood, salivary glands, lung, liver, stomach, small intestine and tumour was detected, indicating specific uptake of the conjugate in vivo. The biodistribution of the conjugate was studied at 3 and 24 h pi (Table 4). At 3 h pi, the blood activity concentration was not higher than 0.53 %ID/g and further decreased with time, which was in accordance with previously tested technetium-, indium- and gallium-labelled variants. Over time, a significant decrease in the activity concentration was observed in salivary glands, lungs, liver, tumour and kidneys.

For the LS174T model, the tumour uptake was 1.9±0.1 %ID/g at 24 h pi, which exceeded the liver uptake of 1.5±0.2 %ID/g, thus, the tumour-to-liver ratios were increased over time from 0.90±0.06 at 3 h pi to 1.31±0.08 at 24 h pi. Images that were acquired at 3 and 24 h pi for both xenograft models reflected the ex vivo biodistribution results, as a decreasing hepatic activity accumulation over time as well as a low background activity were observed, confirming the rapid blood clearance (Figure 12).

![Figure 11.](image-url)

*Figure 11. In vivo specificity of $^{57}$Co-Z08698 to HER3-expressing DU145 (A) and LS174T (B) xenografts and mErbB3-expressing tissues. *Organs where receptor blocking by excess of non-labelled conjugate resulted in significant reduction of the radioactivity uptake (n=3-4, p<0.05). Data are presented as mean values from 3-4 samples ± SD.*
Table 4. Biodistribution of $^{57}\text{Co-Z08698}$ in tumour-bearing Balb/c nu/nu mice after iv injection of 2 μg of conjugate (presented as %ID/g, gastrointestinal tract (GI) and carcass as %ID/sample). The results are presented as the average of 3-4 animals ± SD.

<table>
<thead>
<tr>
<th></th>
<th>DU145 3 h pi</th>
<th>DU145 24 h pi</th>
<th>LS174T 3 h pi</th>
<th>LS174T 24 h pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.27±0.02</td>
<td>0.096±0.007$^a$</td>
<td>0.53±0.05</td>
<td>0.23±0.01$^a$</td>
</tr>
<tr>
<td>Tumour</td>
<td>0.8±0.1</td>
<td>0.58±0.03$^a$</td>
<td>2.6±0.2</td>
<td>1.9±0.1$^a$</td>
</tr>
<tr>
<td>Salivary glands</td>
<td>1.1±0.2</td>
<td>0.62±0.02$^a$</td>
<td>1.4±0.2</td>
<td>0.9±0.1$^a$</td>
</tr>
<tr>
<td>Lung</td>
<td>1.19±0.05</td>
<td>0.36±0.03$^a$</td>
<td>1.28±0.09</td>
<td>0.59±0.05$^a$</td>
</tr>
<tr>
<td>Liver</td>
<td>2.2±0.3</td>
<td>0.88±0.06$^a$</td>
<td>2.9±0.3</td>
<td>1.5±0.2$^a$</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.28±0.04</td>
<td>0.27±0.04</td>
<td>0.39±0.05</td>
<td>0.40±0.03</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.2±0.3</td>
<td>0.5±0.2$^a$</td>
<td>1.4±0.2</td>
<td>1.0±0.4</td>
</tr>
<tr>
<td>Intestine</td>
<td>3.1±0.3</td>
<td>1.6±0.5$^a$</td>
<td>4.7±0.4</td>
<td>2±1$^a$</td>
</tr>
<tr>
<td>Kidney</td>
<td>310±32</td>
<td>231±15$^a$</td>
<td>190±12</td>
<td>158±5$^a$</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.099±0.009</td>
<td>0.068±0.008$^a$</td>
<td>0.14±0.01</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td>Bone</td>
<td>0.14±0.03</td>
<td>0.09±0.01$^a$</td>
<td>0.23±0.02</td>
<td>0.22±0.02</td>
</tr>
</tbody>
</table>

$^a$ Significant difference from that at 3 h post injection (pi) (n=3-4, p<0.05).

Discussion

HER3 has an endogenous expression in many organs, one of which is the liver. Since liver is the most common metastatic site, this fact complicates the characterization and imaging of the actual cancerous tissue. Previous studies have shown that a positively charged N-terminus on the affibody molecule increases the hepatic uptake (63). We are aware of the possible dual nature of the hepatic uptake of the anti-HER3 affibody molecule, and by exchanging the label for radiocobalt and neutralizing the charge of the metal-NOTA complex, we hoped to decrease the unspecific activity accumulation. This modification resulted in the lowest hepatic uptake among the previously tested variants previously reported for anti-HER3 affibody molecules (46,53,61,64)
In conclusion, we observed a faster decrease in activity uptake in healthy organs than in the tumour, and at 24 h pi, the uptake in the liver was lower than that in the tumour, contributing to a positive imaging contrast in the liver. Since we obtained high contrast images at 24 h pi, our hypothesis that imaging HER3 expression could be improved over time and by using a neutral labelling complex was confirmed.

The presented $^{57}$Co-labeled anti-HER3 affibody molecule can bind to the same epitope as the previously discussed therapeutic agent 3A3 in paper I. This point on the possible application of the radiocobalt labeled conjugate as a monitoring tool to observe the changes in receptor expression level during therapy. However, to make best use of imaging, a better contrast in liver would be highly desirable. Therefore, we continued our studies with a trimer anti-HER3 affibody molecule. We believed that a coinjection of an unlabeled trimer molecule together with a radiolabeled anti-HER3 affibody molecule would saturate liver uptake and by that enable an easier transport of the monomer affibody molecule to the tumour.
Paper V

*Improved contrast of affibody-mediated imaging of HER3 expression through co-injection of affibody trimer for in vivo blocking of hepatic uptake*

**Background and aim**
Radiocobalt labelled anti-HER3 affibody molecule has been shown to have the most favourable biodistribution profile among the previously tested variants involving the HER3-targeting affibody molecule (65). It resulted in the lowest unspecific hepatic uptake and a high and stable uptake in HER3-expressing tumours. In paper III, we demonstrated that protein dose optimization of the injected affibody imaging agent could suppress (but not completely saturate) the hepatic uptake (53).

Affibody molecules targeting HER1/EGFR and HER2 have a significantly higher tumour uptake when used in their monomeric form compared to that achieved when they are used in their dimeric form (60,66). This increased uptake is due to the small size of the affibody monomer and better tumour penetration (67).

The liver is a highly vascularized organ, and we hypothesized that we could saturate the endogenous HER3 hepatic uptake using a large, multimeric and nonlabelled affibody molecule coinjected with its radiolabelled monomeric analogue.

A trimer consisting of three linked anti-HER3 affibody molecules ($Z_{08699}$)$_3$ (~24 kDa) was thus generated.

The aim of this study was to investigate the influence of coinjection of the nonlabelled trimeric anti-HER3 affibody molecule on the biodistribution and tumour targeting of a radiolabelled monomer.

**Results**
The affibody trimer was labelled with $^{111}$In with a yield greater than 98% (determined by radio-ITLC) and was stable under challenge with 500-fold molar excess of EDTA. The specificity of the trimer to HER3-receptors was confirmed *in vitro* by pre-saturation of receptors with nonlabelled trimer, resulting in a significant decrease in the cell-associated radioactivity (p<0.05).

Cellular processing was performed in BxPC-3. Rapid binding with constant increases in cellular activity uptake and internalized fraction over time was demonstrated, and the internalised fraction reached 60% of the total cell associated radioactivity at the 24 h time point. The total cell bound radioactivity increased by 2-fold from 1 to 24 h during continuous incubation (Figure 13).
Molar equivalents of the monomer, \(^{57}\text{Co}-Z_{08699}\) (0.5 µg), and the trimer, \(^{111}\text{In}-(Z_{08699})_3\) (1.5 µg), were coinjected into BxPC-3 tumour-bearing mice. The radiolabelled trimer had significantly faster blood clearance than the radiolabelled monomer (Table 5). Notably, despite the rapid blood clearance, the activity uptake in almost all the studied normal organs and tissues was significantly higher for the trimer except in the lungs and kidneys. However, the tumour activity uptake was the same for the monomer and trimer.

Table 5. Biodistributions of \(^{57}\text{Co}-Z_{08699}\) (0.5 µg) and \(^{111}\text{In}-(Z_{08699})_3\) (1.5 µg) at 4 h after coinjection into BxPC-3 xenograft mice. The organ uptake values are expressed as the percentage of injected dose per gram of tissue weight (% ID/g). * value was significantly higher (paired, two-tailed t-test, \(p<0.05\)).

<table>
<thead>
<tr>
<th>Organs</th>
<th>(^{57}\text{Co}-Z_{08699})</th>
<th>(^{111}\text{In}-(Z_{08699})_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.51±0.02*</td>
<td>0.20±0.02</td>
</tr>
<tr>
<td>Lung</td>
<td>0.70±0.04</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>1.6±0.1</td>
<td>21.1±0.2*</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.69±0.04</td>
<td>3.0±0.4*</td>
</tr>
<tr>
<td>Kidney</td>
<td>303±31*</td>
<td>131±14</td>
</tr>
<tr>
<td>Tumour</td>
<td>1.5±0.2</td>
<td>1.4±0.4</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.22±0.05</td>
<td>0.40±0.09*</td>
</tr>
</tbody>
</table>

Further, the biodistributions of \(^{57}\text{Co}-Z_{08699}\) (fixed labelled protein mass of 0.5 µg/animal) coinjected with unlabelled affibody trimer \((Z_{08699})_3\) at monomer:trimer molar ratios of 1:0, 1:1, 1:3 and 1:6 were studied at 4 h pi (68). The activity uptake of the monomer in normal organs decreased with the increase in the molar ratio. A significantly lower hepatic activity uptake was
found for the 1:6 ratio compared to that observed for the other tested ratios, but tumour activity uptake was the highest for the 1:3 ratio. The maximum tumour-to-liver ratio (1.0±0.1) was observed at the 1:3 ratio, which was significantly higher than those observed at the 1:0 (0.44±0.08), 1:1 (0.7±0.1) and 1:6 (0.67±0.01) molar ratios (Figure 14).

Previously, our group published the biodistribution of $^{57}$Co-Z$_{08699}$ (2 µg) at 24 h pi, which was considered the best performing dose and time point (65). These injection conditions were compared to the results obtained from the biodistribution of $^{57}$Co-Z$_{08699}$ (0.5 µg) coinjected with (Z$_{08699}$)$_3$ at the molar ratio of 1:3, which, in turn, was the dose that performed the best in the present study.

![Figure 14. Tumour-to-liver ratios depending on monomer:trimer molar ratios at 4 h after the coinjection of $^{57}$Co-Z$_{08699}$ (0.5 µg):(Z$_{08699}$)$_3$ into BxPC-3 tumour-bearing mice.](image)

The comparison demonstrated that the tumour activity uptake was significantly higher in the case of trimer coinjection. The activity uptake in the other tested organs and tissues did not differ significantly. The tumour-to-normal organ ratios were significantly better for the probe coinjected with the trimer except for that in the small intestine (Figure 15).

Imaging was performed at 24 h pi for $^{57}$Co-Z$_{08699}$ (2 µg) and $^{57}$Co-Z$_{08699}$ (0.5 µg):(Z$_{08699}$)$_3$ (molar ratio 1:3), and tumours were clearly visualised (Figure 16).

**Discussion**

In this study, we hypothesized that imaging HER3-expressing tumours could be improved by *in vivo* blocking of the liver uptake by coinjecting a trimeric anti-HER3 affibody construct (Z$_{08699}$)$_3$.

We demonstrated that the binding of the trimer was specific to HER3 after radiolabelling with indium-111.
Figure 15. Comparison of tumour-to-organ ratios at 24 h pi of $^{57}$Co-$Z_{08699}$ (2 µg) or $^{57}$Co-$Z_{08699}$ (0.5 µg):$(Z_{08699})_3$ (molar ratio 1:3) in BxPC-3 tumour-bearing mice.

Figure 16. microSPECT/CT images at 24 h pi of mice bearing BxPC-3 xenografts. The animals were injected with (A) $^{57}$Co-$Z_{08699}$ (0.5 µg):$(Z_{08699})_3$ (molar ratio 1:3) or (B) $^{57}$Co-$Z_{08699}$ (2 µg). The arrows point at the kidney (K), tumour (T) and liver (L).

To test our hypothesis, we made a direct comparison of the biodistribution patterns of the monomer and trimer, and the results were analysed after coinjection of equimolar amounts of monomer and trimer in mice bearing BxPC-3 tumour xenografts. We observed higher amounts of trimer (13–fold higher) as compared to the monomer hepatic accumulation. At the same time, the tumour activity accumulation was the same for both the trimer and monomer. This accumulation was unexpected since we believed that tissue penetration would be better for the monomer.
The trimer dose was further optimized by analysing the coinjection of unlabelled trimer and radiolabelled monomer at different molar ratios. The 1:3 ratio demonstrated the highest tumour uptake of 1.9±0.1 %ID/g, and a hepatic uptake of 1.81±0.09 %ID/g, which resulted in the highest tumour-to-liver ratio among the tested injection conditions. The imaging contrast was further improved with time, and the tumour-to-liver ratio at 24 h pi was more than 2-fold higher than any results previously published (Figure 14).

In conclusion, we demonstrated that imaging of HER3 expression can be improved by coinjection of a radiolabelled monomeric affibody with an affibody trimer. Coinjection of an anti-HER3 affibody trimer with a radiolabelled affibody monomer at an optimal ratio significantly decreased the hepatic activity uptake while significantly increasing the activity uptake in HER3-expressing tumours and tumour-to-organ ratios, including those in the liver.

The high vascularization in the liver together with its endogenous HER3 expression complicates the HER3 imaging procedure of malignant tumours. We were able to saturate endogenous liver uptake using a multimeric protein. Furthermore, the tumour accumulation of a monomeric, radiolabeled anti-HER3 affibody molecule was increased. This finding strongly contributes to imaging of HER3-expressing tumours.
Concluding remarks

This thesis has focused on the development, evaluation and characterization of affibody molecules for theranostic applications in HER3-expressing malignant tumours. HER3-targeting affibody molecules were used as imaging agents to personalize available therapy strategies and for therapy of HER3-expressing tumours.

The results presented in this thesis demonstrated the therapeutic potential of the bivalent construct 3A3 in vivo, followed by a step-by-step optimization of the imaging properties of a HER3-targeting affibody molecule.

We demonstrate that by exchanging radiolabels (and modifying the overall charge of the conjugate), optimizing the injected protein dose, implementing the strategy of coinjecting a trimer molecule together with a radiolabelled monomer and performing imaging at later time points, the tumour activity uptake can be increased, hepatic uptake can be decreased and the imaging contrast can be improved.

The major findings of this thesis are as follows:

- Circulation time of anti-HER3 affibody molecule in bloodstream was extended when ABD was fused to the affibody based construct 3A3 making this scaffold protein suitable for therapeutic applications.

- The 3A3 construct did not demonstrate any toxicity in normal tissue in vivo and was capable of delaying HER3-expressing tumour growth when injected into mice for 30 days.

- Small differences in the amino acid sequences of HER3-targeting affibody molecules with picomolar affinities (Z08698 and Z08699) influenced their biodistribution. The Z08698 variant had higher tumour-to-blood ratios and was selected as the superior variant for future imaging studies.

- The NOTA chelator conjugated to the HER3-targeting affibody molecule provided stable labelling with radiometals ($^{111}$In, $^{68}$Ga, $^{55}$Co) for PET and SPECT imaging.

- By using $^{111}$In as a label for Z08698 and Z08699, we were not only able to study the biodistribution over a longer period of time but we also
demonstrated that good imaging contrast could be obtained at 4 h pi, enabling the use of short-lived nuclides.

- The activity uptake of an anti-HER3 affibody molecule in tumours was proportional to the level of HER3 expression in the tested cell lines. The $^{68}$Ga-labelled anti-HER3 affibody molecule could distinguish between malignant tumours with low and high HER3 expression as early as 3 h pi.

- Injected protein dose markedly changed imaging contrast. Optimal injected protein dose saturated the receptors expressed in normal organs but not in the tumours.

- The neutral Co-NOTA complex for labelling of the anti-HER3 affibody molecule significantly decreased the unspecific radioactivity accumulation in normal organs, particularly in liver. This resulted in the lowest hepatic uptake among imaging probes based on the anti-HER3 affibody molecule ($^{99m}$Tc-$Z_{08699}$, $^{111}$In-$Z_{08698}$ and $^{68}$Ga-$Z_{08698}$) tested by our group.

- In the case of $^{57}$Co-$Z_{08698}$, a faster decrease in activity uptake was observed in healthy organs compared to that in the tumour, and at 24 h pi, the liver uptake was lower than the tumour uptake, contributing to a positive imaging contrast in the liver.

- It was possible to saturate the receptor-specific hepatic uptake of anti-HER3 affibody molecule with a nonlabelled trimeric anti-HER3 affibody molecule, which resulted in increased tumour accumulation of the radiolabelled monomeric molecule.

- The approach of coinjecting HER3-targeting affibody trimer with a radiolabelled affibody monomer strongly contributed to the improvement of imaging of HER3-expressing tumours.

- We were able to obtain high-contrast images of HER3 expression in xenografts 24 h pi, which confirms our hypothesis that imaging of HER3 expression can be improved with time and optimal injection dosages.
Future studies

Cancer is a common designation for a variety of different diseases which can originate from most organs of the body. It is the leading cause of death worldwide. Since cancer is a genetic disease with high inter- and intra-tumoural heterogeneity as well as inter-patient heterogeneity, it is essential to identify genetic aberrations and use them as targets for imaging and targeted personalised therapy. This thesis demonstrates that affibody molecules with high affinity and binding specificity are very promising targeting vectors that can be used in and imaging of HER3-expressing malignant tumours.

The main goal of cancer therapy is to remove the tumour, kill cancer cells using cytotoxic drugs or stabilize the cancer progression using cytostatic drugs. The results from paper I clearly demonstrated that the fused affibody construct 3A3 had a cytostatic effect a murine model bearing HER3-expressing tumours. Importantly, 3A3 was well-tolerated and did not show any toxicity. Therefore, it would be logical to continue the development of affibody-based therapeutic agents.

The therapeutic efficacy of the 3A3 construct should be tested in a properly designed therapeutic study. Our group recently published the results of such study where an affibody construct similar to 3A3 (with minor modifications) was compared with the anti-HER3 monoclonal antibody seribantumab (MM-121) (69).

Seribantumab blocks the PI3K and Akt signalling pathways by binding to HER3 (70). However, while Seribantumab has not yet been fully clinically investigated, it has been involved in several phase 2 clinical trials as an addition to standard cancer therapies in which a therapeutic benefit was demonstrated (70). The results demonstrated that the affibody construct and Seribantumab had equal efficiencies in terms of tumour growth inhibition and longer survival of mice xenografted with HER3-expressing malignant tumours. However, the affibody construct offers is cheaper production and lower immunogenicity.

The bispecific format of the affibody construct described in paper I (Z08699-ABD-Z08699) enabled efficient tumour targeting and therefore served as a starting point for further investigations into the molecular format. Earlier, it was reported that even anti-HER3 monomers were able to inhibit HRG-induced signalling in vitro (58). Additionally, the placement/order of the affibody molecule and ABD in the construct might influence its binding
affinities to both targets. Therefore, investigations to further develop the anti-HER3 therapeutic construct could compare different combinations, such as 3A, 33A, A33, and A3, with 3A3. This study has already been initiated. Based on the results of this study, more advanced constructs, such as 33A3 and 3A33, could be considered. The targeting approach towards HER3 could be modified and also involve other members of the HER family, mainly EGFR/HER1 and HER2. HER2 is a preferable heterodimerizing partner of HER3, and a format blocking such dimerization would be of great interest.

It would be of great interest to investigate if it is possible to achieve a cytotoxic effect on the cancer cells by fusing the HER3-targeting affibody molecule with a cytotoxic drug or agent. One possible drawback to this approach would be the endogenous HER3 expression, which could lead to lower tolerability, higher toxicity and undesirable side effects.

Another direction for future studies is the development of an affibody imaging probe for in vivo detection of HER3 expression. Imaging quality is dependent on both specific and nonspecific interactions with the imaging agent, on the density of the molecular target in pathological tissues, and on the presence of endogenous receptor expression in critical organs, such as normal tissue organs with high frequencies of metastatic growth, e.g. lung and liver.

The affinity of the HER3-targeting molecule is already at a low picomolar level, meaning that it is difficult to improve the targeting ability. Therefore, it is important to focus on the process of further reducing the nonspecific uptake in normal organs to improve imaging quality. In papers III and V, we were able to demonstrate that by optimizing the injected protein dose, it was possible to reduce the receptor-mediated hepatic activity uptake and increase the tumour uptake.

In papers III and IV, we investigated the effect of the radiolabel on the biodistribution and demonstrated that the metal-chelator complex had a noticeable influence on off-target interactions with the anti-HER3 affibody molecule. Using a NOTA chelator and bivalent radiometal, cobalt, (resulting in a neutral overall complex charge), we were able to reduce unspecific hepatic uptake and obtain a higher activity uptake in the tumour than in the liver. A continuation of the study could be to investigate other chelators (NODAGA, DOTA, DOTAGA) together with different radiometals suitable for PET and SPECT, both bivalent and trivalent. Further investigations of structure-property relationships would be desirable to design an optimal imaging probe for HER3.

We believe that theranostic applications for current cancer therapy approaches will improve the possibilities to monitor the disease progression from the stated diagnose, as well as during and after therapy. This approach will hopefully contribute to reaching our aims and goals of improved patient stratification and personalisation of cancer therapy.
Acknowledgements

This thesis work was carried out at the Department of Medicinal Chemistry, Theranostics, Uppsala University. The PhD-years have been a scientific journey and I would like to thank the ones of you supporting me through it, making this day possible. Especially I would like to thank:

My supervisor Professor Anna Orlova for giving me the possibility to enter the world of cancer research and become a part of it. Thank you for always believing in me, being supporting, calm, rational and caring whenever I needed it the most. Thank you for patiently teaching me everything I know about cell studies, animals (mice, mostly) and radioactivity. Thank you for always sharing funny stories, great food recipes and advices about how to keep flowers alive. Thank you for making my PhD-journey into such a memorable time.

My co-supervisor, Professor Vladimir Tolmachev, for always having many interesting project ideas to share and for the guidance and support along the way. Thank you for always making time for me whenever I needed your help.

My co-supervisor, Olof Eriksson for valuable discussions about imaging and PET/SPECT cameras.

My group members (both past and present):
Bogdan and Sara – where should I begin? Thank you for all the fun and inappropriate discussions we had about everything in general, for sharing ideas about food, movies, parties, life and science. I bet no one will be able to beat our google history. From only being colleagues and master students you have now become very close and dear friends to me.
Mohamed, thank you for all of the interesting discussions we had about science, religion, politics and many others. Also, thank you for always offering your help.
Javad, it has been a pleasure to have you as a group member, thank you for the talks we have had over the years.
Hadis, thank you for your all the talks, fikas and train rides we had together. Your energy has always motivated me to work harder. Also, thank you for
always helping and supporting me, there was a time in life when that was invaluable. 

Joanna, thank you for being there for me. Thank you for all the support you have given me. It was invaluable.

To my more recent group members, Anzhelika and Maryam, thank you for turning out to be such great colleagues and for bringing delicious cakes and cookies to our meetings.

Our collaborators at KTH:

Ken, Tarek, Stefan and John, thank you for being such great partners. You have been invaluable. Thank you for all the hard work you have done.

Kollegorna på PPP:

Sergio, Veronika och Ola, thank you for all the great lunch discussions. We always have a good time when we are together.

Vännerna utanför labbet:

Sara, thank you for being there for me. Thank you for all the支持 and confidence you have shown me.

Ottilia, thank you for being there for me. Thank you for all the good advice you have given me.

Veronica, thank you for being one of the most caring people I know. Thank you for always being there for me.

Restaurangklubben Svettiga Räven, David och Josefine, Daniel och Malin, thank you for the good food and good times.

Lena och Pecka, thank you for all the support and confidence you have shown me. Thank you for being there for me.
Min familj – **mamma, pappa, mormor** och **morfar**, tack för ni har varit en oändlig källa till kärlek, omtanke, styrka och goda råd under dessa år och genom livet. Tack för att ni inspirerade och motiverade mig till att disputera, jag hade aldrig kommit såhär långt utan ert eviga stöd.

References

14. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO J. 1997 Apr 1;16(7):1647–55.


A doctoral dissertation from the Faculty of Pharmacy, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Pharmacy”.)