Radioimmunodiagnosis of Head and Neck Squamous Cell Carcinomas

Preclinical Studies

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**Abstract**


Despite improvements in treatment, the prognosis for patients with advanced head and neck squamous cell carcinomas (HNSCC) has only improved to a minor degree. To raise the success rate and minimize morbidity further developments in diagnostics are highly desired. Radioimmunodiagnosis could offer a more specific and sensitive diagnostic method. Herein, we have evaluated different radioimmunoconjugates directed against CD44v6 and epidermal growth factor receptor (EGFR) for imaging of HNSCC. The studies were performed in a murine HNSCC xenograft model.

Initially, the $^{111}$In-labeled anti CD44v6 chimeric monoclonal antibody U36 (cMAb U36) was evaluated. The novel radioimmunoconjugate showed high and accumulating tumor uptake. Since small molecules might be advantageous for imaging, due mainly to their shorter circulation half-life in the bloodstream, we then investigated antibody fragments F(ab')$_2$ and Fab' derived from cMAb U36. The highest tumor-to-blood ratio was achieved with the dimeric antibody fragment F(ab')$_2$, compared with both the intact anti-body and monomeric Fab'.

Furthermore, the possibility of improving EGFR-targeted imaging was explored by pre-blocking EGFR. The liver uptake of injected labeled human epidermal growth factor (hEGF) was significantly reduced when an excess of unlabeled hEGF was injected 30 minutes in advance. However, as hEGF stimulates cell proliferation it may be inadvisable to treat cancer patients with large amounts. Alternatively, pre-blocking with an anti-EGFR Affibody molecule ($Z_{EGFR,955}$); demonstrated similar decrease in liver uptake as unlabeled hEGF. Finally, ($Z_{EGFR,955}$)$_2$ was compared with other Affibody molecules with higher affinity to EGFR, $Z_{EGFR,1907}$ and ($Z_{EGFR,1907}$)$_2$, as pre-blocking agents. In addition, a novel hEGF radioimmunoconjugate, $[^{68}$Ga]$^{[G}$NOTA-Bn-NCS-hEGF was used for EGFR targeting. The dimeric ($Z_{EGFR,1907}$)$_2$ showed greatest reduction in non-tumor uptake, and highest tumor-to-organ ratio in EGFR expressing organs, when injected in advance of the radioimmunoconjugate.

To summarize, the results presented here demonstrate how different radioimmunoconjugates as well as pre-blocking EGFR can improve the radioimmunodiagnosis of head and neck squamous cell carcinomas.

**Keywords:** Head and neck squamous cell carcinoma, radioimmunotargeting, radioimmunodiagnosis

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“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world...”

Louis Pasteur

To Anja, Sigrid and Einar
Original papers

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


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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td><strong>18FDG</strong></td>
<td>$[^{18}F]$-2-fluoro-2-deoxy-D-glucose</td>
</tr>
<tr>
<td><strong>AR</strong></td>
<td>Autoradiography</td>
</tr>
<tr>
<td><strong>cMAb</strong></td>
<td>Chimeric Monoclonal Antibody</td>
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<tr>
<td><strong>CT</strong></td>
<td>Computed Tomography</td>
</tr>
<tr>
<td><strong>DOTA</strong></td>
<td>1,4,7,10-tetraazacyclododecanetetraacetic acid</td>
</tr>
<tr>
<td><strong>EGFR</strong></td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td><strong>EGF</strong></td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td><strong>Fab’</strong></td>
<td>Antigen-binding fragment</td>
</tr>
<tr>
<td><strong>Fab’$_2$</strong></td>
<td>Antigen-binding fragment, dimer</td>
</tr>
<tr>
<td><strong>FDA</strong></td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td><strong>FDG</strong></td>
<td>Fluorodeoxyglucose</td>
</tr>
<tr>
<td><strong>hEGF</strong></td>
<td>Human Epidermal Growth Factor</td>
</tr>
<tr>
<td><strong>HER2</strong></td>
<td>Human Epidermal growth factor Receptor 2</td>
</tr>
<tr>
<td><strong>hMAb</strong></td>
<td>Humanized Monoclonal Antibody</td>
</tr>
<tr>
<td><strong>HNSCC</strong></td>
<td>Head and Neck Squamous Cell Carcinomas</td>
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<tr>
<td><strong>HPV</strong></td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td><strong>IHC</strong></td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td><strong>i.p.</strong></td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td><strong>i.v.</strong></td>
<td>Intravenous</td>
</tr>
<tr>
<td><strong>K$_D$</strong></td>
<td>Dissociation constant</td>
</tr>
<tr>
<td><strong>kDa</strong></td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>**MAb</td>
<td>Monoclonal Antibody</td>
</tr>
<tr>
<td><strong>MRI</strong></td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>Number of subjects/samples</td>
</tr>
<tr>
<td><strong>NOTA</strong></td>
<td>1,4,7-triazacyclononanetriacetic acid</td>
</tr>
<tr>
<td><strong>p.i.</strong></td>
<td>Post-injection</td>
</tr>
<tr>
<td><strong>PET</strong></td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td><strong>RID</strong></td>
<td>Radioimmunodiagnosis</td>
</tr>
<tr>
<td><strong>RIT</strong></td>
<td>Radioimmunotherapy</td>
</tr>
<tr>
<td><strong>s.c.</strong></td>
<td>Subcutaneous</td>
</tr>
<tr>
<td><strong>SCC</strong></td>
<td>Squamous Cell Carcinomas</td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td>Standard deviation</td>
</tr>
<tr>
<td><strong>SPECT</strong></td>
<td>Single Photon Emission Computed Tomography</td>
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</table>
Introduction

Head and neck squamous cell carcinomas

More than 550,000 new cases of head and neck cancers are diagnosed annually, worldwide. In Europe and the USA they account for ~3% of the overall cancer incidence, skin cancers excluded [1]. The majority, over 90%, of head and neck cancers are squamous cell carcinomas (SCC), originating in the epithelium of the mouth, nose, throat, or ear.

Established risk factors for head and neck squamous cell carcinomas (HNSCC) are use of alcohol and tobacco. Notably, human papilloma virus infection has also arisen during recent decades as a significant and increasing risk factor for oral SCC [2].

HNSCC is a complex disease, characterized by clinical, pathological, phenotypical, and biological heterogeneity. The main prognostic factors for head and neck cancer are the tumor size/stage, presence of locoregional metastasis, and anatomic sub site [3-5].

Diagnostics of head and neck carcinomas

The clinical work-up of a suspected head and neck malignancy includes clinical investigation with endoscopy, fine needle aspiration cytology of neck masses, biopsies of suspicious mucosal lesions, and CT and/or MRI for staging of the tumor [6]. Since CT and MRI diagnostics is based on morphology (with its known limitations), benign lesions cannot invariably be distinguished from malignant tumors. Small tumors or metastases are often overlooked due to their small volume. In addition, detection of recurrent disease can be difficult due to changed anatomy following treatment.

Positron emission tomography (PET) is an imaging technique that offers functional information on biochemical processes by uptake and retention of radiopharmaceuticals in the tissue. PET with the positron-emitting glucose analog $^{18}$F-FDG (FDG-PET) provides information on glucose metabolism. In many malignancies, including squamous cell carcinomas, the tumor cells have increased glucose metabolism, compared with neighboring tissue, rendering contrast in images. FDG-PET, in conjunction with morphological diagnostics, has proven valuable in the management of head and neck cancers. Due to its higher sensitivity to smaller disease and added functional information, FDG-PET in combination with CT is superior for the staging of
lymph nodes compared to CT alone [7, 8]. This is important, as the presence or absence of lymphatic metastases has a major impact on the expected survival and the choice of treatment [5, 9]. FDG-PET also appears to be of additional value in unknown primary disease (e.g. in patients demonstrating only with a neck mass) [10, 11] as well as for the detection of distant metastases [12]. It may also be useful in response evaluation and the detection of residual and recurrent disease following treatment [13-15]. Additionally, a recent study showed that FDG tumor uptake values has significant relationship with recurrence and survival in patients with HNSCC [16]. A drawback with FDG-PET is that it shows glucose metabolism and can therefore give false-positive results in inflammatory lymphadenitis or after any surgical intervention [17].

Treatment of head and neck carcinomas

Although therapy recommendations for head and neck carcinomas vary not only between countries but also between institutions within a country, consensus has increased during recent years. Curative treatment aims at complete removal of the tumor and, if present, loco regional metastases, while preserving vital structures and function.

For patients who present with small tumors, i.e. early stage I or II disease, single modality treatment with surgical resection or radiotherapy are common strategies giving good results. However, most patients present with locally advanced disease (stage III or IV) requiring a combination of surgery, radiation, and/or chemotherapy. Due to the application and improvement of free micro neurovasculature tissue grafts during recent decades, resections that are more extensive have become possible [18].

In recent years, immunological treatment with monoclonal antibodies has arisen as a complement to chemotherapy and radiation. Cetuximab™, a chimeric monoclonal antibody directed against the epidermal growth factor receptor (EGFR), is approved for use in head and neck cancer. Clinical studies have shown improved local control when using Cetuximab™ combined with radiation therapy [19].

Despite advances in treatment, the prognosis for patients with advanced HNSCC only improves slowly, with 5-year survival rates of 30–35% [20]. Surgery, external radiation and chemotherapy all imply severe morbidity for the patient. Consequently, improved diagnostics and treatments are highly desirable for “individually designed treatment”, to increase the success rate and minimize morbidity.
Radioimmunotargeting

The technique of radioimmunotargeting uses radioimmunoconjugates, i.e. a targeting molecule labeled with a radionuclide, for imaging or therapy. As targeting molecules, antibodies, antibody fragments or other affinity molecules can be used. The targeting molecule delivers the radionuclide to the targeted antigen and the corresponding cell (Fig.1).

![Figure 1. Schematic illustration of radioimmunotargeting. A targeting molecule (e.g. an antibody) labeled with a radionuclide binds to the target antigen on the cell.]

Radioimmunodiagnosis

The emitted radioactivity from the radionuclide can be used to visualize the targeted structure or for quantification of the antigen, in order to obtain both functional and physiological information, e.g. radioimmunodiagnosis (RID). Both gamma (γ) and positron (β⁺) emitting radionuclides can be used. Gamma emitters are used with a gamma camera as single photon emission computed tomography (SPECT). Positron emitters are used, as described above for ¹⁸F-FDG, in a PET camera. Currently the PET technique is more sensitive and provides better spatial as well as temporal resolution, although it is still more expensive than SPECT. One advantage of SPECT is easier dual-tracer imaging, i.e. two tracers are administered simultaneously with different radionuclides, thanks to the possibility of multiple energy windows. Both PET and SPECT imaging technologies are currently undergoing intense development, as regards both hardware and the image reconstruction software design [21]. For dual-modality imaging, the numbers of installed systems are increasing for both PET/CT and SPECT/CT.

A few radiolabeled antibody tracers have been approved by the American FDA for cancer diagnostics. They are labeled with γ-emitting radionuclides for use with SPECT. For example, ¹¹¹In-labeled antibody capromab pendetide (ProstaScint™) is approved for detection of lymph node metastases and for staging in prostate cancer [22]. ⁹⁹mTc-labeled Fab’ nofetumomab mepenatan (Verluma™) is approved for imaging of small-cell lung cancer. So far, however, the overall clinical impact of radio-labeled antibodies as tracers for
cancer diagnostics has not been so impressive. New targets and tracers as well as improved technology may change this. One example is a recent small
PET-study in patients with known lesions of recurrent metastatic breast can-
cer, where PET with a $^{68}\text{Ga}$-labeled anti-HER2 Affibody molecule added
information to $^{18}\text{F}$-FDG PET/CT. In one patient a lesion in a muscle could
not be distinguished from an inflammatory change by $^{18}\text{F}$-FDG PET, where-
as PET with $^{68}\text{Ga}$-labeled anti-HER2 Affibody molecule showed distinct
overexpression of HER-2 [23].

Table 1. Some radionuclides of interest for imaging.

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>Element</th>
<th>Physical half-life</th>
<th>Emission</th>
<th>Main application</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{11}\text{C}$</td>
<td>Carbon</td>
<td>20 min</td>
<td>Positron</td>
<td>PET</td>
</tr>
<tr>
<td>$^{18}\text{F}$</td>
<td>Fluoride</td>
<td>110 min</td>
<td>Positron</td>
<td>PET</td>
</tr>
<tr>
<td>$^{67}\text{Ga}$</td>
<td>Gallium</td>
<td>78 h</td>
<td>Gamma</td>
<td>SPECT</td>
</tr>
<tr>
<td>$^{68}\text{Ga}$</td>
<td>Gallium</td>
<td>68 min</td>
<td>Positron</td>
<td>PET</td>
</tr>
<tr>
<td>$^{86}\text{Y}$</td>
<td>Yttrium</td>
<td>15 days</td>
<td>Positron</td>
<td>PET</td>
</tr>
<tr>
<td>$^{99m}\text{Tc}$</td>
<td>Technetium</td>
<td>6 h</td>
<td>Gamma</td>
<td>SPECT</td>
</tr>
<tr>
<td>$^{111}\text{In}$</td>
<td>Indium</td>
<td>2.8 days</td>
<td>Gamma</td>
<td>SPECT</td>
</tr>
<tr>
<td>$^{124}\text{I}$</td>
<td>Iodine</td>
<td>4 days</td>
<td>Positron, gamma</td>
<td>PET</td>
</tr>
</tbody>
</table>

Another possible use of RID is quantitative imaging of target expression for
prognosis and guidance of optimal dosage in antibody therapy. Moreover,
targeted radionuclide imaging is superior to biopsy analysis, as it is a non-
invasive technique that can be repeated many times. Thus, errors caused by
biopsy sampling inaccuracy and any disparity with receptor expression in
primary tumors and metastases can be avoided. Additionally, information on
change in target expression over time might be valuable, particularly when
the antibody therapy is combined with other treatments modalities.

Radioimmunotherapy

The emitted radioactivity from the radionuclide can also be used to treat
tumor cells, e.g. radioimmunotherapy (RIT). The objective is to impair DNA
and hence eliminate the tumor cells. Currently, two radiolabeled antibodies,
$^{131}\text{I}$-tositumomab (Bexxar™) and $^{90}\text{Y}$-ibritumomab tiuxetan (Zevalin™), are
used with good results for treatment of non-Hodgkin’s lymphoma [24, 25].
Against solid tumors, clinical results have not yet been as successful. How-
ever, a tumor necrosis binding antibody labeled with iodine 131 ($^{131}\text{I}$-
chTNT) showed promising results for the treatment of advanced lung cancer, but is only approved for treatment in China [26]. In the head and neck area, preclinical studies as well as some small clinical studies have shown encouraging albeit limited results [27-29].

To allow the confirmation of tumor targeting, RID can be performed before RIT with the same antibody. It also gives an estimate of radiation dose delivery to both tumor and normal tissues, making it possible to maximize efficacy of RIT while minimizing organ (and especially bone marrow) toxicity [30].

Molecules for radioimmunotargeting
The choice of targeting molecule and conjugated radionuclide has a major effect on the in vivo behavior of the radioimmunoconjugate. The molecules addressed in this thesis are described briefly, followed by a discussion of important physiological properties of targeting molecules.

Antibodies
Antibodies are a natural component of the immune system; they are heavy (~150 kDa) glycoproteins with unique affinity for a target (antigen). Each antibody contains two identical antigen-binding domains, one on the end of each Fab’ (fragment antigen binding) arm (Fig. 2). Monoclonal antibodies can be produced by mammalian cell cultures, ovarian cell lines, recombinant myelomas, and hybridomas [31].

Antibody fragments
Antibody fragments are enzymatic cleavage products of an intact antibody, or non-native bioengineered antibody-based structures. Enzymatically produced antibody fragments contain either of the fragment antigen-binding arms (Fab’) or both (Fab’)_2 (Fig. 2). They retain their function and orientation of the original antibody antigen-binding regions, in contrast to bioengineered antibody fragment that may suffer from a loss of antigen-binding strength. The size of Fab’ is ~50 kDa and ~100 kDa (Fab’)_2.
Affibody molecules
Affibody molecules (Affibody AB) are small (monomeric form ~7 kDa) triple helix bundle high affinity proteins. They are based on a scaffold derived from a mutated form of the B-domain of staphylococcal protein A [32, 33]. Both the monomeric form and the dimeric form can be constructed.

Peptides
Naturally occurring peptides regulate cellular function and growth in both normal tissue and tumors. One of the natural ligands of EGFR, the human epidermal growth factor (hEGF, molecular weight of 6.2 kDa), has high affinity and a high internalization of the receptor-ligand complex [34].

Comparison of physiological properties of targeting molecules
Antibodies can be produced with high affinity and selectivity of the target antigen. However, these large molecules are also accompanied with some weaknesses in tumor localization and penetration, due to their size. Heterogeneous blood supply limits the delivery of blood-borne molecules to less perfused regions of a tumor. Increased interstitial pressure reduces extravasation of fluid and macromolecules in the high interstitial pressure regions of tissues having abnormal physiology, such as tumors. Further, the large transport distances in the interstitium increase the time for slowly moving macromolecules to reach distal regions of a tumor [35, 36].

Small targeting proteins, on the other hand, have better extravasation, diffusion in intracellular space and tumor penetration, compared with intact antibodies [37-39]. If the size is less than ~65 kDa the targeting molecule will be inclined to first pass renal elimination via the glomerulus [40]. The unbound fraction is then rapidly excreted, typically within hours, leading to higher tumor-to-organ ratios and contrast, favorable from an imaging point of view.
One drawback is that total tumor uptake is less, but in radioimmunodiagnosis, contrast between tumor and neighboring tissue is more important than total tumor uptake. In addition, if the unbound fraction is rapidly excreted, the time between injection and examination is reduced, which is beneficial in the clinical setting. The absolute lower uptake of antibody fragments in normal tissue also makes it possible to inject a larger dose compared with intact antibodies [39]. For RIT, on the other hand, a longer circulation time is desirable to maximize tumor uptake. Therefore, tracers with a molecular weight over ~100 kDa (as intact antibodies or (Fab’)2) can be more suitable. Intact antibodies have typically very long circulation half-lives in plasma, ranging from days to weeks.

The affinity (antigen-binding strength) of the ligand and its internalization are also important properties in targeted imaging and treatment. The affinity should typically be in the nanomolar range; higher affinity could lead to less tumor uptake due to saturation in the tumor periphery [41-43].

![Figure 3. Schematic illustration of the fate of radiometals vs. radiohalogens after internalization of the radioimmunoconjugate.](image)

A high internalization rate of the tracer and its radionuclide is of value especially when the radionuclide is a metal, as $^{111}$In and $^{68}$Ga. Radiocatabolites of radiometal-labeled antibodies usually have a long intracellular retention time, so-called residualizing properties (Fig. 3). Due to their charge and hydrophilic nature, the radiocatabolites have difficulty in penetrating the cell membranes and escaping the cell [44, 45]. Longer intracellular retention times can lead to higher tumor-to-organ ratios, especially if the unbound fraction is rapidly excreted from the blood. However, one should also take into account that high internalization of residualizing radiometals increases radioactivity not only in the tumor, but also in healthy tissue. Renal uptake is of some concern when residualizing radiometals are used with small targeting vectors. Due to their high renal elimination, a significant part may be reabsorbed in the proximal tubules after glomerular filtration [46, 47]. A high predose of cationic amino acids can diminish renal tubular reabsorption of radiometal-labeled fragments [48]. Radiocatabolites of radiohalogens
such as $^{125}$I are generally lipophilic and can diffuse through the cellular membranes (Fig. 3). A high internalization rate could therefore be disadvantageous if the tracer-radionuclide complex is quickly degraded and eliminated from the cell [49].

Target antigens in head and neck squamous cell carcinomas

Ideally, the target antigen should be readily accessible, with high and preferably homogeneous expression in the tumor tissue and metastases. Expression in healthy tissue should also be low or absent in order to obtain a high tumor to healthy tissue uptake ratio (Fig. 4). In this thesis, the focus has been on targeting CD44v6 and EGFR; other possible target antigens are also discussed [vide infra].

![Figure 4. Schematic illustration of radioimmunotargeting with a radiolabeled antibody. The overexpression of a target antigen on the tumor cells can be used to achieve a greater concentration of radionuclides in the tumor compared with healthy tissue.](image)

**CD44v6**

The CD44 glycoproteins are a family of transmembrane cell adhesion molecules, mediating contact between cells and between cells and the extracellular matrix. Members of the CD44 family differ in the extracellular domain by insertion of variable regions via alternative splicing, giving rise to approx. 20 known CD44 isoforms [50].

CD44v6, CD44 splice variants containing the v6 domain, have been suggested to be involved in aggressive tumor behavior [51], and as a tumor-promoting protein [52]. CD44v6 is associated with a poorer prognosis in various cancers [53]. The mechanism by which CD44v6 promotes metastasis may be related to its co-receptor function of c-Met and VEGFr-2 [54]. In primary HNSCC, strong expression of the CD44v6 isoform has been associated with advanced T stage and decreased disease-free survival [55]. Homogeneous and strong expressions have been observed in many primary
squamous cell carcinomas, including their corresponding metastases, though only in a subset of epithelial tissues [56-58]. However, its expression in normal mucosa can hamper tumor detection of small tumors in the upper aero-digestive tract. But the weak expression in lymphoid tissue and absent expression in muscle tissue enables possible contrast in the surrounding tissues in the head and neck area [59].

**EGFR**

The epidermal growth factor receptor (EGFR, also known as HER1 and ErbB-1) is a transmembrane protein in the epidermal growth factor receptor family. EGFR is activated by the binding of EGF, TGF-α and a few other ligands, regulating essential cellular functions such as cell proliferation, motility, survival, and differentiation [60]. Following ligand binding and activation, the receptor dimerizes with either an identical receptor or another receptor in the same family. The activation leads to downstream signaling via tyrosine kinase activity, followed by internalization of the ligand-receptor complex. The receptor is then either recycled to the cell surface, or degraded.

Dysregulated signaling of EGFR is common in many malignancies [60]; in HNSCC it is due mainly to over-expression, as EGFR mRNA is over-expressed in up to 90% of cancer cells. In contrast to other malignancies, mutations of the EGFR kinase in head and neck cancer are relatively rare, at least in “western” countries [61, 62]. Metastases originating from HNSCC have displayed a high EGFR expression, similar to that in primary tumors [63]. EGFR expression in normal tissue is weak with the exception of liver hepatocytes and to a lesser degree the G.I.-tract and salivary glands. In HNSCC, overexpression of EGFR is associated with a more rapid tumor progression and early metastases [64-67]. EGFR expression in a HNSCC tumor can also be used to predict local-regional relapse after radiotherapy [68] and to select patients who might benefit from accelerated radiotherapy [69, 70].

Other targets in head and neck squamous cell carcinomas (not included in this thesis).

**CAIX**

Tumor hypoxia is associated with a poorer prognosis in several tumor types, with increased metastatic potential and increased resistance to treatment [71]. Hypoxia can stabilize and increase the levels of the transcription factor hypoxia-inducible factor-1 (HIF-1). One of the downstream targets of HIF-1 is carbonic anhydrase IX (CAIX) which has been confirmed as an intrinsic hypoxia-related cell marker [72, 73]. In head and neck cancer, increased expression of CAIX, evaluated by immunohistochemical methods, has been related to poor prognosis [74]. Since CAIX shows relatively strong expres-
sion on the cell surface, while normal tissue expression is restricted to low-levels in the gastric mucosa, bile ducts, and small intestine, it may be used as a target for noninvasive imaging of hypoxia [72]. A recent PET study on mice bearing HNSCC tumor xenografts using $^{89}$Zr-cG250-F(ab$^\prime$)$_2$ directed against CAIX, showed good tumor penetration and accurate microscopic hypoxia localization [75].

cMet

c-Met, the tyrosine kinase receptor for hepatocyte growth factor (HGF), is overexpressed in a variety of tumors and plays a central role in malignant transformation [76]. A recent study in HNSCC has suggested that c-Met could serve as marker for cancer stem cells able to initiate tumor growth, chemotherapy resistance and metastasis [77].

MAb DN30, directed against the extracellular portion of c-Met, has shown promising results inhibiting growth of xenografts and metastatic spread of cancer cells in nude mice [78]. Inhibition of the HGF/c-Met pathway may also be a promising approach for therapy. Semi-quantitative PET imaging of c-Met expression using $^{89}$Zr-MAb DN30, has been performed successfully in nude mice bearing tumor xenografts [79].

VEGF

As with other solid tumors, angiogenesis plays an important role in the pathogenesis of HNSCC. In a study comprising 31 patients with oral HNSCC and 10 healthy individuals, immunohistochemical analysis revealed VEGF overexpression in 50% of the tumors, whereas the healthy oral mucosa was not immunoreactive to an anti-VEGF antibody. The strongest VEGF staining was found at the margin of the invading tumors [80].

The monoclonal antibody Bevacizumab, directed against VEGF, has been approved for the treatment, in combination with chemotherapy, of metastatic colorectal cancer, non-small-cell lung cancer, breast cancer and glioblastoma multiforme. A problem with Bevacizumab therapy is the lack of predictive biomarkers and means to predict the efficacy of anti-VEGF therapy. A preclinical PET study of $^{86}$Y-labeled Bevacizumab demonstrated its usefulness for assessing Bevacizumab uptake and localization [81]. This can be valuable for patient screening, appropriate dosage selection in Bevacizumab treatment as well as dosimetry for RIT with $^{90}$Y-labeled Bevacizumab.
Overall aim

The overall purpose of this thesis was to improve radioimmunotargeting of head and neck squamous cell carcinomas, particularly for diagnosis by SPECT and PET imaging. Thus, more specifically, to evaluate novel and unique radioimmunoconjugates for detection of the antigen CD44v6 and EGFR, as well as ways to reduce non-tumor uptake of labeled hEGF.
The present study

Targeting CD44v6 (paper I and II)

Background

Chimeric monoclonal antibody cMAb U36 recognizes the antigen CD44v6 [82]. The \textit{in vivo} properties of cMAb U36 as evaluated in mice, makes it interesting for both imaging and therapy of HNSCCs [83, 84]. Clinical biodistribution studies on patients with head and neck cancer have shown accumulation of $^{99m}$Tc and $^{186}$Re in the tumors when conjugated with cMAb U36 [27, 85, 86], and the humanized version of cMAb U36 (BIWA 4) [28, 29]. However, small molecules with their better tumor penetration and shorter circulating half-life vs. intact antibodies, are likely to yield better imaging results [37, 38]. Labeled antibody fragments, especially (Fab‘)$_2$, have shown encouraging results for RID, with rapid accumulation in tumor and faster clearance from the blood and non-tumor tissues, compared with the intact antibody [75, 87, 88]. To our knowledge, antibody fragments have not previously been investigated as CD44v6-targeting molecules. F(ab’)$_2$ fragments against a different target in HNSCC (E48) have proved just as effective as the parent antibody for tumor and lymph node detection, using planar gamma camera [86].

Objectives

The aim with paper I was to evaluate the novel radioimmunoconjugate $^{111}$In-cMAb U36, for possible imaging with SPECT. $^{111}$In has residualizing properties and a half-life suitable for radionuclide-based imaging with antibodies (Table 1). We also wanted to evaluate imaging of HNSCC with $^{111}$In-cMAb U36, using a planar gamma camera.

The aim with paper II was to compare the biodistribution of radiolabeled cMAb U36 with the novel cMAb U36 fragments F(ab’)$_2$ and Fab’. We hypothesized that highest tumor-to-blood uptake ratio would be achieved with F(ab’)$_2$, mainly due to its shorter circulation time in the blood. The tumor-to-organ ratio in tissue relevant for head and neck cancer was also investigated. Additionally, we were interested in studying the penetration depth and distribution in tumor tissue by autoradiography.
Results

The *in vitro* experiments in paper I demonstrated that cMAb U36 tolerated the labeling conditions, using the chelator CHXA’’-DTPA, very well. The immunoreactive fraction of the radioimmunoconjugate was very high (>95%), and the blocking experiments with non-labeled antibody verified that the binding was CD44v6-specific. *In vitro* tests also confirmed that no detectable amounts of the negative control, A33 antigen, were present on the HNSCC xenograft cells (data not shown). \(^{111}\)In-hMAb A33 binds to A33, a transmembrane glycoprotein which is expressed in colorectal carcinomas.

The biodistribution of \(^{111}\)In-cMAb U36 in nude mice bearing SCC-9 xenografts, presented as percentage of the injected dose per gram of tissue (% ID/g), is shown in Fig. 5. The mice were euthanized and the organs excised at 6, 24, 48, 72 or 168 h post-injection (p.i.). The organs were weighed and the radioactivity content was measured in a gamma-counter. The uptake (%ID/g) was calculated as the percentage of injected dose per gram of tissue.

![Figure 5. Biodistribution of \(^{111}\)In-labeled cMAb U36 in tumors and selected organs. Animals were sacrificed at 6, 24, 48, 72, and 168 h p.i. The unit %ID/g denotes the activity expressed as a percentage of injected dose per gram of tissue; thyroid is presented as injected dose per organ. Error bars represent the standard deviation (6h \(n=4\), 24h \(n=3\), 48h \(n=4\), 72h \(n=4\), 168h \(n=3\)). Beginning at 24 hours p.i. the radioactive uptake by the tumors was greater than in the examined organs. At 168 h p.i., tumor uptake exceeded uptake in]
blood five-fold (tumor 54.7 SD:±16.6 %ID/g, blood 10.91±1.06 %ID/g), while uptake in all other organs was less than in blood.

The tumor-to-blood ratio of $^{111}$In-cMAb U36 increased continuously during the study, from 0.24±0.10 to 4.45±1.35 168 h p.i., as shown in Fig. 6. At the 72 h p.i. time point the ratio was significantly higher than the negative control, $^{111}$In-hMAb A33. The tumor-to-organ ratio was calculated as activity/g tumor divided by activity/g organ.

![Figure 6.](image)

*Figure 6.* The blood-to-tumor ratio of $^{111}$In-labeled cMAb U36 and hMAb A33 in SCC9 xenograft-bearing nude mice. Animals injected with $^{111}$In-labeled cMAb U36 were sacrificed at 6, 24, 48, 72, and 168 h p.i. Animals injected with $^{111}$In-labeled huA33 were sacrificed at 24 and 72 h p.i. The blood-to-tumor ratio was calculated as activity per gram of tumor divided by the activity per gram of blood. Error bars depict the standard deviation (A33 $n=3$, U36 6h $n=4$, 24h $n=3$, 48h $n=4$, 72h $n=4$, 168h $n=3$).
Images of nude mice with SCC-9 xenograft tumors, obtained with a planar gamma camera 72 h after injection with 1.2MBq $^{111}$In-cMAb U36, are shown in Fig 7. The tumors were small, 220 and 60 mg, and had considerably lower uptake, 7.9 and 11.0 %ID/g, compared with the mice in the biodistribution group. The tumor-to-blood ratios were 0.95 and 1.55 respectively. Despite the low tumor-to-blood ratio and small size, the tumors could be clearly visualized, together with the blood-rich central organs.

Figure 7. Nude mice bearing HNSCC xenograft tumor injected into tail vein with 15 μg (1.2MBq) $^{111}$In-labeled cMAb U36. The planar gamma camera images were taken 72 h p.i. Bright yellow-white areas represent high radionuclide uptake.

In paper II we evaluated the novel cMAb U36 antibody fragments F(ab')$_2$ and Fab', produced by GenScript ® [89]. The antibody and the fragments were conjugated with $^{125}$I, by direct labeling using Chloramine-T. The in vitro experiments demonstrated that the resulting radioimmunoconjugates were stable and were of good radio-chemical purity, with values ranging from 94-100%. The affinity for CD44v6, measured using saturation curves with a fixed quantity of UT-SCC7 cells and different concentrations of labeled antibody fragments, were $2.5 \times 10^{-8} \pm 4 \times 10^{-9}$ M and $3 \times 10^{-8} \pm 8 \times 10^{-9}$ M for F(ab')$_2$ and Fab' respectively.
The biodistribution of $^{125}$I-labeled cMAb U36, F(ab’)$^2$ and Fab’ was studied in nude mice bearing UT-SCC 7 HNSCC xenografts. The mice were euthanized and the organs excised at 16, 24 or 48 h p.i. Organs were weighed and their radioactivity content was measured in a gamma-counter. Fig. 8 shows the proportion of the injected dose per gram of tissue (% ID/g).

The uptake of cMAb U36 in the tumors was clearly greater compared with fragments. In addition, the non-specific retention in the healthy organs, apart from thyroid and kidney, was also greater for cMAb U36. In the kidneys Fab’ showed a tendency for greater uptake, though only significantly higher compared with F(ab’)$_2$ at 16 h p.i. The antibody fragments displayed a tendency to maximum activity at the first time point; 16 h p.i., F(ab’)$_2$ uptake was 0.4 ± 0.1 %ID/g (blood) and 0.9 ± 0.2 (tumor), and Fab’ uptake was 0.5 ± 0.1 (blood) and 0.4 ± 0.1 (tumor). For cMAb U36 there was instead a tendency to highest activity at the second time point, 24 h p.i. in both the 12.5 ± 3.4 (blood) and 8.8 ± 3.7 (tumor).

![Figure 8. Biodistribution of $^{125}$I-labeled cMAb U36 and the antibody fragments F(ab’)$_2$ and Fab’ in tumors and selected organs. Animals were sacrificed at 16, 24 or 48 h p.i. Data are presented as percentages of injected activity per gram of tissue (% ID/g), except for the thyroid which is expressed as percentages of injected activity per organ. Error bars depict the standard deviation (n=4).](image-url)

The tumor-to-organ uptake ratios of $^{125}$I-labeled cMAb U36, F(ab’)$_2$ and Fab’ in selected organs are presented in Fig. 9. The tumor-to-blood ratio of F(ab’)$_2$ exceeded that of cMAb U36 already at 16 h and increased during the period studied; at 48 h it exceeded both cMAb U36 and Fab’. In the tongue, no differences in tumor-to-organ ratio were observed between the different
formats. The tumor-to-salivary ratio showed a clear tendency to higher values for cMAb U36, though significant only for 24 h (F(ab')₂ and Fab') and 48 h (only Fab'). The tumor-to-thyroid ratio was clearly higher for cMAb U36 than for F(ab')₂ and Fab'. Further, in bone, cMAb U36 displayed a higher tumor-to-organ ratio, though significantly higher only at 24 and 48 h p.i.

Figure 9. Tumor-to-organ ratio of ¹²⁵I-labeled cMAb U36 and the antibody fragments F(ab')₂ and Fab' in blood and selected organs. Animals were sacrificed at 16, 24 or 48 h p.i. The tumor-to-organ ratio is expressed as activity/g₄₅ tumor divided by activity/g₄₅ organ. Error bars depict the standard deviation (n=4).

Three mice were injected intravenously with an approx. 7-fold larger dose of labeled cMAb U36, F(ab')₂ and Fab', respectively. Three 10-µm consecutively sectioned samples from each tumor were mounted on glass slides. These were heat-fixed, deparaffinized and then processed for either autoradiography (AR), immunohistochemistry with primary CD44v6 antibody (IHC), or Mayers hematoxylin counterstain. IHC of the tumors showed clearly that the tumor cells expressed CD44v6 in the cell membranes (Fig. 10). The difference in total tumor uptake is also obvious in the AR images. The cMAb U36 tumor showed intense staining, compared with the F(ab')₂ and Fab' tumors.
Figure 10. 10-μm tumor sections with Mayers hematoxylin counterstain, immunohistochemistry with primary CD44v6 antibody, or autoradiography of ¹²⁵I-labeled cMAb U36 and the antibody fragments F(ab’)_2 and Fab’. Arrows indicate identified blood vessels. Images were obtained with a Spot Insight CCD-camera connected to a Nikon Eclipse E400 microscope equipped with a 20x lens.

By using the consecutively sectioned tumor samples, IHC and Mayers hematoxylin counterstain could be employed to identify the blood vessels in the AR-staining. Five uptake regions in the AR image, each measuring 20x40 μm, were then determined along manually selected directions perpendicular to the identified blood vessels. Normalized uptake gradients were calculated as staining intensity normalized to the intensity near the vessel (0-20μm). The uptake gradients from the edges of the vessel down to depth of 100 μm are presented in Fig. 11. The cMAb U36 tumor showed a pattern of more pronounced decrease in uptake related to distance from the vessel, compared with the fragments. The F(ab’)_2 tumor appeared to have the smallest decrease in uptake.
Discussion

The chimeric monoclonal antibody U36 recognizes the CD44v6 antigen [82] which is consistently expressed in many primary squamous cell carcinomas but only in a subset of normal tissues [56-58]. Its potential as a targeted nuclear imaging agent have been shown by PET-studies in head and neck cancer patients [85, 90].

In papers I and II the potential of cMAb U36 and its fragments as tracers for radioimmunodiagnosis was further evaluated. In paper I, we labeled cMAb U36 with the gamma-emitting radiometal $^{111}$In, suitable for SPECT. In clinical use $^{111}$In is combined with various ligands and is readily available; the labeling procedure is also relatively easy. The half-life of 68 h is suitable for radionuclide targeted imaging with intact antibodies. $^{111}$In can also be used as a surrogate marker to represent pure beta emitters, e.g. yttrium-90 for radioimmunotherapy [91]. In paper II, we used the gamma-emitting radiohalogen $^{125}$I (half-life 60 days) as a surrogate for radionuclides more suitable for imaging, such as $^{124}$I, so as to be able to perform autoradiography of the selected tumors. $^{125}$I is readily available and direct labeling of the radionuclide is rapid and simple, usually with a high immunoreactive fraction yield.

The antibody tolerated labeling with $^{111}$In very well. The novel radioimmunoconjugate $^{111}$In-cMAb U36 showed a very high immunoreactive fraction and intact and specific binding to CD44v6 in vitro. Moreover, labeling
the antibody and its fragments with $^{125}$I produced stable radioimmunoconjugates with high radiochemical purity \textit{in vitro}. The \textit{in vitro} studies also showed equal affinity of F(ab$'$)$_2$ and Fab$'$ to CD44v6, with an affinity in the same range as reported for cMAb U36 [92]. In paper I, the targeting specificity of cMAb U36 to CD44v6 was supported by the significantly higher tumor-to-blood ratio after 3 days, compared with the negative control huA33.

The biodistribution study with $^{111}$In-cMAb U36 showed specific, high, and accumulating tumor uptake. The tumors accumulated radioactivity throughout this study, in contrast to other works with $^{111}$In-labeled immunoglobulins where the highest tumor uptake occurred somewhere between 24 and 72 h p.i. [93, 94]. Speculatively, this may be attributed to a stable radioimmunoconjugate \textit{in vivo} and a high internalization rate.

The $^{125}$I-labeled version of cMAb U36 showed a significantly lower uptake in the tumors as well as in liver, kidney and spleen, compared with $^{111}$In-cMAb U36. The tumor-to-blood ratio was therefore inferior for $^{125}$I-cMAb U36. In addition, $^{125}$I-cMAb U36 – as well as the $^{125}$I-labeled fragments – also displayed different kinetics, with decreasing tumor activity over time. The lower uptake can probably be attributed to the larger tumors in paper II and difference in residualizing properties of radiocatabolites from $^{125}$I-labeled and $^{111}$In-labeled antibodies [95]. The activities of $^{125}$I-labeled cMAb U36 in blood, liver, kidney and spleen were in the same magnitude as previously reported with $^{124}$I- and $^{125}$I-labeled cMAb U36 [84, 96].

Of the radioimmunoconjugates studied in papers I and II, $^{125}$I-F(ab$'$)$_2$ achieved the highest tumor-to-blood ratio at comparable time points, despite considerably lower total tumor uptake. The ratio was highest due to a substantially lower blood activity. Even at the first studied time-point, 16 h, the tumor-to-blood ratio exceeded 2. Due to quicker blood clearance, the activity in blood was almost 1/20 for both $^{125}$I-F(ab$'$)$_2$ and $^{125}$I-Fab$'$, compared with $^{125}$I-U36. At 48 h p.i. the $^{125}$I-labeled F(ab$'$)$_2$ had a tumor-to-blood ratio of 4. The second best ratio was achieved with $^{111}$In-cMAb U36, although it only reached values around 4 at 168 h p.i. These results are similar to those found in a study with $^{131}$I-labeled antibody and fragments in nude mice with lung carcinoma tumors [87].

In addition to the tumor-to-blood ratio, the tumor-to-tissue contrast of muscle, salivary glands, and thyroid - and to some extent bone - is also important for imaging in head and neck cancer. There was no significant difference in tumor-to-tongue ratio between the $^{125}$I-labeled radioimmunoconjugates studied. There was however a definite tendency toward higher ratios with cMAb U36 and F(ab$'$)$_2$, with values around 2 offering a possible contrast in imaging.

In the salivary glands, no differences in uptake activity between $^{125}$I-labeled and $^{111}$In-labeled cMAb U36 were observed. The lower tumor-to-
organ ratios of F(\(ab\)')\(_2\) and Fab' in the salivary glands – and bone too – make them less valuable in delineating a tumor in that this area. However, the relatively low uptake in bone of both \(^{111}\)In- and \(^{125}\)I-labeled radioimmunoconjugates indicates a lack of active uptake from the bloodstream and hence limited myelotoxicity. In the kidneys lowest uptake values was achieved with F(\(ab\)')\(_2\). With a size \(\approx\)100 kDa, F(\(ab\)')\(_2\) is less prone to first pass renal elimination via the glomerulus [40], compared with Fab’, hence limiting its toxicity to the kidneys. The low uptake in bone and kidneys may allow larger amounts to be injected, thus possibly improving the results even further.

The \(^{125}\)I-labeled radioimmunoconjugates showed greater thyroid activity compared with \(^{111}\)In-cMAb U36, most likely due to uptake of the free lipophilic iodine catabolites following proteolytic degradation of the immunoglobulin. The activities were of the same order as previously reported for direct-labeled \(^{124}\)I and \(^{125}\)I antibody conjugates [83, 84]. The tumor-to-thyroid ratio was significantly higher for the intact antibody due to much greater tumor activity. Blocking the thyroid with 0.1\% NaI before injecting \(^{125}\)I-labeled tracer can reduce this uptake to some extent. Using other labeling strategies may also improve the targeting potential of halogenated tracers [84, 97].

Since cMAb U36 binds only to human CD44v6, the uptake of the antibody and its fragments in murine tissue must be due to unspecific binding. The extrapolation of the results to targeting HNSCC in humans is therefore rather limited, at least for organs with known CD44v6 expression, such as the thyroid and epithelia of the oral cavity [58, 98]. However, a comparison between the studied agents’ tumor targeting capabilities is still feasible, as they have an equal affinity for CD44v6.

In the study of tumor penetration depth, cMAb U36 showed a more prominent decrease in uptake, related to the distance from blood vessel, compared with the fragments. In addition, IHC with primary CD44v6 antibody confirmed that the tumors expressed CD44v6. One should note that the AR data in this study is merely descriptive, since only three tumor was analyzed. However, the results are consistent with previous studies of penetration depth of macromolecules in tumors [35, 99]. The rate of extravasation of intact antibodies is very low; dense extracellular matrix and high interstitial pressure also limit tumor penetration. Smaller molecules usually exhibit superior extravasation and diffusion in intracellular space, compared with their parental antibody [37, 38].

In the planar gamma camera images of the mice injected with \(^{111}\)In-cMAb U36, the tumors were clearly visible despite the limited spatial resolution. This result is encouraging, since the tumors were small, had low uptake and also a relatively low tumor-to-blood ratio, compared with the biodistribution
group. It is conceivable that imaging with a $^{125}$I-labeled F(ab’)$_2$, with a higher tumor-to-blood ratio at 72 h p.i., would produce even better results.

Chapter summary

In these papers (I and II) we have studied the \textit{in vitro} and \textit{in vivo} tumor targeting characteristics of $^{111}$In and $^{125}$I-labeled cMAb U36, as well as the $^{125}$I-labeled antibody fragments F(ab’)$_2$ and Fab’, derived from cMAb U36. The novel radioimmunoconjugates $^{111}$In-cMAb U36, $^{125}$I-F(ab’)$_2$ and $^{125}$I-Fab’ was stable \textit{in vitro} and showed intact affinity for CD44v6. $^{111}$In-cMAb U36 produced higher tumor activity compared with $^{125}$I-cMAb U36. The antibody fragments had less tumor activity, but also lower blood activity, compared with the antibody conjugates. Thus, the highest tumor-to-blood ratio, in comparable time points, was obtained with $^{125}$I-F(ab’)$_2$ – even higher than $^{111}$In-cMAb U36. $^{125}$I-F(ab’)$_2$ also showed a relatively high tumor-to-tongue ratio and low kidney activity. However, the comparatively high uptake in the thyroid is a concern. A radiometal-labeled F(ab’)$_2$ would probably produce an even higher tumor-to-blood ratio due to residualizing properties, and also less thyroid uptake. Radioimmunodiagnosis with radiolabeled F(ab’)$_2$ directed against CD44v6 can probably improve the diagnostics of HNSCC.
Blocking the EGF receptor (paper III and IV)

Background

Overexpression of EGFR in tumor biopsy tissue samples is associated with faster tumor progression, early metastases [64-67] and may have an impact on the treatment of patients with HNSCC [69, 70, 100]. One of the natural ligands of EGFR, the human epidermal growth factor (hEGF), seems to be a suitable tracer for targeted radionuclide imaging, due to its small size, high affinity for and high internalization of the receptor-ligand complex [34]. Radiolabeled hEGF has demonstrated desirable pharmacokinetics in SPECT and PET imaging studies [93, 101-103]. However, there are concerns regarding targeted radionuclide imaging of the EGFR due to its natural expression in normal organs such as the liver and GI-tract [104]. Even more critical and problematic for imaging of head and neck cancers is the physiological uptake in the salivary glands and oral mucosa, since it may mask the signal from malignant lesions in the head and neck area. It has previously been suggested and demonstrated that the relative uptake in the healthy organs and lesions can be modulated. By varying the tracer amount administered the tumor-to-organ ratios can be improved, most probably due to receptor saturation in the healthy organs [105, 106].

A different approach is pre-targeting, i.e. unlabeled tracer is injected before the radiolabeled tracer. Earlier studies have shown that pre-injection of a large excess of non-labeled EGF causes a significant decrease in subsequently injected radiolabeled EGF uptake in EGFR expressing organs [107, 108].

Objectives

The aim of paper III was to investigate the optimal timing of injected hEGF as a pre-targeting agent. In addition we wanted to explore the use of an anti-EGFR Affibody molecule (Z_{EGFR.955})_2 as a pre-targeting agent. As a blocker it possesses advantageous characteristics of longer circulating time, lower degree of internalization and less EGFR activation, compared with hEGF [34, 109].

In paper IV, our aim was to compare the pre-targeting characteristic of (Z_{EGFR.955})_2 with two other Affibody molecules having higher affinity for EGFR. In addition, we wanted to evaluate the tumor targeting properties of a novel radionuclide conjugate, [^{67}Ga]Ga-NOTA-Bn-NCS-hEGF.
Results

In paper III, the effect of different time intervals (0.5-48h) between pre-administered hEGF and the radionuclide tracer $^{125}$I-hEGF in nude mice bearing A431 squamous cell carcinoma xenografts was evaluated. The control group received only $^{125}$I-hEGF. The mice were euthanized and the organs excised 30 min after the $^{125}$I-hEGF injection. The organs were weighed and the radioactivity content was measured in a gamma-counter. The biodistribution is shown in Fig. 12. With the 30 min interval, the liver uptake of $^{125}$I-hEGF was reduced to 5.8 ± SD:0.4 % of the total organ radioactivity uptake per gram of tissue (%TU/g), compared with 34±4.8 %TU/g for unblocked animals. The tumor uptake was unaffected, resulting in tumor-to-liver ratio of 2±0.9, compared with 0.25±0.1 for unblocked animals. A significant reduction in $^{125}$I-hEGF uptake was also seen in stomach, intestine, and pancreas at this time point (data not shown). Extending the time interval between pre-administered hEGF and $^{125}$I-hEGF to 2½ h abolished this effect.

*Figure 12. Biodistribution of $^{125}$I-labeled hEGF injected intravenously at different time points after i.p. injection of non-radiolabeled hEGF. Inset: Tumor to liver ratio of $^{125}$I-hEGF at different times after injection of non-radiolabeled hEGF. Data are expressed as percentages of total organ activity per gram of tissue. Unblocked = no non-radiolabeled hEGF was injected. Error bars depict the standard deviation ($n = 3$ at 0.5h, 12h, 48h. $n = 4$ at 2.5h and 6h. $n = 6$ at unblocked data point).*
Encouraged by the results with hEGF as a pre-blocking agent, we wanted to investigate the suitability of an Affibody molecule as a blocking agent. The anti-EGFR Affibody molecule \((Z_{\text{EGFR,955}})^2\) competes with hEGF for an overlapping binding site [110]. We also wanted to assess the effect of different amounts of \((Z_{\text{EGFR,955}})^2\) and different pre-targeting time points. In this dual isotope study we labeled the targeting agent \(^{111}\text{In}-\text{hEGF}\), as well as the blocking agent \(^{125}\text{I}-(Z_{\text{EGFR,955}})^2\), and were therefore able to evaluate the biodistribution of both. Nude mice bearing UT-SCC7 squamous cell carcinoma xenografts were injected with either 50, 100 or 200 μg \((Z_{\text{EGFR,955}})^2\), 30 or 60 min later the mice were injected with \(^{111}\text{In}-\text{hEGF}\). A control group received only labeled \(^{111}\text{In}-\text{hEGF}\). The mice were euthanized and the organs excised 1 h after the \(^{111}\text{In}-\text{hEGF}\) injection. The organs were weighed and the radioactivity content was measured in a gamma-counter. As shown in Fig. 13, pre-administration of 50 μg \(^{125}\text{I}-(Z_{\text{EGFR,955}})^2\) 30 min before the administration of \(^{111}\text{In}-\text{hEGF}\) reduced the liver uptake and caused a significant increase in tumor uptake (6.3±0.8 vs. 2.1±1.0 %TU/g for the unblocked group). Larger amounts of \(^{125}\text{I}-(Z_{\text{EGFR,955}})^2\) did not further improve results; on the contrary it reduced tumor uptake (5.7±1.9 and 3.8±1.4 %TU/g for 100 μg and 200 μg respectively).

**Figure 13.** Biodistribution of \(^{111}\text{In}-\text{labeled hEGF}\) injected intravenously 30 min after i.p. injection of various amounts of the anti-EGFR Affibody molecule \(^{125}\text{I}-(Z_{\text{EGFR,955}})^2\). 0 μg = no blocking Affibody molecule was injected. *Inset:* Enlarged graph of tumor uptake of \(^{111}\text{In}-\text{labeled hEGF}\) at different amounts of \(^{125}\text{I}-(Z_{\text{EGFR,955}})^2\). Data are expressed as percentage of total organ activity per gram of tissue, %IA/g. Error bars depict the standard deviation (\(n = 4\)).
Extending the time between $^{125}$I-$(Z_{EGFR,955})_2$ and $^{111}$In-hEGF to 60 min produced results similar to the 30 min group, except for a slightly lower tumor uptake of $^{111}$In-hEGF (data not shown).

Fig. 14 shows the tumor-to-liver ratios, calculated as activity/g$_{tumor}$ divided by activity/g$_{liver}$. With 50 μg $(Z_{EGFR,955})_2$ as blocking agent 30 min before $^{111}$In-hEGF, the tumor-to-liver ratio was six-fold higher in the blocked than in the non-blocked animals. At the higher dose of 100 μg, the uptake of $^{111}$In-hEGF in the tumors was still higher than in the non-blocked mice, though not to the same extent. At 200 μg $(Z_{EGFR,955})_2$, no significant tumor-to-liver ratio was found between groups. When the Affibody molecule was given 60 min before $^{111}$In-hEGF the pattern was similar, though less pronounced (data not shown).

Since the Affibody molecule used for blocking also was labeled $(^{125}$I-$(Z_{EGFR,955})_2$) we were able to study its biodistribution. Fig. 15 shows the uptake values for the tumors and liver. Already at 50 μg of $(Z_{EGFR,955})_2$, the liver uptake of $^{125}$I-$(Z_{EGFR,955})_2$ seemed to have leveled out. With increased amounts of $(Z_{EGFR,955})_2$ there was a tendency, though not significant, toward increased tumor uptake in the 30-min groups.
Figure 15. Biodistribution of $^{125}\text{I}$-labeled $(Z_{\text{EGFR}:955})_2$ injected i.p. 30 min before i.v. injection of $^{111}\text{In}$-hEGF. Data are expressed as percentages of total organ activity per gram of tissue, %IA/g. Error bars depict the standard deviation ($n=4$).

In paper IV, we compared the dimeric $(Z_{\text{EGFR}:955})_2$ (KD 50 nM)) [111] with two Affibody molecules having higher affinity – the dimeric $(Z_{\text{EGFR}:1907})_2$ (KD 1.6 nM) and monomeric $Z_{\text{EGFR}:1907}$ (KD 2.8 nM) [112], as pre-blocking agents. The selected Affibody molecules bind to the same epitope in EGFR and we were therefore able to investigate the effect of off-rate and affinity on the blocking effect. In addition, the targeting was performed using a novel radionuclide conjugate $[^{67}\text{Ga}]\text{Ga-NOTA-Bn-NCS-hEGF}$. The synthesis of $[^{67}\text{Ga}]\text{Ga-NOTA-Bn-NCS-hEGF}$ was a two-step procedure, where hEGF was first conjugated to a bi-functional chelator, $p$-SCN-Bn-NOTA and then labeled with $^{67}\text{Ga}$ at room temperature (Fig. 16). The novel radionuclide conjugate demonstrated high stability in buffer, with constant radio-chemical purity (>95%) and in vitro studies showed a specific binding to EGFR expressing UTSCC-7 cells.

Figure 16. Scheme of $p$-SCN-Bn-NOTA coupling to hEGF.

Nude mice bearing UT-SCC7 squamous cell carcinoma xenografts were injected with non-labeled $(Z_{\text{EGFR}:955})_2$, $Z_{\text{EGFR}:1907}$ or $(Z_{\text{EGFR}:1907})_2$, and 45 min later injected with $[^{67}\text{Ga}]\text{Ga-NOTA-Bn-NCS-hEGF}$. A control group received only labeled hEGF. The mice were euthanized and the organs excised 1 hour after the injection of labeled hEGF. The organs were weighed and the radioactivity content was measured in a gamma-counter. The uptake (%ID/g) was calculated as the percentage of injected dose per gram of tissue.

Results from the biodistribution study are shown in Fig. 17. Generally, all three Affibody molecules were able to reduce the $[^{67}\text{Ga}]\text{Ga-NOTA-Bn-NCS-}$
hEGF uptake in the liver and salivary glands without reducing the tumor uptake. Actually, a tendency toward higher $[^{67}\text{Ga}]\text{Ga-NOTA-Bn-NCS-hEGF}$ uptake in tumor vs. control could be observed for all three blocking groups, though not statistically significant.

![Figure 17](image)

*Figure 17.* Biodistribution of $[^{67}\text{Ga}]\text{Ga-NOTA-Bn-NCS-hEGF}$ in tumors and selected organs. $[^{67}\text{Ga}]\text{Ga-NOTA-Bn-NCS-hEGF}$ was injected i.v. either alone (Control) or 45 min after s.c. injection of indicated Affibody molecules. Data are expressed as percentages of injected activity per gram of tissue (% ID/g). Note the different scale in the two diagrams. Error bars depict the standard deviation ($n=5$).

The tumor-to-organ ratios are presented in Fig. 18. In liver, the dimeric forms of Affibody molecules ($Z_{\text{EGFR:1907}}^2$) (0.27±0.07) and ($Z_{\text{EGFR:955}}^2$) (0.17±0.05) gave higher tumor-to-organ ratio vs. control (0.06±0.01). In salivary glands, significant higher tumor-to-organ ratios were obtain with all the Affibody molecules compared with control (0.32±0.06), the highest tumor-to-organ ratio was obtained with ($Z_{\text{EGFR:1907}}^2$) (1.00 ±0.12).

In tongue and bone, there was no significant difference, although there was a distinct tendency toward higher tumor-to-organ ratios with Affibody molecule block.
Figure 18. The tumor-to-organ ratio of \[^{67}\text{Ga}\]Ga-NOTA-Bn-NCS-hEGF in tumors and selected organs in nude mice bearing UTSCC-7 xenografts. \[^{67}\text{Ga}\]Ga-NOTA-Bn-NCS-hEGF was injected i.v. either alone (Control) or 45 min after s.c. injection of the indicated Affibody molecule. The tumor-to-organ ratio is expressed as activity/g\text{tumor} divided by activity/g\text{organ}. Error bars depict the standard deviation (n=5).

Discussion

In papers III and IV, the potential of hEGF and anti-EGFR Affibody molecules, as pre-administered blockers of labeled hEGF uptake in normal tissue, was evaluated. First we showed that an excess of unlabeled hEGF given 30 min before the radionuclide tracer \(^{125}\text{I}\)-EGF reduced liver uptake without decreasing tumor uptake, and hence provided an excellent tumor-to-liver ratio. A longer period between blocker and tracer gave a poorer result, probably due to the rapid internalization of the natural ligand and replacement of the ligand-receptor complex. Administration of large amounts of hEGF, stimulating cell proliferation, may not be advisable in a patient with cancer. We therefore looked for a more suitable EGFR-binding/blocking molecule.

As a pre-blocking agent of EGFR, the Affibody molecules favor hEGF because of less EGFR activation, longer circulating time in the blood stream and a lower degree of internalization following binding to the receptor [34, 109]. The Affibody molecule \((Z_{\text{EGFR.955}})^2\) compete with hEGF for an overlapping binding site; it have shown to prevent binding of \(^{125}\text{I}\)-hEGF to
EGFR-expressing cell-lines, and hEGF can prevent binding of $^{125}$I-$\text{(ZEGFR:955)}_2$ [113].

By labeling hEGF with $^{111}$In we utilized the residualizing properties of radiometals, hence hopefully improving tumor uptake. Pre-administration of the anti-EGFR Affibody molecule ($\text{(ZEGFR:955)}_2$) did indeed reduce the liver uptake of subsequently injected $^{111}$In-hEGF, and yielded a higher tumor uptake. The tumor-to-liver ratios were six-fold higher than in the unblocked animals. These results were achieved with 50 μg ($\text{(ZEGFR:955)}_2$) injected 30 min before $^{111}$In-hEGF. Higher doses of injected ($\text{(ZEGFR:955)}_2$) did not improve the results, i.e. lowered liver uptake. This can be explained by the fact that $^{125}$I-$\text{(ZEGFR:955)}_2$ uptake in liver reached its maximum level already at 50 μg. The tumor uptake of $^{111}$In-hEGF was greater in the pre-blocking groups compared with control. This is consistent with a previous study where pre-injection of non-labeled hEGF reduced the $^{111}$In-DTPA-hEGF uptake in EGFR-expressing healthy organs, accompanied by increased uptake in the EGFR-expressing tumor xenografts [108].

The reduced $^{111}$In-hEGF tumor uptake in the 30-min groups with increased amounts of $^{125}$I-$\text{(ZEGFR:955)}_2$ could probably be explained by the simultaneous tendency to increased $^{125}$I-$\text{(ZEGFR:955)}_2$ tumor uptake. It is tempting to suggest that with small quantities of injected Affibody molecules, the major uptake is by the liver. With larger quantities, the uptake of Affibody molecules is increased in the tumor as well, thereby blocking the tumor uptake of $^{111}$In-hEGF.

Since the blocking effect of Affibody molecules may be expected to be more prolonged, compared with non-radiolabeled hEGF, we also evaluated a longer time period between blocker and tracer, viz. 60 min. The results were similar although not as good as for the 30-min groups. This may be explained by dissociation of the ($\text{(ZEGFR:955)}_2$)-EGFR complex, or rapid degradation of the tracer-radionuclide complex, followed by elimination of the radionuclide and recycling of the EGFR to the cell surface.

In paper IV, a novel hEGF radionuclide conjugate [$^{67}$Ga]Ga-NOTA-Bn-NCS-hEGF was developed and used as tracer in the in vivo study. We also compared three Affibody molecules, with slightly different properties, as pre-blocking agents. [$^{67}$Ga]Ga-NOTA-Bn-NCS-hEGF demonstrated high stability in vitro and the cellular binding study clearly demonstrated a specific binding to EGFR. Previously, hEGF has been conjugated to DOTA moiety and labeled with $^{68}$Ga under elevated temperature, required by the complex reaction with DOTA. The resulting $^{68}$Ga-DOTA-hEGF conjugate has demonstrated successful localization of tumor xenografts with micro PET [102]. The NOTA complexing moiety offers advantages such as greater stability of the resulting complex and the possibility of performing the labeling synthesis at room temperature [114]. The mild labeling conditions are advan-
tageous for fragile, temperature-sensitive macromolecules. Easy access to $^{68}$Ga from a generator system and kit type labeling at room temperature is of great interest to clinical PET. We used the gamma-emitting $^{67}$Ga as a surrogate for positron-emitting $^{68}$Ga in this study, to take advantage of the longer half-life of the former, thus allowing enough time for the handling of a large group of animals.

In order to achieve high tumor-to-organ contrast, a high uptake of blocker in the liver is desirable thereby increasing blood concentrations of targeting hEGF. As expected from the paper III, all three Affibody molecules were able to reduce the $[^{67}$Ga$]$Ga-NOTA-Bn-NCS-hEGF uptake and improve the tumor-to-organ ratio in the liver. For imaging of head and neck cancers, it is also important for the blocker to saturate EGFR in other organs such as the salivary glands and tongue (mucosa). The ideal blocker should have a high affinity for EGFR and slow off-rate in order to effectively bind and saturate the EGFR in healthy tissue.

The dimeric ($Z_{\text{EGFR:1907}}$)$_2$ with a slower off-rate in vitro and slightly higher affinity than the monomeric $Z_{\text{EGFR:1907}}$ [112], achieved the best results both in liver and salivary glands. The dimeric ($Z_{\text{EGFR:955}}$)$_2$ with much lower affinity [111] produced the poorest results. The lack of a significant reduction in uptake of $[^{67}$Ga$]$Ga-NOTA-Bn-NCS-hEGF in the tongue may be due to its composition of muscle with low EGFR expression and mucosa with high expression. There was however, a tendency toward a lower uptake with all three blocking Affibody molecules, compared with control. The small actual difference in size between monomer $Z_{\text{EGFR:1907}}$ and dimer ($Z_{\text{EGFR:1907}}$)$_2$ seems less important than affinity and off-rate. These results are consistent with a study comparing $^{111}$In-labeled $Z_{\text{EGFR:1907}}$ with $^{111}$In-labeled ($Z_{\text{EGFR:1907}}$)$_2$ in nude mice with xenograft tumors. The dimeric ($Z_{\text{EGFR:1907}}$)$_2$ displayed a greater uptake in both high EGFR-expressing organs and in the low EGFR-expressing organs such as muscle and bone in vivo [112].

In the kidneys, pre-blocking with unlabeled hEGF as well as Affibody molecules gave a greater uptake of radiolabeled hEGF compared with control. The increase in uptake of radiolabeled hEGF in the kidney, is due to partial saturation of EGFR in the liver. Similar results has been reported for unlabeled hEGF [105, 107, 108] and anti-EGFR Affibody molecules [112]. The lack of reduction in tumor activity (paper IV) as well as the greater tumor activity (paper III) in the pre-blocking groups also supports the hypothesis, that partial blocking of EGFR in liver should increase bioavailability of the radiolabeled ligand. The lack of significantly greater activity in blood in the present studies is probably due to fact that animals were sacrificed 1 h p.i. radiolabeled hEGF. The majority of radiolabeled hEGF molecules are probably distributed to EGFR expressing organs within the first 10 minutes [102].
The Affibody molecules used in these studies have been selected to bind human EGFR. Previous studies have shown that the uptake of radiolabeled $(Z_{EGFR:955})_2$, $Z_{EGFR:1907}$ and $(Z_{EGFR:1907})_2$ was significantly higher in murine tissues expressing EGFR than uptake of a non-EGFR-specific control Affibody molecules [112, 115]. Additionally, the uptake in these tissues was significantly reduced when an excess of non-labeled tracer was injected. These are strong indications of cross-reactivity of the anti-EGFR Affibody molecules with murine EGFR. However, the affinity of these Affibody molecules to murine EGFR is probably less compared with human EGFR. The fact that we were able to produce such positive results in these settings is very encouraging – an even more effective blocking might occur in a clinical setting. One should note that anti-EGFR Affibody molecules have not been used in humans yet. However, Anti-HER2 Affibody molecules have been used without adverse events [23].

Chapter summary

Both pre-administration of hEGF and anti-EGFR Affibody molecules reduced the uptake and improved the tumor-to-organ ratio of subsequently injected radiolabeled hEGF in high EGFR expressing organs. Since hEGF stimulates cell proliferation, it may not be advisable to give large amount as a pre-blocker to a patient with cancer. Therefore, the most promising format for blocking is the dimeric high-affinity $(Z_{EGFR:1907})_2$ Affibody molecule. Pre-blocking with anti-EGFR Affibody molecules, especially $(Z_{EGFR:1907})_2$, is a promising method that could improve the outcome of hEGF-based radioimmunodiagnosis. Furthermore, the kit type labeling at room temperature of the novel radionuclide conjugate $[^{67}\text{Ga}]\text{Ga-NOTA-Bn-NCS-hEGF}$ is promising for further $^{68}\text{Ga}$ studies using PET.
Conclusions

In this work, different approaches to improve radioimmunodiagnosis of head and neck cancer have been investigated. Novel and unique radioimmunoconjugates directed to CD44v6 and EGFR were studied in tumor xenograft-bearing mice. In addition, methods of reducing the organ uptake of radioabeled EGF was evaluated.

More specifically, we have shown that:

- cMAb U36 can be labeled with $^{111}\text{In}$, using the chelator CHXA”-DTPA, with no adverse labeling effects.
- The novel radioimmunoconjugate $^{111}\text{In}$-cMAb U36 displays a favorable biodistribution with specific, high, and accumulating tumor uptake.
- The enzymatically derived cMAb U36 antibody fragments F(ab’)$_2$ and Fab’ can successfully be labeled directly with $^{125}\text{I}$.
- The novel radiolabeled antibody fragment $^{125}\text{I}$-F(ab’)$_2$ displays an improved tumor-to-blood ratio, compared with both $^{111}\text{In}$- and $^{125}\text{I}$-labeled cMAb U36.
- The non-specific organ uptake of $^{125}\text{I}$-F(ab’)$_2$ is lower than that of both $^{111}\text{In}$- and $^{125}\text{I}$-labeled cMAb U36, allowing larger amounts to be administered.
- Radioimmunodiagnosis with labeled F(ab’)$_2$, targeting CD44v6, may improve the outcome of HNSCC tumor imaging.
- hEGF can be conjugated to the bi-functional chelator p-SCN-Bn-NOTA.
- NOTA-Bn-NCS-hEGF can be labeled effectively with $^{67}\text{Ga}$ at room temperature.
- The novel radioimmunoconjugate $[^{67}\text{Ga}]$Ga-NOTA-Bn-NCS-hEGF is stable and shows specific binding to EGFR-expressing cells.
- Easy kit type labeling at room temperature of the novel radionuclide conjugate $[^{67}\text{Ga}]$Ga-NOTA-Bn-NCS-hEGF is promising for further $^{68}\text{Ga}$ studies using PET.
- Liver uptake of injected labeled hEGF can be reduced substantially by pre-blocking with an excess of unlabeled hEGF, given 30 minutes earlier.
- Pre-blocking with unlabeled hEGF does not reduce tumor uptake of subsequently injected labeled hEGF, resulting in a high tumor-to-liver ratio.
- Pre-blocking with the anti-EGFR Affibody molecule (Z$_{\text{EGFR:955}}$)$_2$ gives a similar reduction in liver uptake, as with unlabelled hEGF.
- 50 μg of (Z$_{\text{EGFR:955}}$)$_2$ given 30 minutes before injecting labeled hEGF seems to be an optimal dose in mice.
- With the high-affinity anti-EGFR dimeric Affibody molecule (Z$_{\text{EGFR:1907}}$)$_2$ as a pre-blocker, improved tumor-to-organ ratios in the liver and the salivary glands of labeled hEGF can be achieved.
Pre-blocking EGFR with \((Z_{\text{EGFR, 1907}})_2\) may improve the outcome of HNSCC tumor imaging with labeled hEGF.
Future projects

With the long term goal to find radioimmunoconjugates suitable for radioimmunodiagnosis of HNSCC in humans, primarily with PET, it would be interesting to further study targeting of the antigens CD44v6 and EGFR.

Thus, in this work, halogenated (Fab’)_2, derived from cMAb U36, was the most promising radioimmunoconjugate for targeting CD44v6, with superior tumor-to-blood ratio. Future studies should focus on evaluation of radiometal labeled (Fab’)_2-cMAb U36, since a radiometal labeled version would probably produce even greater tumor-to-blood ratio. Moreover, such studies should also include novel humanized antibody fragments targeting CD44v6. Further, since radioimmunodiagnosis with Affibody molecules against other targets have shown encouraging results, it would be interesting to extend the present studies to novel Affibody molecules directed against CD44v6.

The easy labeling, and tumor targeting properties of the novel radioimmunoconjugate [^{67}Ga]Ga-NOTA-Bn-NCS-hEGF is promising for PET-studies with ^{68}Ga. Thus, the tumor targeting properties and kinetics of [^{68}Ga]Ga-NOTA-Bn-NCS-hEGF in non-human PET studies should be further studied. Furthermore, the present studies prompt further evaluation of pre-blocking with the Affibody molecule (Z\text{EGFR:1907})_2.

Provided these studies are successful, clinical PET-studies in patients with HNSCC might be initiated.
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// Karl
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