Towards Personalized Cancer Therapy

New Diagnostic Biomarkers and Radiosensitization Strategies

DIANA SPIEGELBERG
Abstract


This thesis focuses on the evaluation of biomarkers for radio-immunodiagnostics and radioimmunotherapy and on radiosensitization strategies after HSP90 inhibition, as a step towards more personalized cancer medicine. There is a need to develop new tracers that target cancer-specific biomarkers to improve diagnostic imaging, as well as to combine treatment strategies to potentiate synergistic effects. Special focus has been on the cell surface molecule CD44 and its oncogenic variants, which were found to exhibit unique expression patterns in head and neck squamous cell carcinoma (HNSCC). The variant CD44v6 seems to be a promising target, because it is overexpressed in this cancer type and is associated with radioresistance. Two new radioconjugates that target CD44v6, namely, the Fab fragment AbD15179 and the bivalent fragment AbD19384, were investigated with regard to specificity, biodistribution and imaging performance. Both conjugates were able to efficiently target CD44v6-positive tumors in vitro and in vivo. PET imaging of CD44v6 with 124I-AbD19384 revealed many advantages compared with the clinical standard 18F-FDG. Furthermore, the efficacy of the novel HSP90 inhibitor AT13387 and its potential use in combination with radiation treatment were evaluated. AT13387 proved to be a potent new cancer drug with favorable pharmacokinetics. Synergistic combination effects at clinically relevant drug and radiation doses are promising for both radiation dose reduction and minimization of side effects, or for an improved therapeutic response. The AT13387 investigation indicated that CD44v6 is not dependent on the molecular chaperone HSP90, and therefore, radio-immunotargeting of CD44v6 in combination with the HSP90 inhibitor AT13387 might potentiate treatment outcomes. However, EGFR expression levels did correlate with HSP90 inhibition, and therefore, molecular imaging of EGFR-positive tumors may be used to assess the treatment response to HSP90 inhibitors.

In conclusion, these results demonstrate how tumor targeting with radiolabeled vectors and chemotherapeutic compounds can provide more specific and sensitive diagnostic tools and treatment options, which can lead to customized treatment decisions and a functional diagnosis that provides more precise and safer drug prescribing, as well as a more effective treatment for each patient.

Keywords: tumor targeting, radionuclide targeting, HSP90 inhibition, AT13387, radiosensitization, molecular imaging, combination treatment, EGFR, CD44v6

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I don’t want to believe. I want to know.

Carl Sagan
All of the figures in the introduction have been designed and drawn by D. Spiegelberg and C. Malmberg.
List of Papers

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


* Contributed equally

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List of Papers not Included in this Thesis


* Contributed equally
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<td>17-AAG</td>
<td>17-allyl-17-demethoxygeldanamycin</td>
</tr>
<tr>
<td>17-DMAG</td>
<td>17-(dimethylaminoethylamino)-17-demethoxygeldanamycin</td>
</tr>
<tr>
<td>ALK</td>
<td>Anaplastic lymphoma kinase</td>
</tr>
<tr>
<td>AKT</td>
<td>V-akt murine thymoma viral oncogene homolog</td>
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<tr>
<td>AT13387</td>
<td>2,4-dihydroxy-5-isopropyl-phenyl-[5-(4-methylpiperazin-1-ylmethyl)-1,3-dihydro-isindol-2-yl] thanone, l-lactic acid salt</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
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<td>bFGF</td>
<td>Bovine fibroblast growth factor</td>
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<td>BIWA</td>
<td>Bivatuzumab</td>
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<td>CAT</td>
<td>Chloramline T</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CD44s</td>
<td>Standard CD44</td>
</tr>
<tr>
<td>CDv</td>
<td>CD exon variant</td>
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<tr>
<td>cmAb</td>
<td>Chimeric monoclonal antibody</td>
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<tr>
<td>CPS</td>
<td>Counts per second</td>
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<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
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<tr>
<td>CT</td>
<td>Computed tomography</td>
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<tr>
<td>Da</td>
<td>Dalton</td>
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<tr>
<td>DARPins</td>
<td>Designed ankyrin repeat proteins</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
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<tr>
<td>DSB</td>
<td>Double-strand break</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ErbB</td>
<td>Erythroblastic leukemia viral oncogene homolog</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen binding fragment</td>
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<tr>
<td>FACS</td>
<td>Fluorescent-activated cell sorting</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FAR</td>
<td>Fraction of activity released</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
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<td>FDG</td>
<td>Fluorodeoxyglucose</td>
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GIST  Gastrointestinal stromal tumors
Gy  Gray
HER2, 3, 4  Human EGFR 2, 3, 4
HOP  HSP-organizing protein
HSP  Heat shock protein
HNSCC  Head and neck squamous cell carcinoma
HPV  Human papilloma virus
LET  Linear energy transfer
mAb  Monoclonal antibody
MET  Hepatocyte growth factor receptor
MIP  Maximum intensity projection
mM  Millimolar
MRI  Magnetic resonance imaging
MTT  (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Na(TI)  Sodium iodide (activated with thallium)
nM  Nanomolar
NRG1, 2, 3, 4  Neuregulins 1, 2, 3, 4
IC50  Median inhibitory concentration
%ID/g  Percentage of the injected dose measured per gram of tissue or organ
IgG  Immunoglobulin, class G
IHC  Immunohistochemistry
iodogen  1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril
i.v.  Intravenous injection
p53  Tumor protein 53
PET  Positron emission tomography
PFGE  Pulsed-field gel electrophoresis
p.i.  Post injection
PTK  Protein tyrosine kinase
RECIST  Response evaluation criteria in solid tumors
scFvs  Single-chain antibody variable fragments
SD  Standard deviation
SEM  Standard error of the mean
SPECT  Single-photon emission tomography
SPR  Surface plasmon resonance
TGFα  Transforming growth factor alpha
TKI  Tyrosine kinase inhibitor
VEGFR  Vascular endothelial growth factor receptor
µM  Micromolar
Introduction

Cancer

Statistically, more than 1 in 9 of everyone reading this text will die due to cancer, and virtually everyone will come into close contact with the disease in their lifetime through family and friends [1]. It is not surprising that fighting cancer, or finding “the cure for cancer”, is considered by many as the holy grail of biomedical research - the very epitome of our struggles as medical scientists.

However, cancer is not a single disease. Rather, the term cancer summarizes a very broad class of diseases defined by a common mechanism: loss of control of the own cells of the affected organism, which involves uncontrolled cell proliferation, invasion into normal unaffected tissues and the capability for the development of metastases in other parts of the body. Over 100 different types of cancer exist, with an overwhelmingly diverse flora of subtypes and molecular mechanisms. It is clear that the probability of a single, universal cancer cure is exceedingly small.

Cancer has been with us since the dawn of multicellular organisms, and throughout human history. Relatively recently, cancer has emerged as a leading cause of death in the developed world, following the near elimination of infectious and parasitic diseases, malnourishment, and the “neglected diseases of the developing world”: homicides, suicides, and war. The same transition is expected to increase the cancer mortality in the developing world in the following decades, as the people there progress into transitional or developed societies. In 2008, 7.6 million people (approximately 13% of all recorded deaths this year) died due to cancer, and deaths from cancer worldwide are projected to continue to rise to over 13.1 million in 2030 due to this shift [1]. The economic impact of cancer on both the healthcare system and society as a whole is severe; in 2008, it was estimated to be 201.5 billion US dollars in the USA alone. This figure includes both direct treatment costs (77.4 billion) and indirect costs to society as a result of lost productivity due to premature death (124 billion). The great cost in human life as well as economic resources makes cancer treatment a highly important field of research [2, 3].

Most instances of cancer derive from a single abnormal cell. These abnormalities can be caused randomly by mistakes in DNA replication, by exposure to carcinogens such as radiation or a plethora of carcinogenic
chemical substances, by viruses or by inherited genetic disorders [2-4]. The transformation from normal cells to cancer cells is generally attributable to mutations or alterations in two different groups of genes: activation of onco-genes and inactivation of tumor suppressor genes. Still, a single mutation is not sufficient to cause cancer; typically the affected cells gradually accumulate mutations over time and spiral increasingly out of control. This process is constantly ongoing, but in the vast majority of cases, the abnormal cells are swiftly cleared by the immune system or undergo apoptosis. Eventually however, one abnormality will escape the multiple layers of protective systems, which is why the cancer risk increases with age. The first step in this process is an abnormal proliferation of tissue, called a neoplasm, which typically forms a tumor. There are three general forms of tumors that can be distinguished: benign, pre-malignant (carcinoma in situ) or malignant. Benign tumors are not cancerous; they do not grow in an infinite or aggressive way, nor do they invade other parts of the body. Nevertheless they can be dangerous, e.g., if the tumor imposes pressure upon vital organs, particularly the brain. The term pre-malignant tumor refers to a pre-cancerous condition defined by the absence of invasion of surrounding tissues, which seems to be the interstate between benign and malignant tumors. If pre-malignant tumors remain untreated the condition can lead to malignant growth. Malignancies or malignant tumor growth are generally referred to as cancer. These tumors invade and destroy the surrounding tissue and may potentially spread to other parts of the body to form metastases; at this stage of the disease the cancer is life-threatening, and the likelihood of successful treatment is very low [5-7].

It is important to understand that tumors are highly heterogeneous, and there is evidence that a large proportion of tumors consists of relatively inactive and sensitive bulk cells together with small populations of more aggressive tumorigenic cells. Interestingly, these subpopulations also seem to be more resistant to many cancer drugs and to possess an elevated tolerance to radiation, indicating that they are more important for treatment outcomes. They are often called cancer initiating cells or “cancer stem cells” (CSC), due to their functional similarities to the stem cells of the body: the capability to undergo self-renewal and differentiation [8].

This study focuses on two cancer types, originating from the head and neck and the colorectal area, which are presented in the following section.

**Head and neck squamous cell carcinoma**

Head and neck cancer comprises tumors of diverse origin, such as the oral cavity, sinonasal cavity, salivary glands, pharynx, larynx and lymph nodes in the neck. More than 95 % of head and neck cancers are squamous cell carcinomas (HNSCC) rising from the epithelial cells of in these regions [9]. HNSCC represents the fifth most common solid cancer worldwide, with
approximately 0.5 million new cases diagnosed every year [1]. Tobacco and alcohol use, as well as infection with high-risk types of human papilloma virus (HPV), are important risk factors for head and neck cancers [9]. Most patients with HNSCC present with advanced-stage locoregional disease with metastases that are located primarily in regional lymph nodes in the neck area. Current treatment options consist of multiple-modality therapy with surgery, radiation, and chemotherapy. Despite significant improvements in these modalities, the overall 5-year survival rates are less than 50 %, a figure that has remained relatively unchanged for the past 30 years [10]. This indicates a need for earlier diagnosis and additional treatment options that target the disease more effectively and with reduced toxicity. Several aberrant signaling pathways and abnormal expression oncogenes and oncoproteins have been identified in HNSCCs, including CD44v6, epidermal growth factor receptor (EGFR) and heat shock 90 protein 90 (HSP90) [9-12], which are described later in the section Targets of the present study.

**Colorectal adenocarcinoma**

After lung and breast cancer, colorectal cancer is the third most frequent type of cancer worldwide and the second cause of all cancer deaths, with approximately 1 million newly diagnosed cases every year [1, 13]. Colorectal cancer occurs in the colon, rectum and/or cecum with appendix. At diagnosis many patients have already developed metastases in the liver or lung, which is one reason why colorectal cancer is associated with a poor prognosis in late stages and has a 5-year survival rate of less than 10 % [14]. Because the progression is quite slow, the risk of acquiring colorectal cancer increases with age; most patients are 50 years or older. Other factors that increase the risk for developing colorectal cancer are related to the general lifestyle: for example, an unbalanced diet that is high in fat and with a high intake of red meat, alcohol and small amounts of fresh fruits and vegetables, together with sparse exercise [15].

Multiple signaling pathways and constitutively activated signaling proteins are involved in the pathogenesis of colorectal cancer, e.g., APC, PTEN, KRAS, BRAF, PI3K and NRAS [16]. KRAS, BRAF and NRAS mutations can all activate the RAS/RAF/MAP kinase pathway, which is located downstream of growth factor receptors such as EGFR. EGFR plays a key role in the activation of these pathways and is commonly overexpressed in metastatic colorectal cancer [17, 18]. Furthermore, metastatic progressing of colorectal cancer is initiated by cancer cells expressing CD44v6, which can function both as a biomarker and as a therapeutic target [19].
Detection

The early detection, diagnosis and treatment of cancer increases the patient’s chance for a full recovery. Cancer is most commonly discovered during a physical exam or as a result of routine tests. Detection methods include endoscopy of the neck, esophagus and gastrointestinal tract, histological analysis of tissue samples (biopsies), laboratory tests of body fluids including blood and urine as well as various imaging techniques. Imaging methods that are in clinical practice include X-ray, ultrasound, magnetic resonance imaging (MRI), computed tomography (CT) and radionuclide based techniques such as single-photon emission tomography (SPECT) and positron emission tomography (PET) [20]. Molecular imaging using SPECT and PET is further described in the Radio-immunodiagnosics section.

Treatment

Alternatives for the treatment of cancer depend on several factors, such as the type and stage of the cancer, location, whether the cancer has recurred subsequent to earlier treatment, and the general health condition of the patient. However, the most common treatment is surgical removal of the tumor together with chemotherapy and/or radiation therapy, and/or support provided by biologic therapy after surgery [6, 21].

Radiation therapy

At present, treatment with ionizing radiation is one of the most successful treatment modalities for solid cancers and is applied to over 50% of all cancers at one stage of their management [3, 22]. Radiation in the form of high energy X-rays, γ-rays and charged particles is used for adjuvant, neoadjuvant or palliative therapy to treat nearly all types of cancer. The common mechanism of action is ionization of molecules in the cells. Although all molecular constituents of the cell are affected, the desired effect of applied radiation is DNA damage. The rays/particles can either directly ionize the DNA molecule, or more likely, indirectly affect it via the formation of free hydroxyl radicals through the ionization of water molecules. The induced effects are manifested as base damage and single or double-strand breaks (DSB) in the DNA [22]. The goal is to disrupt the genetic material to such an extent that repair and replication cannot proceed, thus inactivating or killing the cell.

However, radiation induces DNA breakage in both normal and cancer cells. To some extent this damage can be mitigated by moving and focusing the beam to minimize the exposure to surrounding tissue. Furthermore, most cancer cells have lost or have deficient apoptotic or cell cycle control, as well as poor or lack of DNA repair responses. When normal cells with a functioning cell cycle checkpoint system are affected by DNA damage the
cell cycle phase does not transition to allow the repair of the breaks. The rapid growth and abnormal cell cycle of tumor cells instead sensitizes them to radiation. DNA damage is not corrected, and errors are inherited through subsequent cell divisions, resulting in accumulating damage to the cancer cells and finally leading to reduced growth and cell death [23].

Chemotherapy
Drug therapies, which are applied to reduce tumor size and growth, as well as to suppress both the development and spread of metastases, are referred to as chemotherapy. Chemotherapy treatment can be used as an adjuvant, meaning after surgical intervention, or as a neo-adjuvant before surgery. Chemotherapy is even administered as the primary therapy for palliative treatment to reduce the severity of symptoms and disease progression. The application depends on the progression of the cancer; for example, an adjuvant treatment is usually only given if the cancer has started to spread to lymph nodes or formed metastases in other tissues [6]. Many drugs used in chemotherapy are directed against the growth and division of cells in the entire organism, with effects similar to radiation therapy but generally without the targeting possibilities, which greatly increases the risk for side effects [6]. Thus, the concept of personalized medicine and targeted therapy has become increasingly clinical relevant.

Personalized medicine
The concept of personalized cancer medicine is based on customized treatment decisions after functional diagnosis. Because cancerous diseases are very heterogeneous, not all patients benefit from the same treatment. Patients with a different genetic background may only partially respond or not respond at all to the treatment, or in the worst cases, they may develop adverse effects. Therefore, it is important to tailor healthcare to the individual characteristics, needs, and preferences of the patient. The growing understanding of molecular mechanisms and genetics provides more precise diagnoses, safer drug prescribing, and more effective personalized treatment. Ideally, personalized medicine is practiced during all stages of disease, including prevention, diagnosis, therapy, and follow-up [24-26].

The concept of personalized cancer medicine is gradually integrated in the clinical practice, and during the last decade, more than 50 new targeted drugs for the treatment of solid and hematological malignancies have been approved by the FDA [26]. However, personalized medicine is not yet a commonly used cancer treatment strategy, potentially due to the lack of experience, relatively high costs of the treatments and the accompanying diagnoses [27]. The concept of personalized medicine is presented in Figure 1.
Figure 1. The concept of personalized medicine. Molecular diagnostic tools are used to match patients with treatments that provide them with the best outcomes, while traditionally treated patients with the same diagnosis do not necessarily benefit from the same treatment.

Tumor targeting

Tumor targeting relies on the usage of highly specific targeting agents, such as monoclonal antibodies (mAb), which can target specific molecular abnormalities in the tissue of concern. These targets are antigens, which are specific or overexpressed on tumor cells but are absent or expressed at a low level on normal cells, to avoid damaging the healthy tissue. This concept can be utilized for diagnostic imaging and therapy purposes. The cancer cells either can be selectively destroyed by the targeting agents themselves when they are also designed as effectors, or a payload of cytotoxic substances can be delivered to the malignancy with a lower risk of affecting healthy tissues. This strategy of delivery is used, e.g., in radio-immunotargeting, in which a
Figure 2. The concept of radio-immunotargeting. A radiolabeled compound binds to a target that is overexpressed on the surface of tumor cells, while normal cells do not (or to very small extent) express that target.

Radio-immunodiagnostics aim to non-invasively visualize, characterize and quantify the molecular interactions of biological processes in real time; in living cells, tissues and intact subjects [20, 29]. The wide range of applications of molecular imaging includes diagnostics, drug discovery and devel-
Development, theranostics and personalized medicine. It can be used to precisely locate diseased cells in the body, which allows for appropriate treatment planning and treatment response monitoring [29]. Radio-immunodiagnostic imaging techniques are most useful in combination with CT or MRI scans, which allow co-localization of the tracer and the precise anatomical position, so called "multimodality imaging" [20, 29]. Table 1 summarizes the features of the central imaging modalities that are used preclinically and clinically.

Molecules that are suitable for imaging are e.g., antibodies, antibody fragments, scaffold proteins, ligands and peptides. Small molecules (below 100 kDa) are preferable for imaging since they provide high contrast images in shorter time intervals due to better tissue penetration, shorter circulation and biological half-lives and efficient clearance from the body [29]. Imaging vectors are further described in the section Targeting agents.

Table 1. Features of the central imaging modalities used preclinically and clinically.

<table>
<thead>
<tr>
<th>Modality</th>
<th>Temporal Resolution</th>
<th>Spatial Resolution</th>
<th>Sensitivity</th>
<th>Cost</th>
<th>Safety Profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>Minutes</td>
<td>50-200 µm (preclinical) 0.5-1 mm (clinical)</td>
<td>ND</td>
<td>+</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>MRI</td>
<td>Minutes-hours</td>
<td>25-100 µm (preclinical) ~1 mm (clinical)</td>
<td>10^{-3}-10^{-5} M</td>
<td>++</td>
<td>No ionizing radiation</td>
</tr>
<tr>
<td>SPECT</td>
<td>Minutes</td>
<td>1-2 mm (preclinical) 8-10 mm (clinical)</td>
<td>10^{-10}-10^{-11} M</td>
<td>+</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>PET</td>
<td>Seconds-minutes</td>
<td>1-2 mm (preclinical) 5-7 mm (clinical)</td>
<td>10^{-11}-10^{-12} M</td>
<td>++</td>
<td>Ionizing radiation</td>
</tr>
</tbody>
</table>

**Single-photon emission computer tomography (SPECT)**

In SPECT, tracers labeled with radionuclides (e.g., $^{99m}$Tc, $^{111}$In, $^{123}$I, $^{67}$Ga) that emit gamma ray photons or high-energy X-ray photons are used, with an energy range of 100-300 keV [30]. Here, one photon is detected at a time by a single or a set of collimated radiation detectors. Today, most sensors are based on single or multiple NaI(Tl) scintillation detectors.

SPECT imaging is cheaper than PET but it is less sensitive [31].

**Positron emission tomography (PET)**

In PET, the radioisotope attached to a targeting molecule undergoes positron emission decay and two oppositely directed (180°) 511 keV photons are emitted that can be registered by a circular scanner via coincident detection. By tracking the photons, computer simulations reconstruct three-dimensional images of the source of the photons. Radioisotopes that can be used for PET imaging include $^{11}$C, $^{13}$N, $^{15}$O, $^{18}$F, $^{64}$Cu, $^{62}$Cu, $^{124}$I, $^{76}$Br, $^{82}$Rb,
^{89}Zr and ^{68}Ga and ^{18}F which is the most commonly used isotope [32-34]. ^{18}F-labeled fluorodeoxyglucose (^{18}F-FDG) is used to assess the metabolic activity of tumors.

PET imaging has many advantages compared with SPECT, in particular its high sensitivity and spatial resolution. The disadvantages of PET are the relatively high costs, limited use for dual isotope imaging and that the majority of the compatible radionuclides must be produced with a cyclotron, preferably on site because of the short half-life of the radionuclides [29, 35].

Radio-immunotherapy

Targeting cancer with radiolabeled molecules for therapy purposes has not achieved the same success as radionuclide targeting as a diagnostic tool. However, treatment of lymphoma patients with CD20 targeting antibodies (e.g., ^{131}I-Tositumomab and ^{90}Y-Ibritumomab tiuxetan) has realized great clinical success [36]. The clinical outcomes of radio-immunotherapy of solid disease have been moderate but are currently undergoing broad preclinical and clinical investigations [37-40].

The radiation used for therapy can be of alpha (e.g., ^{211}At, ^{225}Ac, ^{212/213}Bi, ^{227}Th) or beta type (e.g., ^{90}Y, ^{67}Cu, ^{131}I, ^{186/188}Re, ^{177}Lu), or it can be applied as Auger electrons (e.g., ^{125}I, ^{111}In), resulting in cytotoxic, genotoxic and apoptotic effects [41]. The range of negatively charged beta emitters is in the order of a few millimeters in tissue, which is suitable for larger tumors and metastases. Higher radionuclide concentrations of beta emitters compared with alpha emitters are required for comparable cell killing. However, crossfire of the associated penetrating radiation along the beta particle path length decreases the need to target each cancer cell with a radionuclide emitter. Targeting of the nucleus is crucial for effective tumor cell killing with Auger emitters because of the nanometer range of the tracks. By using short-range high-LET radiation, the effect on the closely situated target cell is maximized in comparison to the distal healthy tissue. Factors that influence the clinical success of radio-immunotherapy are the amount and quality of the delivered radioactivity, biological and radionuclide half-life and the oxygenation state of the tumor cells by simultaneous competition against enzymatic DNA repair mechanisms [42].

Theranostics

The youngest field of nuclear medicine is the field of theranostics, which combines the modalities of diagnostic imaging and therapy. Theranostics deliver therapeutic drugs and imaging vectors concomitantly within the same dose [43]. Therefore, diagnostic imaging including response monitoring of the disease can be followed by personalized treatment utilizing the same
agent. Therapeutic radionuclides that can be used for molecular imaging, e.g., $^{177}$Lu or $^{90}$Y are of particular interest in this approach [43, 44].

Targets and targeting agents

Targets of the present study

**CD44 and its variant CD44v6**
The CD44 cell-surface glycoprotein plays a role in the facilitation of cell-cell and cell-matrix interactions through its affinity for hyaluronic acid. In addition, it is known to impart adhesion and is also involved in the assembly of growth factors on the cell surface, for example, EGFR and HER4 [45, 46]. Dysfunction and/or altered expression of the protein causes various pathogenic phenotypes. Additionally, CD44 has been shown to be highly expressed in cancer cell subpopulations with CSC-like properties, e.g., in HNSCC and colorectal tumors [47-50].

![Figure 3. A) Gene map of human CD44. CD44s does not contain variable exons. The exons v2-v10 are alternatively spliced. B) Simplified overview of CD44. CD44 is a transmembrane molecule, which consists of a cytoplasmic domain, and extracellular hyaluronan binding domain and variable domain.](image)

CD44 is encoded by a single gene consisting of 20 exons. The standard form (referred to as CD44s) is encoded by exons 1-5 and 15-20. The exons that are lacking in CD44s are called CD44 exon isoform variants (referred to as CD44v1-10) [51]. While evidence of exon v1 expression exist in many spe-
cies, in humans the exon contains a stop codon and has not been found expressed, see also Figure 3.

Additionally, 19 different splice variants have been found that are generated by alternative splicing of the CD44 mRNA, all of which are expressed at various levels in different tissues, and the roles of these variants are not fully understood [52]. Certain CD44 splice variants, in particular those containing CD44 exon variant 6 (CD44v6), have been associated with disease progression, tumor cell invasion and metastasis. CD44v6 expression has been found in several cancers, including breast, gastrointestinal, colorectal and HNSCC [46, 51, 53, 54]. The large selection of CD44 variants combined with different expression patterns in different tissues, normal cells and cancer types, may allow for precise tumor targeting. Several CD44v6 binders are described in the section CD44v6 targeting agents.

**EGFR**

The 170 kDa (mass of the monomer) large transmembrane glycoprotein EGFR (also called ErbB1 or HER1) is a member of the HER/ErbB family, which in addition to EGFR includes three members: HER2/ErbB2/neu, HER3/ErbB3 and HER4/ErbB4. EGFR has several known natural ligands, such as the epidermal growth factor (EGF), transforming growth factor alpha (TGFα), amphiregulin, betacellulin, heparin-binding EGF-like growth factor (HB-EGF) and neuregulins (NRG1, NRG2, NRG3 and NRG4) [17, 55]. EGFR is a receptor tyrosine kinase (RTK) connecting the extracellular space and intracellular signal transduction. After ligand binding to the extracellular domain, the receptor undergoes a shift from the inactive monomeric to the active hetero- or homodimeric form followed by subsequent auto-phosphorylation and induction of several intracellular pathways. PI3K/AKT, JAK/STAT, SRC/Focal adhesion kinase (FAK) and MAPK/ERK are signal cascade pathways that can be activated through EGFR, regulating processes in the nucleus, which lead to proliferation, inhibition of apoptosis, angiogenesis, migration, differentiation, adhesion or metastatic invasion [14, 56]. Important players in the EGFR mediated signal transduction are presented in Figure 4A.

EGFR has been found to be up-regulated and/or modified in multiple cancer types, promoting carcinogenesis and disease progression. Additionally, the expression of EGFR has been associated with resistance to radiation and standard chemotherapy [30, 56], which is why it has become an important target in cancer therapy and is of great interest in cancer research. EGFR gene expression has been shown in 25-77 % of colorectal neoplasms and 80-100 % of HNSCCs, indicating that many patients could benefit from EGFR targeting pharmaceuticals [57].

Two approaches for EGFR targeting are used today; the intracellular suppression of the tyrosine kinase activity, and blocking of the extracellular
domain by specific antibodies or antibody-like structures [58]. Several EGFR targeting molecules are described in the section EGFR targeting agents.

**HSP90**

In recent years, there has been rapid progress in the identification of new molecular targets that could be useful for cancer therapies. Some of these promising targets are members of the heat shock protein family, which is a group of proteins that are induced in response to cellular stress. Their primary function is to establish protein-protein interactions, stabilize three-dimensional protein structures and assist newly synthesized proteins to fold into their correct confirmation. HSP90 is one member of the very diverse HSP group and is named based on its size [59, 60]. Several isoforms of HSP90 have been found in the cytoplasm and in the nucleus, including HSP90alpha and HSP90beta. Although HSP90alpha and HSP90beta show 85 % sequence identity, HSP90beta is constitutively expressed and often referred to as the major form. Both isoforms can be overexpressed in malignant disease [61]. HSP90 contains three functional domains, the ATP-binding, the protein-binding and the dimerizing domain. It uses a complex chaperone regulation cycle by binding and hydrolysis of ATP and co-chaperones, e.g. HSP70, p23, HSP-organizing protein (HOP) and CDC37 [62]. Currently, more than 200 HSP90 client proteins have been found; they are involved in various cellular responses and many are activated in malignancy, as summarized in Figure 4 (e.g. cell cycle progression, migration, growth, cell signaling, activation of transcription factors) [60, 63, 64]. While HSP90 is required in all cells, an increased expression level of HSP90 has been found in several hematologic and solid tumors [11, 65]. Furthermore, tumor cells are particularly sensitive to anti-HSP90 drugs because they are ‘‘oncogene addicted’’ and require especially high levels of the chaperone. This is mainly due to the microenvironment within solid tumors, which includes chromosome and/or microsatellite instability, hypoxia, a low pH and an insufficient nutrient supply. HSP90 overexpression in cancer cells is associated with increased tumor cell survival, an effect that is probably due to stabilization of oncogenic cell signaling proteins, which ultimately prohibits apoptosis. HSP90 client proteins include mutated p53, MEK, FAK, PDGFR, VEGFR2, KIT, ATM, ATR and MET [66-68]. In addition, HSP90 stabilizes proteins which are known to be associated with cell cycle checkpoints like CDK2, CDK3 or CDK4, with constitutive activated signaling proteins like AKT and ERK, or with protection against radiation-induced cell death like HER2, EGFR, AKT and RAF-1. Another example is the stabilization of the constitutively auto-phosphorylated EGFR variant EGFRvIII, which lacks the extracellular ligand-binding domain [64, 69, 70]. EGFRvIII has been shown
to enhance tumorigenicity by increasing proliferation and decreasing apoptosis.

Targeting and inhibition of HSP90 provides the unique possibility of overcoming mutations in multiple downstream signaling proteins, disrupting

Figure 4. A) A schematic overview of select signal transduction proteins and signaling pathways connected with HSP90 and tumorigenesis. HSP90 client proteins (red circle) are present at several levels and key junctions of the oncogenic signaling cascades, implying that HSP90 targeting could affect multiple pathways simultaneously and constitute an effective treatment strategy. B) Schematic illustration of several hallmark traits of cancer which depend on HSP90 client proteins and which are abrogated by HSP90 inhibition.
feedback loops and shutting down several pathways simultaneously. The downregulation of DNA repair proteins could lead to the sensitization of tumor cells to radiation and ultimately be utilized for combination treatment with radiotherapy or radio-immunotherapy, an approach that was investigated in Paper IV.

Figure 5. Schematic overview of the development process of targeting agents.

Development of radiolabeled targeting agents

The development path of new cancer drugs and targeting agents is long and winding, and each phase involves an increase in costs and efforts [71]. Generally, the first phase consists of basic medical science studies of patient material, leading to the identification of a pathology or biochemical process of interest. In the second phase, a specific molecular target is sought via molecular biology and biochemistry, which can either visualize or affect the

Figure 6. Selection of targeting agents: schematic structure and properties.
pathology or the process. This phase is followed by the selection of a specific targeting agent and payload (signaling or therapeutic, as described previously), which involves labeling chemistry and synthesis. After this process has led to a viable targeting compound, the following phase consists of in vitro and in vivo studies of, e.g., the specificity, selectivity and toxicity of the compound. Based on these tests, either the targeting compound or labeling chemistry must be re-visited to optimize the parameters. The final phases move from the biomedical into the clinical domain, where the compound is evaluated in terms of toxicity (Phase 0 and I clinical trials) compared with existing drugs/targeting compounds (Phase II, III clinical trials) [29, 72]. This process is summarized in Figure 5. The majority of the work presented in this thesis focuses on processes 2-5 (discovery, definition and preclinical validation).

Targeting agents
The most studied molecules for imaging and radio-immunotherapy are full size antibodies (~150 kDa). The use of antibodies as radio-immunotargeting agents has many advantages. They have a fairly high affinity and are easily and economically produced. Initial problems of, e.g., murine monoclonal antibodies causing severe immune reactions have been eliminated by routine humanization, or through the de novo production of human antibodies via display methodology (e.g., phage, yeast) [34, 73]. Large targeting vectors, like antibodies, possess desirable pharmacokinetics for radio-immunotherapy due to their slow clearance from the bloodstream and long duration in the circulation during the targeting phase of the tumor, properties that may cause problems for radio-immunodiagnostics for which small molecules are preferable. The structures and characteristics of several targeting agents are displayed in Figure 6.

To further reduce immunoreactivity, Fc interactions with complement and Fc receptors on immune system cells have been removed by depletion of the Fc region, either through the generation of conventional enzymatically derived Fab (~50 kDa) or F(ab’)2 fragments (~100 kDa) or by producing recombinant fragments that lack the immunoglobulin Fe or CH2 domains [34, 74]. These fragments retain the specificity and affinity of the parental antibody. Furthermore, agents with molecular weights of less than 60 kDa are secreted via the kidneys and provide rapid kinetics and circulating half-lives that are measured in hours rather than days. Single-chain antibody variable fragments (scFvs) are even smaller (~30 kDa) than Fab fragments and provide fast clearance and tissue penetration. Depending on the application, the monovalency of scFvs can be a substantial disadvantage. Consequently, scFvs are coupled to generate bivalent single-chain formats, including scFv-Fc fusion proteins (105-110 kDa), minibodies (~80 kDa), and diabodies.
As an alternative to the engineered antibody molecules, recombinant DNA technology enables the development of new binding proteins and the creation of large libraries of potential binders. One example of a new class of binders is the group of scaffold proteins, which includes the designed ankyrin repeat proteins (DARPins), Fynomers, Affibody molecules and Knottins [77]. A major advantage of these molecules is, despite very high affinities, their robustness and tolerance to harsh labeling conditions (e.g., pH, high temperature). Another class of targeting molecules is the natural peptide ligands (5-20 amino acids) and their analogues, which are almost exclusively used for molecular imaging. Peptides can provide excellent high-contrast images, yet not all target receptors (e.g., HER2) possess natural ligands. Moreover, peptides can trigger antagonistic effects [77].

**CD44v6 and EGFR targeting agents**

**CD44v6 targeting agents**

Several CD44v6-targeting conjugates have been investigated preclinically and clinically for therapeutic and imaging applications, mainly in HNSCC [51]. These compounds displayed selective accumulation in the tumor tissue and only minimal uptake in normal tissues like squamous epithelia, oral mucosa, lung, spleen, kidney, bone marrow and the scrotal area [78]. The most frequently studied anti-CD44v6 molecules are variants of the mAbs BIWA and U36 [79-81]. However, in clinical trials of bivatuzumab (BIWA 4) attached to a highly toxic anti-microtubule agent (mertansine), the agent bound to skin keratinocytes and resulted in severe skin toxicity with a fatal outcome in one patient. After this Phase I clinical trial, all development of bivatuzumab mertansine was stopped [82, 83]. In its place, radio-immunotargeting of CD44v6 appeared to be a more promising strategy because radionuclides with the appropriate half-life and energy can avoid skin toxicity [84]. A radio-immunotherapy Phase I trial with the $^{188}$Re-labeled chimeric mAb (cmAb), cU36, resulted in promising anti-tumor effects and stabilized the disease without skin toxicity [85]. PET studies in HNSCC patients with $^{89}$Zr-cU36 permitted the visualization of primary disease as well as metastases in the neck region and was well tolerated by all of the subjects [86].

In addition, the use of radiolabeled antibody fragments, e.g., F(ab’)$_2$ and Fab’ fragments, was even superior to the parental U36 mAb in terms of tissue penetration and tumor to blood ratios at early time points [87]. The current in vitro design and selection based on recombinant combinatorial libraries is a promising strategy for the development of new CD44v6 binders with novel, superior characteristics [88].
**EGFR targeting agents**

The EGFR serves as an attractive target for molecular imaging and therapy, mainly due to the strong correlation between its deregulation and the aggressiveness of the disease [89]. Numerous EGFR targeting compounds are approved by the FDA or in ongoing clinical trials. These drugs either target the extracellular domain of the cell surface receptor or the intracellular tyrosine kinase domain, the so-called tyrosine kinase inhibitors (TKIs). Both the targeting of the extra- and intracellular domains has been used for therapy and molecular imaging.

The most commonly used EGFR targeting drug is cetuximab (Erbitux, ImClone System Inc.), a chimeric IgG1 mAb that blocks receptor activation and downstream signaling by binding to the extracellular ligand-binding domain and inducing internalization [32, 89]. Cetuximab is used as a single agent or in combination with radiotherapy in several solid cancers, e.g., HNSCC and colorectal cancer. Still, the therapeutic potential of the targeting of EGFR remains to be refined and optimized [57]. Two major approaches have been undertaken for the development of EGFR tracers for molecular imaging: labeling of mAbs and their derivatives and small organic compounds.

Cetuximab labeled with various radionuclides has been used in SPECT and PET cameras [90]. $^{89}$Zr-cetuximab and $^{177}$Lu-cetuximab investigated by PET before radio-immunotherapy confirmed the tumor targeting and allowed the estimation of radiation dose delivery to tumors and normal tissues [91]. The expression and quantification of EGFR has been investigated with $^{86}$Y-labeled and $^{64}$Cu-labeled cetuximab [90, 92]. Furthermore, $^{111}$In-cetuximab-F(ab$'$)$_2$ imaging allowed the effects of EGFR inhibition to be monitored in combination with radiation treatment in HNSCC models [93]. Another approach to anti-EGFR antibodies and their derivatives is imaging with natural ligands such as human epidermal growth factor (hEGF, approximately 6.4 kDa) [94]. Also EGF has been labeled with various SPECT radionuclides, including $^{99m}$Tc [95] and $^{111}$In [96]. Small animal PET scans with $^{68}$Ga-labeled DOTA-hEGF or $^{18}$F-FBEM-EGF could visualize EGFR expressing tumors within 5 min to 2 h p.i. [97, 98]. Furthermore, radiolabeled small scaffold molecules [77] or tyrosine kinase inhibitors are interesting anti-EGFR targeting vectors. For example, several Affibody molecules targeting EGFR have demonstrated preferable biodistribution patterns despite a high kidney uptake, as well as superior imaging properties [99, 100]. Moreover, targeting of mutated EGFR variants with different targeting vectors, especially EGFRvIII, is receiving increasing interest in nuclear medicine [73, 101].
HSP90 inhibitors

At present, HSP90 inhibitors are gaining increasing preclinical and clinical attention due to their unique potential for combined targeting of multiple oncogenic protein pathways. Since the initial discovery of the first natural product HSP90 inhibitors, including geldanamycin and radicicol, almost two decades ago, remarkable progress has been achieved in the development of effective and selective drugs against this chaperone [102, 103]. The mode of action for all developed anti-HSP90 compounds is very similar. They bind to the regulatory ATP/ADP pocket in the amino-terminal portion of HSP90, blocking the unfolded client protein from binding and thus causing its ubiquitination and proteasomal degradation, which may lead to apoptosis. Additionally, HSP90 inhibition can trigger the recognition and destruction of cancer cells by the immune system of the patient because the accumulation of misfolded proteins leads to digestion into small peptides, which are presented on the cell surface by MHC class I molecules [104].

It has been found that instead of complex molecules such as radicicol, smaller molecules are more efficient [105]. The synthetic geldanamycin analogs, e.g., 17-Allyl-17-Demethoxygeldanamycin (17-AAG), 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), NVP-AUY922 and NVP-BEP800, have been intensely characterized in vitro and in vivo [106-109]. However, the clinical outcomes of the previously described drugs have been modest due to poor pharmaceutical properties, limited solubility in water, difficulties associated with their formulation and high hepatotoxicity [110]. New synthetic small molecules, second- and third-generation HSP90 inhibitors, are currently developed via the combined use of high-throughput screening and structure-based drug design. These high-affinity HSP90 inhibitors may overcome the limitations of the previously described drugs because they are easier to administer and may have reduced toxicity. One of these promising, novel and high-affinity HSP90 inhibitors is AT13387 (2,4-dihydroxy-5-isopropyl-phenyl)-[5-(4-methyl-piperazin-1-ylmethyl)-1,3-dihydro-isoindol-2-yl] thanone, l-lactic acid salt) [64, 111]. AT13387 is now undergoing evaluation in Phase I and II clinical trials that include patients with prostate carcinomas, refractory tumors, gastrointestinal stromal tumors (GIST) and ALK positive lung cancer [112, 113]. Studies on AT13387 are presented in Paper IV and Paper V.

HSP90 inhibition in combination with other treatments

The main aims of treatment combinations are to achieve synergistic therapeutic effects that account for the potential tumor heterogeneity and provide dose and toxicity reductions and minimize the induction of drug resistance. The development of resistance in particular is a major problem in cancer
patient management and is frequently induced by rapid genetic and epigenetic changes during adaptation to the microenvironment in the tumor or induced by the treatment itself [63]. Targeting and inhibiting HSP90 represents a novel approach to overcome resistance, especially in combination with other treatment options. Preclinical and clinical studies have demonstrated additive and synergistic effects when combining HSP90 inhibitors with other anti-cancer drugs in solid and hematologic tumors [63, 114]. In HER2-positive breast cancer patients who are progressing on the HER2-targeting drug trastuzumab (Herceptin™), proof-of-concept was demonstrated by first-generation HSP90 inhibitors in combination with trastuzumab [114].

Furthermore, the inhibition of HSP90 is an interesting strategy to sensitize tumors towards irradiation. The misfolding, degradation and finally depletion of HSP90 client proteins is associated with antitumor activity and may also radiosensitize cells, because the cell cycle control and DNA repair machineries, among others, are affected [115-118]. Interestingly, several preclinical studies have demonstrated that first generation HSP90 inhibitors do not radiosensitize normal cell lines [116, 119].

In effect, this approach could lead to lower radiation dosages or fewer radiation treatments and potentially reduce systemic exposure and undesirable side effects in normal tissues. Furthermore, this strategy may be useful for restoring efficacy in chemotherapy and radiotherapy-resistant cancers and/or reducing the recurrence of disease.

Current preclinical findings, including the results in Paper IV, indicate that HSP90 inhibitors have promising efficacy as an adjunct to radiation therapy in several types of cancer [105, 117, 118]. Although the radiosensitizing effects of HSP90 inhibitors have been shown in vitro, this concept has not been investigated in a clinical setting. The next logical step is to translate these findings to Phase 0/Phase I clinical studies investigating this therapeutic combination.
Aim

The overall aim was to characterize the heterogeneity of tumor cells and to evaluate new biomarkers for radio-immunodiagnostics and radio-immunotherapy. There is a need to develop new tracers that target these biomarkers and combine treatment strategies to potentiate the synergistic effects between new cancer drugs and radio-immunotherapy, which could lead to improved treatment protocols.

The specific goals were as follows:

- Evaluate new potential targets for radio-immunotherapy and diagnostic imaging, with a focus on the expression fingerprints of CD44 variants
- Investigate radiolabeled CD44v6-targeting fragments as targeting agents for radio-immunodiagnostics (PET and SPECT imaging) of CD44v6-expressing tumors
- Characterize the novel HSP90 inhibitor AT13387 in vitro and in vivo with regard to its potential radiosensitizing effects
- Evaluate biomarkers for imaging and radio-immunotherapy in vivo in combination with HSP90 inhibition
Results

Paper I
New targets for imaging and radio-immunotherapy: CD44 variants

Aim and background
Several studies have found high expression of the molecules CD133 and CD44 and low expression of CD24 to be markers for aggressive subpopulations in various malignancies [8].

The aim of the present study was to identify specific expression fingerprints of these markers, with a special focus on CD44 and its variants CD44v3, v4, v5, v6, v7, v8 and v10, in squamous cell carcinoma cell lines, to identify suitable targets for molecular radiotherapy or diagnostic imaging. Furthermore, we wanted to determine whether the expression of these variants changes under different growth conditions, such as a lack of nutrients (as commonly exists inside solid tumors, simulated by serum starvation), and whether any of the variants is associated with a certain phase of the cell cycle, increased proliferation, migration potential and radioresistance. Such molecular fingerprints could, e.g., be used for patient stratification, therapeutic monitoring, or selective targeting of specific tumor subpopulations.

Table 2. Expression pattern of CD44, CD44 exon variants, CD133, and CD24. The expression levels were measured by flow cytometry and graded on a relative scale to the isotope control (expression baseline).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Expression of the marker</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CD133</td>
</tr>
<tr>
<td>UT-SCC7</td>
<td>+</td>
</tr>
<tr>
<td>UT-SCC12</td>
<td>+</td>
</tr>
<tr>
<td>SCC-25</td>
<td>+</td>
</tr>
<tr>
<td>H314</td>
<td>+/-</td>
</tr>
</tbody>
</table>

- low expression; + moderate expression, ++ high expression; n = 2-6.
Methods

The HNSCC cell lines were characterized in vitro under standard and low serum culturing conditions. Cell proliferation and radiation resistance were studied using clonogenic survival assays. The migratory activity of starved and non-starved cells was assessed using a cell migration scratch (wound-healing) assay. Morphology, antigen expression and cell cycle distribution were assessed by flow cytometry and fluorescence-activated cell sorting (FACS) using DNA binding dyes and fluorescently labeled antibodies.

Results

The investigated HNSCC cell lines lacked or displayed very low expression patterns for CD24. CD133 was moderately expressed in three of the four cell lines, whereas all of the cell lines were highly positive for CD44 expression (see Table 2 and Figure 7A). High and uniform expression levels of the CD44 standard variant (CD44s) and the variants CD44v6 and CD44v7 were measured in all of the cell lines. CD44v3 was highly expressed in some of the studied cell lines, whereas CD44v5 and CD44v8 demonstrated low or no expression. In several cell lines, an interesting subpopulation of very high antigen expression was identified for the variant CD44v4, which was independent of the cell cycle phase. In the cell starvation experiments that were designed to simulate the environment within a large tumor and to increase the numbers of aggressive cancer cells, a major population with dramatically increased expression of CD44, CD44v6 and CD44v7 was identified. Additionally, the morphology of the starved cells was altered, rendering fewer cells that grew as a monolayer and a larger proportion of cells that grew
three-dimensionally. Previously starved CD44++, CD44v6++ and CD44v7++ cells displayed enhanced motility and increased resistance to radiation compared with non-treated cells (Figure 7B).

Conclusion and discussion
By analyzing novel molecular tumor biomarkers, we hope to elucidate the molecular and biological mechanisms responsible for tumor progression and aggressiveness and to identify suitable tumor-exclusive targets that can distinguish aggressive, resistant, and re-growing cancer subpopulations. Successful selective targeting of these subpopulations could lead to improved therapeutic responses and a reduced recurrence of cancer.

The characterization of CD44 and its variants revealed that CD44, CD44v6 and CD44v7 are highly expressed in cultured HNSCC cells, in which they were found to mediate migration, proliferation, and radiation resistance. The markers CD44v6 and CD44v7 are very interesting because they are more specific anti-tumor targets than the commonly assessed CD44s, which is also present to a high extent in normal tissue. We conclude that targeting splice variants containing v6 and/or v7 could provide a basis for more personalized medicine in the future, hopefully enabling improved treatment outcomes together with a better quality of life and longer lifetime expectancies in patients with HNSCC.
Paper II and III
Radiolabeled CD44v6-targeting fragments for radio-immunodiagnostics

Aim and background

Paper I demonstrated that the targeting of CD44v6 could be a viable strategy for radio-immunodiagnostics in HNSCC. For molecular imaging, small tracers such as antibody fragments directed against cell surface molecules appear to be a particularly favorable class of molecules due to the higher contrast and earlier optimal imaging time compared with the full-length parental antibodies.

Therefore, we have developed a fully human, anti-CD44v6 Fab fragment, AbD15179, and a bivalent antibody fragment, AbD19384, engineered from two AbD15179 Fab-fragments corresponding in size to a F(ab')2.

The objective of this study was to evaluate, for the first time, the in vitro and in vivo properties of radiolabelled AbD15179 (Paper II) and of the bivalent antibody fragment AbD19384 (Paper III) as novel targeting tracers for radio-immunodiagnostics of CD44v6-expressing tumors.

Methods

In Paper II the Fab fragment AbD15179 was labeled with 111In and 125I as models for radionuclides that are suitable for molecular imaging with SPECT or PET, respectively. In vitro studies resulted in the characterization of AbD15179 in terms of the species and antigen specificity as well as the internalization properties. The in vivo specificity and biodistribution were then evaluated in a mouse xenograft model using a dual-tumor and dual-isotope approach.

In Paper III, the specificity, binding properties, and interaction analysis of the bivalent antibody fragment AbD19384 were assessed in vitro. 125I-labeled AbD19384 was then studied in mouse xenografts bearing two tumors with different expression levels of CD44v6. Small animal PET/CT scans revealed the uptake and distribution of 124I-labeled AbD19384 and 18F-FDG in mice bearing tumors with both low and high CD44v6 expression at three different time points.

Results

AbD15179 was successfully labeled with both 111In and 125I using the chelator CHX-A"-DTPA [120, 121] and a direct labeling approach with chloramine T (CAT) [88], respectively. Both the radiolabeled AbD15179 conjugates 111In-Fab and 125I-Fab demonstrated selective tumor cell binding in
Figure 8. A) Biodistribution of $^{111}$In-Fab and $^{125}$I-Fab as percentages of injected activity per gram of tissue (%ID/g), excluding the thyroid (inset), which is expressed as percentages of injected activity per organ. B) Tumor-to-organ ratios for A431 tumors and inset: tumor to blood ratios of radiolabelled Fab for both A431 and H314 tumors. The target antigen CD44v6 was expressed moderately in H314 tumors and at high levels in A431 tumors. The animals were sacrificed at 6 h (black bars), 24 h (dark grey), 48 h (light grey) and 72 h (white) post injection. A431 tumors were used as reference for tumor to organ ratio calculations. The animals were sacrificed at 6 h (black bars), 24 h (dark grey), 48 h (light grey) and 72 h (white) post injection. Error bars represent SD, n = 5.
vitro and good tumor targeting properties in vivo (Figure 8A). However, $^{111}$In-Fab displayed higher tumor uptake, slower dissociation as well as higher tumor to blood ratio compared to $^{125}$I-Fab (Figure 8B). On the other hand, uptake in non-target organs like liver, spleen and kidneys was lower for iodinated Fab.

Figure 9. Representative data from SPR analyses of A) AbD15179 and B) AbD19384 binding to CD44v3–10. Five concentrations of each fragment ranging from 3 to 50 nM were injected over a surface with immobilized CD44v3–10 on a ProteOn XPR36 protein interaction array system Bio-Rad. Data are double referenced by subtraction of simultaneous responses from a blank surface and a buffer injection. Experimental data are plotted together with curves drawn from a fitted 1:1 Langmuir isotherm.

AbD19384 was successfully conjugated with either $^{125}$I (using direct chloramine T labeling) or with $^{124}$I (using iodogen labeling (1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril)). SPR analysis confirmed specific binding of AbD15179 and of AbD19384, where AbD19384 displayed a higher affinity combined with improved dissociation rate compared to AbD15179, see Figure 9. Ligand Tracer measurements showed that $^{125}$I-labeled AbD19384 was able to distinguish between CD44v6 high, moderate and low expressing cancer cells (Figure 10A). The labeling method (CAT or iodogen) did not influence the binding properties of the tracer (Figure 10B).

Figure 10. Representative Ligand Tracer measurements of real-time binding of radioiodinated AbD19384. Binding traces using three subsequent concentrations (10, 30, 90 nM) were obtained for at least 1 h per concentration (marked by dotted lines), followed by a dissociation measurement for at least 15 h. A) Binding of $^{125}$I-AbD19384 to A431, H314, UM-SCC-74B, or negative MDA-MB-231 cells. Signal is shown in cps. B) Comparison of $^{125}$I-AbD19384 (labeled using CAT), $^{125}$I-AbD19384 (labeled using iodogen) and $^{124}$I-AbD19384 (labeled using iodogen). Curves were normalized to the percentage of maximum binding.
Furthermore, $^{125}$I-AbD19384 displayed a favorable biodistribution and tumor-specific uptake, which was consistent with the small animal PET/CT study investigating $^{124}$I-labeled AbD19384, as summarized in Table 3. $^{124}$I-AbD19384 clearly allowed the visualization of high CD44v6-expressing tumors, while $^{18}$F-FDG was neither able to detect nor distinguish between CD44v6 high and low-expressing tumors. The contrast and tumor to blood ratios increased with time (up to 72 h) though 48 h p.i. was optimal for imaging (Figure 11).

Conclusion and discussion

We conclude that CD44v6 expression profiling has the potential to be a valuable diagnostic tool in HNSCC patients. We could prove, for the first time, that the novel radiolabeled fragment AbD15179 was able to efficiently target CD44v6-positive tumors in vitro and in an in vivo setting. In particular, $^{111}$In-Fab showed favorable tumor to blood ratios. Furthermore, the results of Paper II demonstrate that radiolabeling can change the kinetic properties of the tracer, emphasizing the importance of repeated characterization of the protein even after radiolabeling.

Table 3. Comparison of tumor uptake of $^{124}$I-AbD19384 relative to blood (heart), as obtained by PET imaging and ex vivo organ distribution. 24 h, 48 h and 72 h refer to time points post administration of $^{124}$I-AbD19384.

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Mode</th>
<th>24 h Mean</th>
<th>24 h SD</th>
<th>24 h N</th>
<th>48 h Mean</th>
<th>48 h SD</th>
<th>48 h N</th>
<th>72 h Mean</th>
<th>72 h SD</th>
<th>72 h N</th>
</tr>
</thead>
<tbody>
<tr>
<td>A431</td>
<td>PET</td>
<td>0.83</td>
<td>0.18</td>
<td>4</td>
<td>1.32</td>
<td>0.32</td>
<td>3</td>
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<tr>
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<td>0.38</td>
<td>4</td>
<td>0.90</td>
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The bivalent antibody fragment AbD19384 was also found to be a promising candidate for the imaging of high CD44v6-expressing tumors. Functional anti-CD44v6 imaging with AbD19384 revealed many advantages compared with imaging using the clinical standard $^{18}$F-FDG, although since only one animal was used, the FDG scan should only be viewed as descriptive.

Based on these results, radiolabeled CD44v6 targeting fragments could be used in the future for patient stratification and the early detection of HNSCC in the clinic. Therefore, they could help to improve the management of head and neck malignancies.
Figure 11. Small animal PET/CT imaging of $^{124}\text{I}-\text{AbD19384}$ in CD44v6 high expressing tumors on the left ($T_1$) and low expressing tumors ($T_2$) on the right flank. The best contrast was obtained at 48 h p.i. $^{18}\text{F}-\text{FDG}$ shown on the right at 30 min p.i. $^{18}\text{F}-\text{FDG}$ demonstrates no clear visualization of tumors, no discrepancy between the high CD44v6 expressing tumor and the low expressing tumor, and uptake in brown fat (B).
The HSP90 inhibitor AT13387 potentiates the effects of radiation

Aim and background

HSP90 overexpression has been demonstrated in HNSCC and adenocarcinomas of the colon. Overexpression is associated with increased tumor cell survival due to the stabilization of oncogenic client proteins. Inhibition of HSP90 provides the possibility to target multiple oncoproteins concurrently and to disrupt several cell-signaling pathways, including feedback loops.

The aim of this study was to assess the treatment efficacy of the novel HSP90 inhibitor AT13387, alone or in combination with radiotherapy. A special focus was to investigate the effects of this inhibitor on migration, the cell cycle and the expression of oncogenic client proteins in vitro in SCC and colorectal cancer cells, with the goal of exploring the mechanisms underlying radiosensitization. Furthermore, we aimed to investigate the in vivo treatment response of commonly expressed cell surface receptors, cell signaling and DNA repair proteins in mouse xenografts.

Methods

Colony-forming and multicellular tumor spheroid assays, flow cytometry, scratch (wound-healing) assays, Western blot analysis of target antigen expression and Pulsed-field gel electrophoresis (PFGE) were first used to evaluate the effect of AT13387 on cell survival, motility, cell cycle distribution, radiosensitivity and DNA repair capabilities in vitro. Potential radiosensitizing effects of the inhibitor were studied in 2D and 3D cell cultures after exposure to external beam radiation. Effects on antigen expression were then assessed in vivo in mouse xenografts bearing CD44v6 high and EGFR high tumors. The animals were treated 5 times on 5 consecutive days with 50 mg/kg AT13387. The tumors were dissected and analyzed using immunohistochemistry (IHC).
Figure 12. Clonogenic survival and Multicellular tumor spheroid growth. A) Clonogenic survival assays. Dose response curves of H314, A431 LS174T and HCT116 cells treated with AT13387 (0.5 nM, 5 nM, 50 nM) and radiation (2, 4 and 6 Gy). The cells were pre-plated in triplicates, incubated with AT13387 24 h later and irradiated 1 h after drug incubation. Colonies with >50 cells were counted. (n ≥ 6-12). All curves are normalized to the plating efficiency of the non-irradiated controls. B) Effects of AT13387 and radiation alone as measured by plating efficiencies of the dataset in A), evaluated with Student’s t-test with * p < 0.05, ** p < 0.01, *** p < 0.001. C) Multicellular tumor spheroid growth. H314 cells and HCT116 cells treated with AT13387 (5 nM, 50 nM, 100 nM), 5 times 2 Gy radiation fractions and combination treatment of 5 nM AT13387 and radiation. 1000-3000 cells were pre-plated in agarose coated 96-well plates, incubated with AT13387 after 24 h and irradiated 1 h after drug incubation. The error bars represent SD, n ≥ 3. All curves are normalized to the size of controls at day 1. D) Example of H341 spheroids after 2, 12 and 22 days.
Figure 13. DNA DSB rejoining capacity, migration distance and cell cycle analysis. 
A) PFGE analysis. H314, A431, LS174T and HCT116 cells were exposed to 200 nM AT13387 for 24 h prior 40 Gy radiation. After irradiation, cells were allowed to repair. Kinetics of DSB end rejoining was calculated by fraction of activity released (FAR) corresponding to DNA of sizes < 5.7 Mbp. The error bars represent SD, n = 4. B) Cell migration assay. Left hand images represent photographs of A431 and HCT116 cultures taken at 0 h (immediately after scratching) and at 24 h with and without AT13387 treatment. The graphs show quantification of the wounded area invaded after 24 h, measured in migrated distance in mm. The error bars represent SD, n ≥ 3. Student’s t-test was used to calculate statistics: * p < 0.05, ** p < 0.01, *** p < 0.001. C) Flow cytometry evaluation of cell cycle progression. The histograms on the left show representative data of DNA content stained with DAPI/PI to show the progression from G1 through the S and G2/M phases. Increasing concentrations of AT13387 resulted in a greater G2/M peak and depletion of the S phase. The panel on the right shows the quantification of the flow cytometry data and statistical significance based on ANOVA with Tukey’s post-test.
Results

AT13387 treatment displayed effective cytotoxic activity in colorectal and SCC cells with low nanomolar IC\textsubscript{50} values. AT13387 significantly radiosensitized the tested cells in 2D and 3D cell culture models, as seen in Figure 12. HSP90 client proteins were efficiently downregulated, both \textit{in vitro} and \textit{in vivo}. Moreover, exposure to AT13387 resulted in G2/M phase arrest, depletion of S phase and significantly reduced migration ability (Figure 13). Immunohistochemical staining of tumor tissue is presented in Figure 14. AT13387 treatment efficiently downregulated HSP90, tumor markers like EGFR and MET, and DNA repair proteins like DNA-PKcs and ATM. CD44v6 expression was not significantly altered by the drug, however.

![Immunohistochemical analysis of A431 tumors. Representative images of ex vivo immunohistochemical stainings for HSP90, EGFR, CD44, CD44v6, DNA-PKcs, ATM and MET expression on A431 tumor xenografts (magnification x10). Mice in the treatment group (n = 6) received 5 doses of 50 mg/kg AT13387 on 5 consecutive days before dissection and analysis. The effect of AT13387 was highest on the expression pattern of HSP90, EGFR and MET.](image1)

Conclusion and discussion

We conclude that AT13387 is a potent new cancer drug with improved pharmacokinetics compared with first-generation HSP90 inhibitors. We showed for the first time the excellent anti-tumor effects of AT13387 alone and in combination with radiation in SCC and in colorectal cancer cells both
*in vitro* and *in vivo*. The synergistic combination effects at clinically relevant drug and radiation doses are especially promising for both reduction of the radiation dose and minimization of side effects, or for an improved therapeutic response.

The mechanism underlying this effect is likely related to the detected downregulation of HSP90 client proteins, which are involved in all hallmarks of cancer. Our results strengthen the case for further clinical studies of HSP90 inhibitors and radiation co-treatment.

Furthermore, the varying treatment responses of cell surface proteins could potentially be used either as biomarkers for the monitoring of AT13387 treatment or as potential targets for radio-immunotherapy when unaffected by HSP90 inhibition.
Paper V
Molecular imaging in combination with HSP90 inhibition

Aim and background

Paper IV demonstrated that the novel HSP90 inhibitor AT13387 promotes the degradation of oncogenic proteins upon binding and acts as a radiosensitizer. However, for optimal treatment, there is a need to identify biomarkers for therapeutic response monitoring, patient stratification and to find suitable targets for combination treatments.

Molecular imaging of shifts in biomarker expression after AT13387 treatment could be a suitable approach to assess the treatment response. One marker for treatment response monitoring identified in Paper IV is of specific interest: EGFR. EGFR expression has been associated with tumor progression and the emergence of chemo- and radiotherapy resistance in several cancer types.

Furthermore, combined treatment modalities such as AT13387 together with radio-immunotherapy might potentiate treatment outcomes due to the radiosensitizing effects of the drug. Paper I showed that CD44v6 is an interesting overexpressed biomarker in HNSCC, and Paper IV demonstrated that CD44v6 was not affected by HSP90 inhibition, implicating CD44v6 as a potential marker for targeted radionuclide therapy in combination with AT13387 in HNSCC.

The aim of this study was to target and monitor EGFR and CD44v6 with suitable radiotracers using PET/CT in animals treated with AT13387 to establish whether they are viable biomarkers for this purpose. The expression of EGFR and CD44v6 was investigated using $^{124}$I-labeled Cetuximab and $^{124}$I-AbD19384, respectively. $^{124}$I-AbD19384 is a novel anti-CD44v6 tracer that was initially investigated (Paper III) in mouse xenografts with EGFR/CD44v6 high and low-expressing SCC tumors. For comparison of the tracers and of the specificity, the metabolic activity of the tumors in control and treated mice was concurrently investigated using the clinical standard $^{18}$F-FDG.

Methods

Cancer cell proliferation, cell toxicity (MTT) assays and radio-immunoassays with $^{125}$I-cetuximab and $^{125}$I-AbD19384 were used to quantify the effect of AT13387 on EGFR and CD44v6 expression in vitro. Inhibitor effects were then assessed in vivo in mice xenograft bearing tumors with high EGFR/CD44v6 and low EGFR/CD44v6 expression. Animals were treated 5 times on 5 consecutive days with AT13387 (50 mg/kg), and were
then imaged either with $^{18}$F-FDG (30 min p.i.) or with $^{124}$I-labeled tracer (48 h p.i.), using small animal PET/CT, followed by ex vivo biodistribution measurements and immunohistochemical analysis.

Figure 15. Expression of EGFR and CD44v6 in A) A431 and B) UM-SCC-74B cells using radio-immunoanalysis. Cells were exposed to 0.01 to 60 nM of $^{124}$I-cetuximab or $^{124}$I-AbD19384 and a 100-fold excess of unlabeled antibody was added at the highest concentrations for unspecific binding correction. The number of cells was counted and radioactivity measurements were performed in a gamma counter, n = 3, error bars represent standard error of the mean (SEM).

Results

Cancer cell treatment with AT13387 caused sufficient cytotoxicity and radiosensitization with low IC$_{50}$ values, below 4 nM. In vitro measurements showed specificity of the radiiodine labeled compounds cetuximab and AbD19384 and downregulation of EGFR after AT13387 exposure in high and low expressing cancer cells, while CD44v6 expression was not affected (Figure 15). Quantitative PET analysis of EGFR with the $^{124}$I-labeled mAb cetuximab demonstrated a significant reduction of EGFR in the AT13387 treated group compared to untreated mice (Figure 16A). CD44v6 expression (visualized with $^{124}$I-labeled AbD19384) and $^{18}$F-FDG uptake were not significantly altered by AT13387 treatment (Figure 16B and C).
Figure 16. Representative small animal PET/CT images of EGFR and CD44v6 high expressing A431 tumors (T1 left posterior flank) and EGFR and CD44v6 low expressing UM-SCC-74B tumors (T2 right posterior flank) in nude mice after intravenous injection (i.v.) of A) 124I-cetuximab, B) 124I-AbD19384, C) 18F-FDG. The upper row shows a representative cross section of the xenograft. The tracer uptake was calculated as quotient of high and low expressing tumors. The lower row displays planar maximum intensity projections (MIP) images of the tracer distribution.

Conclusion and discussion

In conclusion, CD44v6 is not dependent on the molecular chaperone HSP90, and therefore, radio-immunotargeting of CD44v6 in combination with the HSP90 inhibitor AT13387 might potentiate treatment outcomes due to the radiosensitizing effects of the drug. A combination approach may allow a decrease in the radiation doses to normal and dose-limited tissues and may overcome resistance to conventional chemotherapeutic and radiation therapies.

In contrast to CD44v6, EGFR expression levels correlate with HSP90 inhibition, and molecular imaging of EGFR-positive SCC may be used to assess treatment responses to HSP90 inhibitors. Our results indicate that molecular imaging may serve as a tool to monitor responses and complement or replace standard tumor size measurements. Currently, treatment outcomes are measured according to RECIST criteria (response evaluation criteria in solid tumors), which relies to a great extent on the size of the tumor. This could be misleading in many ways, e.g., when the main bulk of the tumor consists of non-tumorigenic cells that are more easily killed. PET imaging for the treatment response has many advantages because it is noninvasive, repeatable and permits the investigation of the whole tumor burden in the body. To further investigate the AT13387 treatment response, repeated PET analysis could be beneficial because the drug-induced effects on biomarkers are temporary, as shown in Paper IV. We believe that treatment follow-up with suitable PET tracers, such as mAb Cetuximab or even smaller conjugates, is likely to be an important method for estimating the duration of the treatment effect as well as for defining personalized drug doses and sched-
ules. Furthermore, imaging of response biomarkers has the potential to serve as an early indicator of treatment adaptation and the development of resistance.
Concluding remarks

This thesis has focused on new diagnostic biomarkers and on radiosensitization strategies following HSP90 inhibition as a step towards more personalized cancer medicine.

A specific focus has been the screening for novel molecular targets such as CD44 exon variants, which were found to provide unique expression patterns in squamous cell carcinoma cells. Furthermore, several new radioconjugates that were tailored to target CD44v6 were evaluated in vitro and in vivo in terms of their specificity, biodistribution and imaging performance. The final part of the work presented herein concerns the new targeted chemotherapeutic compound AT13387 and the potential to use it in combination with radiation treatment. The AT13387 investigation provided promising targets for treatment response monitoring and targeted radionuclide therapy.

The main findings of the thesis are as follows:

- High and homogenous expression of the cell surface protein CD44 and its variants CD44v6 and CD44v7 in HNSCC cell lines
- Serum starvation increases the amount of aggressive cancer cells, which also displays an increased expression of CD44, CD44v6 and CD44v7
- Cells enriched under starvation show increased migration and higher radioresistance in comparison to non-starved cells
- The novel Fab fragment AbD15179 is specific for CD44v6 with high affinity and retention
- AbD15179 can be labeled with both $^{111}$In and $^{125}$I using the chelator CHX-A$^\prime$-DTPA or a direct labeling approach with chloramine T, respectively with sufficient yield and serum stability and no detectable internalization
- The labeling chemistry affects performance parameters such as tumor uptake, dissociation rate and tumor to organ ratios. Indium labeling of AbD15179 induces higher uptake with high tumor to blood ratio compared to iodine labeling, which in turn has less uptake in non-target organs like liver, spleen and kidneys.
- The bivalent fragment AbD19384 is specific for CD44v6 with higher affinity compared to AbD15179 and pronounced retention in vitro
• AbD19384 can be successfully labeled with both $^{125}$I and $^{124}$I using direct labeling with chloramine T and iodogen with sufficient yield and serum stability
• $^{125}$I-AbD19384 shows favorable biodistribution with a similar tumor and organ uptake pattern as the previously described Fab fragment $^{125}$I-AbD15179
• $^{124}$I-AbD19384 is a promising PET tracer showing best imaging qualities after 48 h
• $^{124}$I-AbD19384 is able to discriminate between CD44v6 high and low-expressing tumors, which cannot be achieved with the current imaging standard $^{18}$F-FDG
• The HSP90 inhibitor AT13387 is a potent new cancer drug with highly cytotoxic effects on SCC and colon cancer cells
• Co-treatment of radiation and AT13387 triggers synergistic effects, leading to increased efficacy and tumor cell kill
• AT13387 decreases cell mobility rates and causes G2/M cell cycle arrest and simultaneous S-phase depletion, without affecting DNA DSB rejoining capacity after radiation exposure
• AT13387 efficiently downregulates HSP90 and its oncogenic client proteins DNA-PKcs, ATM, EGFR, MET and AKT involved in DNA repair, cell signaling, growth and proliferation
• The effect of AT13387 on the expression levels of client proteins is transient and recurs after 24 h in the absence of drug exposure
• CD44v6 is not stabilized by the chaperone HSP90 and therefore is not affected by AT13387 treatment
• The treatment response of AT13387 can be visualized by targeting EGFR with $^{124}$I-Cetuximab using PET/CT to quantify the expression level, whereas the treatment effect cannot be distinguished using the current clinical standard $^{18}$F-FDG
• CD44v6 is a promising target for patient stratification in HNSCC
• CD44v6 is a promising target for radio-immunotherapy and AT13387 co-treatment due to the lack of downregulation after HSP90 inhibition
Future studies

We believe that future studies should be directed along several lines of research. First, the results from Paper I must be confirmed and expanded upon using a broader variety of tumor strains and patient material. Such a study should be centered on the large-scale expression analysis of CD44 variant isoforms, which display fingerprint-like features that we anticipate to be associated with distinct patient outcomes. These findings could be used for detection or therapy targeting in a variety of cancer types.

All five studies (Papers I-V) presented in this thesis rely on the well-established method of cancer cell line cultivation. The use of immortalized cancer cell lines in cancer research is a valuable tool for the investigation of cancer, but this method has many limitations. These limitations include the loss of heterogeneity and of genetic alterations over time with extensive \textit{in vitro} pasanging. The use of primary cancer cells derived from solid clinical specimens collected at the time of surgery could be a useful technique to avoid such pitfalls. Such primary cells can be used in 2D and 3D cell culture experiments as well as in mouse xenografts. Despite the representation of the original specific clinical specimen, another advantage is that such primary cells could be sampled from different locations in the patient, e.g., the primary tumor and a different metastasis. Therefore, the establishment of primary tumor cell models, e.g., HNSCC patients, is an attractive approach for future investigations.

Paper II and Paper III demonstrated that radio-immunotargeting of CD44v6 is a promising strategy for early non-invasive diagnosis and HNSCC patient stratification. The investigated conjugates AbD15179 and AbD19384 displayed favorable kinetics, but their targeting properties could be improved. It would be valuable to direct focus toward optimizing the choice of radionuclide and the radiolabeling method. Furthermore, the flexible recombinant origin of AbD19384 might facilitate the development of novel tracers that are adapted either for targeted therapeutic or diagnostic applications, which may help improve the management of head and neck malignancies in the future.

The logical continuation of Paper IV is an \textit{in vivo} therapy study investigating co-treatment with AT13387 and radiation to determine an optimal dose and time schedules to potentiate the combination effect. In such a study, radiation treatment could be administered as an external beam of radi-
ation or in the form of radio-immunotherapy. For this purpose, CD44v6 targeting is promising, because the expression of this molecule is not affected by the inhibition of HSP90. CD44v6 expression has been associated with increased radioresistance, and the targeting of CD44v6 high-expressing cells is of particular interest.

Paper V demonstrated that monitoring of EGFR with PET/CT is a novel approach to investigate the treatment response to AT13387. Other EGFR tracers, preferably smaller conjugates could be tested that allow imaging at earlier time points with high tumor uptake rates and improved contrast. Additionally, imaging at different time points could be beneficial.

Another appealing approach is to monitor the response of the constitutively auto-phosphorylated EGFR variant EGFRvIII in combination with the inhibition of HSP90.
I would like to thank everyone who has contributed to this thesis. In particular, I would like to thank the following people:

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References


A doctoral dissertation from the Faculty of Medicine, 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 Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)