Precision medicine and targeted therapy

Turning the tables on cancer

ANJA C MORTENSEN
Abstract

An extended understanding of the molecular characteristics of cancer has led to a revolution within the field of precision medicine. This thesis explores the utilization of two targets for precision medicine, namely, CD44v6 and murine double-minute 2 and X (MDM2/X).

A novel mini-antibody construct targeting CD44v6 (AbD19384), was assessed for possible use in radiodiagnostics, while a recombinant full-length anti-CD44v6 antibody based on the same construct, AbN44v6, was evaluated for radio-immunotherapy (RIT) following labeling with $^{177}$Lu and $^{131}$I. Additionally, normal tissue biodistribution and dosimetry was assessed for radiolabeled AbN44v6. The efficacy and mechanisms behind the observed effects of PM2 therapy, a novel stapled peptide that inhibits MDM2/X, were assessed in vitro and in vivo both as monotherapy and in combination with external beam radiotherapy (EBRT). Lastly, combination therapy using RIT ($^{177}$Lu-AbN44v6) and PM2 was evaluated in an in vitro 3D tumor spheroid model.

AbD19384 successfully visualized CD44v6-positive xenografts. Similarly, radiolabeled AbN44v6 bound specifically to CD44v6, and RIT resulted in antigen-dependent, activity-dependent growth inhibition of in vitro 3D tumor spheroids. Biodistribution and dosimetry revealed low-level accumulation in normal tissues and low total effective doses of 0.1 mSv/MBq of injected radioconjugate. PM2-based therapy increased pro-apoptotic protein levels and caused growth inhibition of wt p53 cancer cell lines, which was amplified in combination with radiotherapy. In vivo studies of wt p53 and p53-knockout xenografts demonstrated the specificity of PM2 towards wt p53 cancers and established the potency of combining PM2-based therapy with EBRT.

The work presented in this thesis exemplifies the potency of combination treatments based on a precise understanding of the targeted cancer. Utilizing not one but several of the molecular characteristics of a specific cancer will help turn the tables on cancer and improve patient outcomes in the future.

Keywords: p53 cancers, PM2, radiosensitization, radio-immunotherapy, MDM2/X inhibition, combination therapy, targeted radionuclide therapy, CD44v6

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Research is what I’m doing when I don’t know what I’m doing.
- Wernher von Braun
List of Papers

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


III Mortensen, AC., Spiegelberg, D., Brown, CJ., Lane, DP., Nestor, M. The stapled peptide PM2 stabilizes p53 levels and radiosensitizes wild-type p53 cancer cells. Submitted manuscript.


*Contributed equally.

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<th>Description</th>
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<tbody>
<tr>
<td>ALL</td>
<td>Acute lymphocytic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>AT13387</td>
<td>2,4-dihydroxy-5-isopropyl-phenyl-[5,(4-methyl-piperazin-1-ylmethyl)-1,3-dihydro-isoindol-2-yl]thanone, 1-lactic acid salt</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology 3</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CDKs</td>
<td>cyclin dependent kinases</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CHOP</td>
<td>Cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate, prednisone</td>
</tr>
<tr>
<td>CHX-A”’-DTPA</td>
<td>[(R)-2-Amin-3-(4-isothiocyanatophenyl)-propyl]-trans-(S,S)-cyclohexane-1,2-diamine-pentaacetic acid</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CVP</td>
<td>Cyclophosphamide, vincristine sulfate, prednisone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBRT</td>
<td>External beam radiotherapy</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen binding fragment</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorodeoxyglucose</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma</td>
</tr>
<tr>
<td>GEP</td>
<td>Gastro-enteropancreatic</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>H&amp;N</td>
<td>Head and neck</td>
</tr>
<tr>
<td>HER2</td>
<td>Human EGFR 2</td>
</tr>
<tr>
<td>HLA-DR10</td>
<td>Peptide anchor motif of human leukocyte antigen</td>
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<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HSP90</td>
<td>Heat shock protein 90</td>
</tr>
<tr>
<td>IHC</td>
<td>Immuno-histochemistry</td>
</tr>
<tr>
<td>MDM2</td>
<td>Murine double-minute 2</td>
</tr>
<tr>
<td>MDMX</td>
<td>Murine double-minute X (MDM4)</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilization</td>
</tr>
<tr>
<td>MUC1</td>
<td>Mucin 1</td>
</tr>
<tr>
<td>NET</td>
<td>Neuroendocrine tumor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Noxa</td>
<td>Phorbol-12-myristate-13-acetate-induced protein 1</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>PEM</td>
<td>Polymorphic epithelial mucin</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PM2</td>
<td>SMTide02</td>
</tr>
<tr>
<td>PRRT</td>
<td>Peptide receptor radionuclide therapy</td>
</tr>
<tr>
<td>PSMA</td>
<td>Prostate-specific membrane antigen</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>RAI</td>
<td>Radio-iodinetherapy</td>
</tr>
<tr>
<td>R/R</td>
<td>Relapsed or refractory</td>
</tr>
<tr>
<td>RIT</td>
<td>Radio-immunotherapy</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SCLL</td>
<td>Small cell lymphocytic lymphoma</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SSTR</td>
<td>Somatostatin receptor</td>
</tr>
<tr>
<td>TRNT</td>
<td>Targeted radionuclide therapy</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
</tbody>
</table>
Why one size does not fit all – the rise of precision medicine in cancer

Cancer, the collective name for more than two hundred diseases that share a specific set of hallmarks, has existed throughout human history (1,2). However, despite shared characteristics and common features, each cancer is unique and heterogeneous and should ideally be treated as such (3). The heterogeneity and complexity of the disease as well as the health status and response to therapy of each unique patient, lower the success rates of standardized cancer therapies (4). The importance of identifying the underlying causes and molecular characteristics of individual cancers is becoming increasingly relevant in the field of cancer medicine (5). Once identified, these characteristics can be utilized for more precise therapeutic strategies, which will ultimately benefit cancer patients by, hopefully, increasing therapeutic success rates (3,6).

The term “precision medicine” can be defined in a variety of ways, all of which are based on the same underlying principles: to tailor the treatment of each patient based on individual genetic profile, lifestyle, and environment (2). Through utilizing and applying precision medicine principles, cancer patients will hopefully have a greater life expectancy. For this strategy to become a reality, an enhanced arsenal of both precise diagnostic tools and therapeutic compounds is essential.

Understanding the disease and selecting a strategy

The therapeutic strategy for any cancer is hypothetically more likely to succeed when based on a collective analysis of the molecular characteristics of the cancer (7). Commonly used diagnostic methods (i.e., [18F]-FDG-PET/CT (FDG-PET) and immuno-histochemistry (IHC) following biopsies) offer valuable information regarding both diagnosis and prognosis. However, more precise assessments such as genetic screening or targeted molecular imaging can provide additional information regarding the best strategy to attack the cancer at the molecular level (8,9).

Molecular imaging offers a non-invasive means of complementing commonly used prognostic and diagnostic methods such as IHC following biopsies of the primary tumor. Molecular imaging of cancer, primarily via FDG-
PET, is currently a broadly applicable method that is used in most industrialized territories (10). Although useful and broadly applicable, imaging techniques can be imprecise and can produce false-positive or false-negative results while offering limited information about treatment strategies (11,12). In comparison, targeted molecular imaging aimed at specific, cancer-associated markers, can assist the selection of the therapeutic strategy to be pursued (8,13,14).

The need for precision

For decades, cytostatic chemotherapeutic compounds have been a workhorse and gold standard of cancer therapy accompanied by radiotherapy and surgery (15). Unfortunately, the nonspecific nature of these compounds and the associated toxicities can wreak havoc on healthy tissues without eradicating the cancer (16,17). Additionally, tumor cell resistance, both intrinsic and acquired, present a challenge that is hard to overcome with the broadly applicable, commonly used treatments (18,19). The need for more precise compounds along with fast-paced technological advances has resulted in an increase in the number of targeted cancer therapies available, with many more underway in preclinical or clinical pipelines (20–23). Essentially, targeted therapies may turn the tables on cancer and utilize the specific characteristics of each tumor to eradicate the cancer.

The role of precision medicine in radiodiagnostics and radiotherapy

One test may tell all

The gold standard in molecular imaging of cancer remains FDG-PET. FDG-PET is an established method and a common step on the path to diagnosis and prognosis (10). Though highly useful, FDG-PET has certain limitations (24). This method relies on the increased glucose uptake of rapidly dividing cancer cells (i.e., the Warburg effect (25)), which is less relevant for slow-growing cancers (26). False-positives from either active immune cells during inflammation or adipose tissue further limit the accuracy of the assessments made based on FDG-PET imaging (11). FDG-PET is likely to visualize the primary tumor as well as metastases but offers no additional information regarding possible therapeutic strategies (27). Targeted radiotracers, on the other hand, offer more precise and perhaps crucial information for diagnosis and prognosis and for selecting a therapeutic course of action (13,14,28–30).

Several novel targeted radiotracers have entered clinical trials. One example is the affibody ABY-025, which targets the human epidermal growth factor receptor 2 (HER2) in breast cancer and is labeled with $^{68}$Ga, a short-lived
positron-emitting radiometal (31). Compared to commonly used diagnostic methods (i.e., FDG-PET and IHC), $^{68}$Ga-ABY-025 provides more precise information on the expression of HER2 throughout both primary tumors and metastases (Figure 1). FDG-PET will likely detect both primary tumors and metastatic burden, while IHC of primary tumors may detect an elevated HER2 expression. However, the heterogeneity of both primary tumors and metastases can result in false-negative assessments of IHC following biopsies. As HER2 expression in breast cancer is crucial for the selection of a therapeutic strategy, it is imperative that the expression level be accurately assessed (32). $^{68}$Ga-ABY025 provides the precise information needed, ultimately increasing the success rates of therapy.

![Figure 1. FDG-PET versus $^{68}$GaABY-025 images of Patient A (HER2-negative): (A1) FDG-PET image, (A2) $^{68}$GaABY-025 image. Patient B (HER2-positive): (B1) FDG-PET image, (B2) $^{68}$GaABY-025 image. From Sörensen et al. “Measuring HER2-Receptor Expression in Metastatic Breast Cancer Using $^{68}$GaABY-025 Affibody PET/CT”. Theranostics, 2016.]

Additionally, targeted molecular imaging can be used for monitoring the response to therapy, another element that is gaining importance in precision medicine (30). The ability to estimate whether a patient is responding to the chosen therapy at an early stage is a crucial part of increasing the success rates of therapies. Targeted radiotracers can improve this assessment in a minimally invasive manner (33).

**An age-old but ever-rising star**

Radio-iodinetherapy (RAI), peptide receptor radionuclide therapy (PRRT), and radio-immunotherapy (RIT) are all examples of targeted radionuclide therapies (TRNT). RAI ($^{131}$I-Nal) has been in use for more than half a cen-
tury against thyroid cancer and utilizes an innate mechanism (the sodium-iodide symporter) that ensures uptake and accumulation of the radionuclide in thyroid cells (34). Similarly, the newly approved Xofigo® (\(^{223}\text{RaCl}_2\)) utilizes another innate process, namely, the uptake of Ca\(^{2+}\) ions during bone formation in bone metastases (35,36). \(^{223}\text{Ra}\), an alpha-emitting radionuclide, replaces the Ca\(^{2+}\) ions needed for bone formation, thus irradiating the newly formed bone-metastases of disseminated prostate cancers from within, with limited exposure to adjacent, healthy tissues (37).

Table 1. *Examples of current targeted radionuclide therapies, either approved or undergoing clinical trials.* PSMA, prostate-specific membrane antigen; CEA, carcinoembryonic antigen; SSTR, somatostatin receptor; HLA-DR10, peptide anchor motif of human leukocyte antigen; MUC1, mucin 1; PEM, polymorphic epithelial mucin (identical target to MUC1); GBM, glioblastoma; CLL, chronic lymphocytic leukemia; NSCLC, non-small cell lung cancer; H&N, head and neck (44).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Nuclide</th>
<th>Cancer(s)</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotara DNA</td>
<td>131I</td>
<td>GBM, anaplastic astrocytoma</td>
<td>Phase III</td>
<td></td>
</tr>
<tr>
<td>J591 PSMA</td>
<td>177Lu</td>
<td>Castrate-resistant prostate cancer with bone metastases</td>
<td>Phase II</td>
<td></td>
</tr>
<tr>
<td>Labetuzumab CEA</td>
<td>90Y/131I</td>
<td>Breast, lung, pancreatic, stomach, colorectal carcinoma</td>
<td>Phase III</td>
<td></td>
</tr>
<tr>
<td>Licartin HAb18G/CD147</td>
<td>131I</td>
<td>Hepatocellular carcinoma</td>
<td>Phase II</td>
<td></td>
</tr>
<tr>
<td>Lutathera® SSTR</td>
<td>177Lu</td>
<td>Metastatic GEP NETs</td>
<td>Approved</td>
<td></td>
</tr>
<tr>
<td>Lymphocide CD22</td>
<td>90Y</td>
<td>Non-Hodgkin’s lymphoma/CLL</td>
<td>Phase III</td>
<td></td>
</tr>
<tr>
<td>MIBG Norepinephrine transporter</td>
<td>131I</td>
<td>Neuroblastoma, pheochromocytoma, paraganglioma</td>
<td>Phase III</td>
<td></td>
</tr>
<tr>
<td>Oncolym HLA-DR10</td>
<td>131I</td>
<td>Non-Hodgkin’s lymphoma/CLL</td>
<td>Phase III</td>
<td></td>
</tr>
<tr>
<td>PAM4 MUC1</td>
<td>90Y</td>
<td>Pancreatic adenocarcinoma</td>
<td>Phase III</td>
<td></td>
</tr>
<tr>
<td>Radretumab Fibronectin</td>
<td>131I</td>
<td>Refractory Hodgkin’s lymphoma, NSCLC, melanoma, H&amp;N carcinoma</td>
<td>Phase II</td>
<td></td>
</tr>
<tr>
<td>Theragin PEM</td>
<td>90Y</td>
<td>Ovarian, gastric carcinoma</td>
<td>Phase III</td>
<td></td>
</tr>
<tr>
<td>Xofigo® None</td>
<td>223Ra</td>
<td>Metastatic castration-resistant prostate cancer</td>
<td>Approved</td>
<td></td>
</tr>
<tr>
<td>Zevalin® CD20</td>
<td>90Y</td>
<td>Non-Hodgkin’s lymphoma</td>
<td>Approved</td>
<td></td>
</tr>
</tbody>
</table>

In contrast to RAI and Xofigo®, PRRT and RIT utilize specific cancer-associated molecular targets in order to deliver cytotoxic radiation (38,39). For these methods to be effective, the targeted antigens must be exclusively or predominantly expressed in the cancerous tissues (39). In 2017 and early 2018, the European Medicines Agency and the US Food and Drug Administration approved the radiolabeled somatostatin analogue Lutathera® (\(^{177}\text{Lu-DOTA-Tyr3-octreotate}\)) for treatment of gastro-enteropancreatic neuroendocrine tumors (GEP NETs) (40,41). Clinical trials with Lutathera® gathered promising results in terms of increased progression free survival and improved the quality of life of otherwise terminal patients (42). The remarkable results of the Lutathera® trials further emphasize the potency of TRNT.
As mentioned earlier, RIT exploits the overexpression of cancer-associated antigens on the cell surface. The most established RIT is Zevalin® (ibrutinomab tiuxetan), an anti-CD20 antibody radiolabeled with $^{90}$Y, a beta-emitting radiometal. Zevalin® was approved for therapy of primarily non-Hodgkin’s lymphoma more than a decade ago and continues to provide positive outcomes (43). Recent successes within the field of TRNT in combination with historical successes such as Zevalin® and RAI have rapidly advanced the field of TRNT. As a result, several new TRNTs are undergoing various stages of clinical trials (Table 1) (44).

The established workhorse

External beam radiotherapy (EBRT) is a non-invasive therapy and remains the most commonly used form of radiotherapy worldwide. Given that more than 50% of cancer patients receive EBRT, this method is not only the most common form of radiotherapy worldwide but also among the most common cancer therapies (45). Despite being widely used across the world, EBRT has its limitations. First, EBRT has little effect other than palliative on disseminated disease (46,47). Second, EBRT has limited applicability in cases of tumor-infiltration into vital tissues such as lungs, esophagus or trachea (48). Third, EBRT can result in both short- and long-term side effects such as acute toxicity and secondary malignancies, which are of great concern (49–51).

Despite being a broadly applicable, commonly used therapy, EBRT can be adapted for the field of precision medicine. To avoid adverse effects, there exist guidelines for radiation absorbed doses and doses tolerated by dose-limiting organs. Although such guidelines are based on empirical evidence, they do not account for the fact that each patient can respond differently to therapy (52). The use of a precision medicine-based approach by monitoring and assessing individual responses to ongoing therapy to adjust therapeutic doses may increase success rates or limit side effects (53).

The strategic targets

An overexpressed cell-surface receptor

One of the primary challenges of RIT is the identification of a suitable target. To avoid unnecessary toxicity in healthy tissues, the targeted antigen should be expressed primarily in the cancerous tissues. In an ideal “magic bullet” scenario, the targeting moiety would course through the body and seek out even the smallest of metastasis, while ensuring that the dose of cytotoxic radiation is delivered solely to the cancer.

Several targets have been investigated for use in RIT in past decades. One of these targets, CD44v6, was successfully utilized in a Phase I trial using
Re against head and neck squamous cell carcinoma, yielding promising results for future RIT applications using this strategy (54). The antigen itself is a splice variant of the glycoprotein CD44 (cluster of differentiation 44) cell surface receptor. While CD44 is commonly found in most tissues, the splice variants (CD44v) are much more tissue specific (55). The splice variants are generated as a result of post-translational modifications and alternative splicing (56). Proteins of the CD44 family are predominantly involved in facilitating cell-cell and cell-extracellular-matrix interactions via their main ligand, hyaluronic acid (57). Several of the splice variants have been the focus of cancer research as possible cancer stem cell-like markers and are often associated with a more aggressive disease. CD44v6, in particular, is associated with a more aggressive, invasive, and radio- and chemoresistant disease, which naturally translates to a poor prognosis (58,59). An overexpression of CD44v6 can be detected in several cancers, and when present, is likely expressed at an extreme level (60,61). The expression of CD44v6 in normal tissues is limited primarily to the basal membrane and suprabasal stratum spinosum epithelial layers (62). The combination of the scarce distribution of CD44v6 in healthy tissues and extreme expression in cancerous tissues is what makes CD44v6 an exciting and promising target for RIT.

The guardian of the genome

At the very center of the innate defense against cancer, lie tumor suppressors (63). These guardians help maintain cellular integrity by for example initiating cell cycle arrest, allowing damaged cells time to repair, or inducing programmed cell death, such as apoptosis (64). Perhaps the most well-known tumor suppressor is the transcription factor TP53, sometimes referred to as the guardian of the genome (65). While p53 affects a variety of cellular mechanisms, this protein is most commonly associated with cycle arrest and apoptosis following DNA damage or cellular stress (66). The importance of p53 as a tumor suppressor is evident in the mutational rate of the gene in malignancies. It is estimated that TP53 is mutated in approximately 50% of all human cancers (64). In addition to neutralizing the native tumor-suppressive role of a functioning wild-type p53 protein (wt p53), the mutated p53 protein can acquire gain-of-function properties, which essentially transform the protein into a malicious oncogene (67). In cancers where wt p53 is retained, dysfunctional activation pathways or overexpression of negative regulators of p53 such as murine double-minute 2 (MDM2) limit the innate function of wt p53 as a tumor suppressor and genomic watchdog (68–70).

p53 and cell cycle arrest

Once DNA damage is detected, cell cycle arrest is initiated by wt p53. Once activated, wt p53 induces the expression of p21cip1/waf1, which inhibits cyclin dependent kinases (CDKs) that are essential for cell cycle progression (Figure
2) (71). By inhibiting CDKs, p21 inhibits both the G1-to-S-phase transition and the G2-to-M-phase transition. Furthermore, wt p53 downregulates the expression of cyclin A, which can result in a secondary pause in cell cycle progression into and through the S-phase (66,71).

Figure 2. Simplified schematic depiction of p53-induced cell cycle arrest via induction of p21cip1/waf1.

**Apoptosis**

The role of wt p53 in apoptosis is complex. However, a simplified explanation can be found in the relationship between wt p53 and members of the B-cell lymphoma 2 (Bcl-2) family (Figure 3) (72). The Bcl-2 family can be classified into three subgroups: a group with anti-apoptotic function and two groups with pro-apoptotic function (73). The pro-apoptotic proteins are sectioned into effectors and sensitizers of apoptosis: Bim, Bid and PUMA (p53 upregulated modulator of apoptosis) are examples of effector proteins, which bind with high affinity to anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-xL (72,73). Sensitizer proteins include Noxa (phorbol-12-myristate-13-acetate-induced protein 1) and Bad. Upon wt p53-induced transcription, Noxa localizes to the mitochondria and assists in liberating pro-apoptotic BH3-only (Bcl-2 homology 3) proteins, thereby inducing further inhibition of anti-apoptotic Bcl-2 proteins (72). Inhibition of anti-apoptotic proteins and activation and stabilization of pro-apoptotic proteins culminates in Bax/Bak activation, resulting in mitochondrial outer membrane permeabilization (MOMP) and initiation of caspase-cascades essential for apoptosis (73,74). While these interactions are far more complex than stated, wt p53 remains at the very center, as Bax, Noxa, Puma, Bid, etc. are all downstream targets of wt p53 (73).
Figure 3. Simplified depiction of p53-mediated apoptosis via interactions with pro- and anti-apoptotic members of the Bcl-2 family following p53 activation.

The highly conserved feedback loop balancing the scales

p53 expression in normal cells is nearly non-existent due to the short half-life of the protein, which is between 5-30 minutes depending on the cell type (75). The degradation of p53 in the proteasome lies at the center of this short half-life, a degradation which is facilitated by the main negative regulator of p53; MDM2 (66,76). Regulation of p53 by MDM2 and its structural homolog MDMX (commonly referred to as MDM4) is a highly conserved autoregulatory feedback loop found in all jawed vertebrates and even in some invertebrate species (77). MDM2 acts in a heteromeric complex with MDMX to negatively regulate p53. MDM2/X-heterodimers are more efficient as well as more stable than MDM2-homodimers in terms of p53-inhibition (68,78). The MDM2/X-complex has potent ubiquitin E3 ligase activity, which marks p53 for degradation. Once the complex binds to p53, it facilitates the relocation of the protein from the nucleus to the cytoplasm and ultimately to the 26S proteasome for degradation (79). The conserved autoregulatory relationship between p53 and MDM2/X maintains the cause-and-effect balance of p53 in response to cellular stress (76).
Table 2. Selection of antagonists of the p53-MDM2 protein-protein interaction undergoing clinical trials. AML; acute myeloid leukemia; ALL, acute lymphocytic leukemia; CML, chronic myeloid leukemia; SCLL, small cell lymphocytic lymphoma; R/R, relapsed or refractory (80,81).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Type</th>
<th>Target cancer(s)</th>
<th>Combination</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>RG7112</td>
<td>Cis-imidazoline MDM2 antagonist</td>
<td>Solid tumors, AML, ALL, CML, refractory CLL/SCLL, liposarcoma</td>
<td>Doxorubicin</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soft-tissue sarcoma</td>
<td>Cytarabine</td>
<td>I</td>
</tr>
<tr>
<td>RG7388</td>
<td>Cis-imidazoline MDM2 antagonist</td>
<td>Multiple myeloma</td>
<td>Ixazomib citrate, dexamethasone</td>
<td>I/I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R/R AML</td>
<td>Cytarabine</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R/R Follicular lymphoma</td>
<td>Obinutuzumab</td>
<td>I/I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Diffuse large B-cell lymphoma</td>
<td>Rituximab</td>
<td>I/I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R/R AML</td>
<td>Venetoclax</td>
<td>I/I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Prostate cancer</td>
<td>Idasanutlin with abiraterone or enzalutamide</td>
<td>I/I</td>
</tr>
<tr>
<td>SAR-405838</td>
<td>Spiro-oxindole MDM2 antagonist</td>
<td>Advanced cancer</td>
<td>Pimasertib</td>
<td>I</td>
</tr>
<tr>
<td>MK-8242</td>
<td>Piperidines MDM2 antagonist</td>
<td>AML</td>
<td>Cytarabine</td>
<td>I</td>
</tr>
<tr>
<td>AMG232</td>
<td>Piperidinone MDM2 antagonist</td>
<td>R/R AML</td>
<td>Trametinib</td>
<td>lb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R/R multiple myeloma</td>
<td>Carfilzomib, lenalidomide, dexamethasone</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced solid tumors and multiple myeloma</td>
<td>Trametinib and dabrafenib</td>
<td>lb/Ia</td>
</tr>
<tr>
<td>DS-303b</td>
<td>Imidazothiazole MDM2 antagonist</td>
<td>AML, ALL, CML, R/R multiple myeloma, advanced solid tumors and lymphomas</td>
<td>LEE011</td>
<td>I</td>
</tr>
<tr>
<td>HDM201</td>
<td>Dihydroisoquinolinone MDM2 antagonist</td>
<td>Liposarcoma</td>
<td>Advanced solid and hematological tumors</td>
<td>I</td>
</tr>
<tr>
<td>CGM097</td>
<td>Dihydroisoquinolinone MDM2 antagonist</td>
<td>Advanced solid tumors</td>
<td></td>
<td>I</td>
</tr>
<tr>
<td>ALRN-6924</td>
<td>Stapled peptide MDM2/X inhibitor</td>
<td>AML or advanced myelodysplastic syndrome</td>
<td></td>
<td>I/lb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AML or advanced myelodysplastic syndrome</td>
<td>Cytarabine</td>
<td>I/lb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Advanced solid tumors or lymphomas</td>
<td></td>
<td>I/lb</td>
</tr>
</tbody>
</table>

As the amount of p53 protein present in cells is defined by the rate of degradation as opposed to the rate of production, the regulatory factors involved are crucial for a functional p53 response (66). In tumors that retain wt p53, irregularities in the autoregulatory feedback loop with the negative regulators MDM2/X are a common occurrence. However, dysfunctional activation pathways, for example epigenetic silencing of p14ARF expression, can disrupt the native function of wt p53. p14ARF binds to MDM2/MDMX and inhibits their activity, leading to increased levels of p53 expression (66).
In recent years, several wt p53-targeted therapies have entered clinical trials (Table 2). Small-molecule antagonists of the p53-MDM2 protein-protein interaction inhibit the binding of MDM2 with wt p53. These MDM2 inhibitors all have tumor-regressive effects; however, to date, the observed effects have been meager and primarily in non-solid tumors (81). A majority of the inhibitors are in clinical trials in combination with either well-known chemotherapeutic agents or new cytostatic compounds (Table 2) (80,81). As wt p53 is central to both DNA repair and radiation response mechanisms, it seems odd that none of these inhibitors are involved in clinical trials with radiotherapy. Utilizing MDM2 inhibitors as radiosensitizers could potentially increase the therapeutic effect of radiotherapy or perhaps lower the radiation doses otherwise required for tumor regression.

Our entities

An anti-CD44v6 mini-antibody construct functionally equivalent F(ab’)_2

AbD19384, a fully human recombinant, bivalent mini-antibody construct, that is functionally equivalent to a F(ab’)_2, is comprised of two Fab AbD15179 peptides linked non-covalently via a spontaneous dimerization domain (82,83). Fab AbD15179 was developed by our group and binds specifically and with high affinity to the CD44v6-receptor (84,85). The mini-antibody construct (AbD19384) was produced with a potential dual-purpose function. First, the smaller size (113 kDa) of this construct compared to a full-length antibody (150 kDa) should result in a more rapid elimination from the bloodstream, albeit slower than that of a Fab (50 kDa) (86). Second, the bivalent nature of the dimer could result in higher affinity and slower dissociation rate than that of Fab AbD15179. These characteristics indicate a possible dual-function of AbD19384 in precision medicine as both an imaging tracer and a possible targeting moiety for RIT (82,83).

A fully human, full-length anti-CD44v6 antibody

The number of antibody-based TRNT is rapidly increasing, as is the number of antibody-based targeted therapies utilized in precision medicine. Previous studies on anti-CD44v6 targeting moieties (i.e., U36, an U36-derived Fab and F(ab’)_2) demonstrated how tumor uptake of the full-length antibody was superior to that of both the Fab and F(ab’)_2 (87). The greater tumor uptake of U36 compared to the smaller moieties illustrates the potential benefit of utilizing a full-length antibody for RIT (87). Therefore, following the evaluation of AbD19384, a full-length anti-CD44v6 antibody (here referred to as AbN44v6) was developed for use in RIT in the hopes of increasing tumor
uptake. Identically to AbD19384, the full-length, fully human recombinant monoclonal antibody originated from the original Fab construct, AbD15179. Thus, the binding sequence was identical throughout all three moieties (Fab AbD15179, AbD19384, and AbN44v6).

A dual MDM2/X-inhibitor
The current generation of MDM2 inhibitors (Table 2) primarily targets MDM2 and not MDMX (78,80,81). The development of resistance following continuous treatment with the inhibitors has been observed and only a meager apoptotic response to MDM2 inhibition-based therapies have so far been reported (78). Relatively short biological half-lives and hematological toxicities are challenges that need to be overcome as this generation of inhibitors proceeds through clinical trials.

PM2 (SMTide02), is a novel stapled peptide targeting the p53-MDM2 protein-protein interaction (70). ‘Stapling’ occurs via a covalent, hydrocarbon linker between two non-adjacent amino acids. The hydrocarbon linker thus connects the turns of the α-helix of the peptide, resulting in greater stability (88,89). However, in addition to binding and inhibiting MDM2, PM2 also binds to and blocks the binding of MDMX to wt p53. This dual inhibition results in a more comprehensive network of interactions with not only MDM2 but also MDMX, which exceeds the interaction network of similar, small molecule MDM2 inhibitors in terms of complexity (90). In fact, recent studies have shown that PM2 can bind to and antagonize otherwise inhibition-resistant MDM2 (90). The increased stability of the secondary structure of PM2, in addition to increasing the affinity for MDM2/X by reducing the entropic cost of binding, also results in an increase in the in vivo half-life of PM2 (89). These factors are all greatly important for the future utilization of this the peptide as an anti-cancer therapeutic.

The importance of radionuclides
Criteria for the suitability of radionuclides for molecular imaging or therapy differ greatly. While some radionuclides possess properties that are important for both applications, most radionuclides are limited in their fields of use. For molecular imaging, gamma- or positron-emitting nuclides are adept; however, additional characteristics such as decay half-life, energy spectra, abundance and availability are essential for the suitability and applicability of these nuclides (91).

As for nuclides suitable for therapeutic use, among the most important properties are range of the therapeutic particle in tissue, decay half-life, and abundance of unwanted radiation (92). Beta and alpha particle-emitting radionuclides are highly valued for radiotherapy. Alpha particles are by far the most powerful, causing devastating DNA damage as they pass through the cell.
(93). However, the short ranges of alpha particles, which are restricted to a few cell diameters, limit their use, particularly in larger tumors and metastases (94). Beta particles on the other hand, have a greater range in tissues and benefit from the so-called cross-fire effect, although the damage of each particle may be less devastating than that of an alpha particle (93,95). The range of beta particles in tissue results in a cross-fire effect via irradiation from nearby cells. The cross-fire effect is thus beneficial for the targeted treatment since not every cancerous cell must express the targeted antigen in order to receive a dose of cytotoxic radiation (96). However, the greater range of beta particles also potentially increases the absorbed dose received by healthy tissues, once again emphasizing the importance of precision, specificity, and stability of the targeting moieties.

This thesis has focused on several radio-iodine isotopes (124I (paper I), 125I (papers I and II) and 131I (paper II)) and one radiometal, 177Lu (papers II and V). All radiohalogenations were a result of direct labeling by electrophilic substitution using chloramine-T (125I and 131I) or [1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril] (iodogen) (124I). 177Lu was conjugated to AbN44v6 by chelation with CHX-A"-DTPA ([R]-2-Amin-3-(4-isothiocyanatophenyl)-propyl]-trans-(S,S)-cyclohexane-1,2-diamine-pentaacetic acid).

**Residualizing and non-residualizing radionuclides**

An element of importance in TRNT is the biological nature of the molecular target. The accessibility, whether it shreds into circulation or the expression is retained in metastases and if the target is affected as a result of therapy are important for therapeutic success. For therapeutic use, cell-surface receptors remain the most common target. Activation of the receptor by ligand binding and consequent internalization of the receptor is crucial to the choice of radionuclide. Radiohalogens are considered non-residualizing labels as they are subject to diffusion following lysosomal degradation due to their lipophilicity (97). Radiometals, on the other hand, are residualizing and are thus retained within the cell (98). Therefore, it is crucial to understand the characteristics of the chosen molecular target, in order to select an appropriate radionuclide. Two of the most commonly used beta-emitting therapeutic radionuclides are 177Lu and 131I. These radionuclides share similar characteristics in terms of decay half-lives (6.7 days and 8.0 days, respectively) and mean range of the beta particle in tissue (0.25 mm and 0.4 mm, respectively) (95). The half-lives of these radionuclides make them both highly suited for conjugation with full-length antibodies for therapeutic use in precision medicine.

**The promise of combination therapies**

Two of the major risks involved with radiotherapy are long-term side effects (i.e., secondary malignancies) and immediate adverse events (i.e., toxicities)
in healthy tissues and cells, such as those of the hematopoietic system (51). While the risks differ among the various forms of radiotherapy, they share a common denominator: radiation-induced toxicities in healthy tissues. Similar issues apply to both existing chemotherapeutic compounds and novel anticancer therapies (99).

Table 3. Examples of approved targeted radionuclide therapeutic compounds partaking in combination studies in clinical trials. CVP, cyclophosphamide, vincristine sulfate, prednisone; CHOP, cyclophosphamide, doxorubicin hydrochloride, vincristine sulfate, prednisone (101).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Nuclide</th>
<th>Combination</th>
<th>Cancer(s)</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOTATATE or octreotide</td>
<td>SSTR</td>
<td>$^{177}$Lu or $^{111}$In</td>
<td>Fluorouracil</td>
<td>NETs</td>
<td></td>
</tr>
<tr>
<td>DOTATATE</td>
<td>SSTR</td>
<td>$^{177}$Lu</td>
<td>Capcitabine with/without temozolomide</td>
<td>Advanced NETs</td>
<td>I/II</td>
</tr>
<tr>
<td>DOTATATE</td>
<td>SSTR</td>
<td>$^{177}$Lu</td>
<td>$^{90}$Y-DOTATATE</td>
<td>Metastatic NETs</td>
<td>II</td>
</tr>
<tr>
<td>DOTATATE</td>
<td>Norepinephrine</td>
<td>$^{177}$Lu</td>
<td>Irinotecan and vincristine</td>
<td>R/R neuroblastoma</td>
<td>I/II</td>
</tr>
<tr>
<td>Xofigo®</td>
<td>None</td>
<td>$^{223}$Ra</td>
<td>Abiraterone, enzalutamide or both</td>
<td>Castration-resistant prostate cancer with bone metastases</td>
<td>IIIb</td>
</tr>
<tr>
<td>Zevalin®</td>
<td>CD20</td>
<td>$^{90}$Y</td>
<td>RIT before high-dose BEAM chemotherapy and autologous stem-cell transplantation</td>
<td>Relapsed, diffuse large B-cell lymphoma</td>
<td>III</td>
</tr>
<tr>
<td>Zevalin®</td>
<td>CD20</td>
<td>$^{90}$Y</td>
<td>Consolidation after first-line CVP, CHOP, CHOP-like regimen, fludarabine or rituximab combination</td>
<td>Stage III/IV follicular non-Hodgkin’s lymphoma</td>
<td>III</td>
</tr>
</tbody>
</table>

For increased success rate and improved quality of life for the patient, it is critical to limit the toxicities of cancer therapies. One way of limiting therapeutic toxicity and possibly avoiding adverse events is via combination therapies, which offer a number of distinct advantages. First, decreasing the incidence of toxicity while retaining the therapeutic outcome by combining therapies that have different toxicity profiles. Second, additive or synergistic effects of combination therapies may reduce the toxicity profiles upon reducing the doses of one or both therapies. Third, the promise of increased success rates without having to increase the doses of either therapy or risking increased toxicity rates (100).

Potentiating radiotherapy

As more than half of all cancer patients undergo radiotherapy, an advantageous strategy involves combining radiotherapy with radiosensitizing compounds (102). Radiosensitization involves potentiating the effects of or sensitizing the cancer cells to the effects of radiotherapy prior to, during or post radiotherapy (103). EBRT is routinely used in combination with approved
chemotherapeutic compounds such as etoposide and fluorouracil in order to enhance therapeutic success (104). However, despite the increase in the number of approved TRNT compounds, few are being assessed as combination therapies with chemotherapeutic compounds (Table 3) (101). Those that are, are tested in combination with chemotherapeutic compounds that have already been approved. Similarly, only a few trials involving approved chemotherapeutic compounds are undergoing clinical assessment for possible combination with experimental (i.e., not yet approved) TRNT (Table 4) (101).

Table 4. Examples of experimental targeted radionuclide therapeutic compounds currently undergoing clinical trials as combination therapies: GI, gastrointestinal (101).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Nuclide</th>
<th>Combination</th>
<th>Cancer(s)</th>
<th>Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5B7</td>
<td>CEA</td>
<td>$^{131}$I</td>
<td>Combretastatin-A4-phosphate</td>
<td>Advanced GI carcinomas</td>
<td>Phase I</td>
</tr>
<tr>
<td>HEDP</td>
<td>Bone</td>
<td>$^{186}$Re</td>
<td>Docetaxel</td>
<td>Prostate cancer with bone metastases</td>
<td>Phase I</td>
</tr>
<tr>
<td>J591</td>
<td>PSMA</td>
<td>$^{177}$Lu</td>
<td>Docetaxel or prednisone</td>
<td>Castrate-resistant prostate cancer with bone metastases</td>
<td>Phase II</td>
</tr>
<tr>
<td>L19SIP</td>
<td>Fibronectin</td>
<td>$^{131}$I</td>
<td>Whole-brain EBRT</td>
<td>Brain metastases</td>
<td>Phase II</td>
</tr>
<tr>
<td>Lintuzumab</td>
<td>CD33</td>
<td>$^{213}$Bi</td>
<td>Cytarabine</td>
<td>AML</td>
<td>Phase I/II</td>
</tr>
<tr>
<td>mAb425</td>
<td>EGFR</td>
<td>$^{125}$I</td>
<td>EBRT and temozolomide</td>
<td>GBM</td>
<td>Phase II</td>
</tr>
</tbody>
</table>

However, the current climate appears to be changing and paints an encouraging picture. Current studies focus on novel therapies that influence pathways involved in cell cycle arrest, DNA damage repair, and apoptosis (102–104). While each strategy is promising, we believe that targeting upstream influencers or effectors is an interesting and promising concept. Upstream of cell cycle arrest, DNA repair and radiation response mechanisms as well as apoptotic pathways, lies wt p53. The potential of targeting all of the abovementioned mechanisms simultaneously via wt p53 activation is a tempting radiosensitization strategy.

PM2 as a radiosensitizer

At the preclinical level, early research into MDM2 inhibitors delved into the correlation with radiation. Upon irradiating cancer cells, a p53 induction occurs, thus resulting in upregulation of the therapeutic target, MDM2. As a result, the term ‘radiosensitization’ was frequently used in early studies of these inhibitors. Once the inhibitors entered clinical trials, their functions as radiosensitizers appeared to have been completely abandoned, leading to combina-
tions therapies with cytostatic compounds (Table 2) (80,81). To date, the therapeutic response to MDM2 inhibition has been lower than anticipated, primarily due to a meager level of apoptotic response (81).

PM2 belongs to a new generation of MDM2/X-inhibitors and has yet to be tested in a clinical setting. Studies have shown that PM2 is able to bind to and inhibit otherwise resistant MDM2, which offers promise for the future application of PM2 or new generations of the peptide. This ability has been attributed to the dual inhibition of both MDM2 and MDMX by PM2 as well as to the increased \textit{in vivo} stability of stapled peptides. Dual inhibition could lead to increased accumulation of wt p53, resulting in a greater apoptotic response to MDM2/X therapy (88,90).

A suitable combination therapy for PM2 has yet to be determined. The preliminary results from preclinical studies involving PM2 and radiotherapy presented in this thesis indicate the best way the drug could be used in a combination therapy trial. Induction of p53 activation by radiotherapy could potentially not only increase the therapeutic response to MDM2/X inhibition but also potentiate the effects of radiotherapy. This hypothesis covers both the promise and the premise of combining PM2 with radiotherapy to achieve greater precision in selection of the most effective and least invasive individualized treatments for cancer patients.
Aim

The overall aim of the work included in this thesis comprises three parts:

First, to evaluate the novel CD44v6 mini-antibody construct, AbD19384, for potential application as a diagnostic tool for the detection of CD44v6-positive xenografts and for monitoring the response to treatment.

Second, to evaluate the novel, full-length anti-CD44v6 antibody, AbN44v6, for potential use in radio-immunotherapy of CD44v6-positive cancers.

Third, to evaluate PM2-based treatment of wt p53 cancers and establish whether PM2-based therapy in combination with radiotherapy, both external beam radiotherapy and radio-immunotherapy, would potentiate the effects of both therapies.
The present study

Treatment response studied via molecular imaging of two overexpressed antigens (paper I)

The epidermal growth factor receptor (EGFR) and CD44v6 are two commonly overexpressed cell surface antigens in several cancers including squamous cell carcinomas (SCC). Therefore, these receptors are potential targets for precision medicine for diagnosis and response monitoring using targeted molecular imaging modalities. In addition, these receptors are potential targets for targeted therapies. By utilizing targeted imaging modalities, as opposed to gold standard tracers such as FDG-PET, useful information regarding diagnosis and response to therapy can be extracted in a non-invasive manner.

EGFR and CD44v6 expression levels were evaluated to test the response to Heat shock protein 90 (HSP90) inhibition via treatment with the novel HSP90 inhibitor AT13387 (onalespib), a promising therapeutic compound and potential radiosensitizer. The expression levels were evaluated in vitro before and after AT13387 treatment using radiolabeled cetuximab (an anti-EGFR antibody) and AbD19384 (an anti-CD44v6 mini-antibody construct functionally equivalent to a F(ab’)_2). An in vivo dual-xenograft study, which included a small animal PET comparison of targeted molecular imaging modalities ^124_I-cetuximab and ^124_I-AbD19384, with the gold standard imaging probe FDG-PET, was conducted.

All experiments, both in vitro and in vivo, were carried out on a high EGFR and high CD44v6-expressing SCC-cell line (A431) and a low EGFR and low CD44v6-expressing SCC-cell line (UM-SCC-74B) and corresponding xenografts. Both radio-iodinated cetuximab and AbD19384 were stable following labeling and retained their specificity for their respective target antigens. AT13387 treatment downregulated EGFR expression levels by 58% on the A431 samples and 64% on the UM-SCC-74B samples (Figure 4). The expression levels of CD44v6 were unaffected by HSP90 inhibition regardless of concentration on both cell lines. The in vitro results correlated with the in vivo xenograft studies. ^124_I-cetuximab clearly distinguished between the high and low EGFR-expressing tumors in control animals. Additionally, ^124_I-AbD19384 clearly visualized the high CD44v6-expressing tumor (A431) in both the control and AT13387 treatment groups. FDG-PET was unable to discriminate between the control and treatment groups. While AT13387 had no
effect on tumor growth, the uptake of $^{124}$I-cetuximab was significantly diminished in the drug-treated animals, with a 67% and 40% reduction in the A431 and the UM-SCC-74B xenografts, respectively. The uptake of neither $^{124}$I-AbD19384 nor FDG-PET was altered following treatment with AT13387 in the A431 or the UM-SCC-74B tumors. *Ex vivo* IHC confirmed the imaging results, i.e., downregulation of EGFR and unchanged CD44v6 expression in both tumor models.

**Figure 4.** EGFR and CD44v6 expression and binding specificity in A) A431 and B) UM-SCC-74B cells. Cells were incubated with increasing concentrations of $^{124}$I or $^{125}$I-labeled cetuximab or AbD19384 (0.01-60 nM). Non-radiolabeled antibody (100-fold molar excess compared to radiolabeled) was added to the highest concentrations to correct for nonspecific binding. n=3, error bars presented as SD.

**Discussion**

As the arsenal of targeted cancer therapies grows, so do the therapeutic options. The ability to assess the initial response to a chosen course of therapy via non-invasive methods is not only an important tool but perhaps a growing necessity. Precision medicine may offer an increased success rate, but even when based on the individual specifications of the patient, precision medicine does not secure a positive outcome. Therefore, monitoring the specific response to a chosen therapy increases the chances of success by evaluating the
treatment at an early stage. Thus, clinicians gain the opportunity to change the chosen course of action.

The targets of paper I offer valuable information on two levels. First, the EGFR expression is clearly affected by the AT13387 treatment, indicating that HSP90 inhibition has an effect on the tumor. Second, the unchanged expression levels of CD44v6, as exemplified by the uptake of radiolabeled AbD19384 following AT13387 treatment, indicates that this particular receptor could also be utilized in a different capacity.

**Figure 5.** Representative images of small-animal PET/CT of a) 124I-cetuximab, b) 124I-AbD19384, and c) FDG-PET. The left posterior flank (T1) represents the high EGFR and CD44v6-expressing xenografts (A431) and the right posterior flank (T2) represents the low EGFR and CD44v6-expressing xenografts (UM-SCC-74B). Cross sections of the xenografts and planar maximum intensity projection images are presented on the upper and lower rows, respectively.

Downregulation of EGFR is a positive clinical outcome (105,106). Targeted molecular imaging for monitoring treatment response could assess the early therapeutic response in real-time in a precise, non-invasive manner that surpasses solely measuring tumor size. Although the tumor size was unaffected by the five AT13387 treatments prior to PET/CT-imaging, this result need not indicate a lack of efficiency of the novel drug. Rather, this result was obtained due to the experimental setup, which was designed to evaluate the expression levels of the receptors directly after the initial treatment with AT13387. In comparison, the lack of downregulation of CD44v6 following AT13387 therapy makes it a possible target for RIT in combination with HSP90 inhibition. However, proceeding with AbD19384 for RIT is unlikely. The bivalent AbD19384 construct is formed through spontaneous dimerization through a non-covalent bond with the monomer and dimer in a state of equilibrium (82). Therefore, the injected 124I-AbD19384 was comprised of both 124I-labeled monomers and 124I-labeled dimers, which may raise future questions concerning in vivo stability. Furthermore, studies on U36, its Fab and F(ab')2, have shown that tumor uptake of a full-length antibody is >20-fold compared to that of either smaller targeting moieties (87). As such, a full-length antibody
is more likely to achieve superior tumor-to-organ ratios and absorbed doses to the tumors and, ultimately, be the more suitable RIT candidate (86).

In conclusion, the application of targeted molecular imaging at an early stage in order to monitor the therapeutic response would greatly benefit the selection of therapeutic strategies. In precision medicine, this strategy is of increasing importance to ensure the best clinical outcome.

In the present thesis perspective, paper I evaluated and characterized AbD19384, functionally equivalent to a F(ab′)_2 (based on the original Fab AbD15179 construct), for potential use in radio-immunodiagnostics. Radiolabeled AbD19384 bound specifically to CD44v6 in vitro and in vivo, as demonstrated by e.g., ¹²⁴I-AbD19384 PET/CT xenograft imaging, where ¹²⁴I-AbD19384 imaging revealed a clear difference between high and low CD44v6-expressing xenografts. Lastly, the lack of downregulation of CD44v6 following HSP90 inhibition indicated a potential future RIT application for CD44v6-binders in combination with radiosensitizers. Furthermore, this may be a promising construct to further develop into a full size antibody for potential RIT applications.

Preclinical assessment of an anti-CD44v6 antibody for RIT (paper II)

CD44v6, with limited expression in normal tissues and high expression in several cancers, is an excellent candidate for RIT. A novel, fully human, full-length anti-CD44v6 antibody, AbN44v6, was developed from the same Fab AbD15179 construct as AbD19384 in paper I.

AbN44v6 was evaluated in vitro and in vivo for suitability of use in RIT with two therapeutic radionuclides, ¹⁷⁷Lu and ¹³¹I. Specificity of the antibody following radiolabeling was assessed prior to in vitro RIT using 3D multicellular tumor spheroids of a moderate CD44v6-expressing cell line (HCT116) and a low CD44v6-expressing cell line (UM-SCC-74B). A dual-nuclide in vivo investigation of normal tissue biodistribution and dosimetry concluded the study.

Stability and specificity were unaffected following all radiolabeling procedures. A clear activity-dependent growth inhibition was measured in the spheroids for both cell lines tested, although the greater response was observed in the moderate CD44v6-expressing spheroids (HCT116). Growth inhibition was mainly caused by non-targeted radiation, i.e., the presence of a therapeutic beta-emitting nuclide in the cell medium for the UM-SCC-74B spheroids, as exemplified in the radiolabeled rituximab samples. HCT116 spheroids presented with a greater response to targeted RIT, which at the higher activities did not regain normal spheroid growth rates even after removal of the activity. In the in vivo study, both radioconjugates presented similar biodistribution
profiles, with rapid clearance from most organs. The $^{177}$Lu-labeled conjugate demonstrated higher uptake in liver and spleen than the $^{125}$I-labeled conjugate, making these organs the dose-limiting organs for $^{177}$Lu-AbN44v6. $^{125}$I-AbN44v6 was not cleared quite as rapidly from the circulation, resulting in a higher absorbed dose to the red marrow, consequently making the red marrow the dose-limiting organ for this conjugate. The total effective dose for both radioconjugates was estimated to be approximately 0.1 mSv/MBq.

Figure 6. 3D in vitro RIT of either $^{177}$Lu-AbN44v6 or $^{131}$I-AbN44v6. A) Growth-response of UM-SCC-74B, normalized to % of untreated controls at each time point. B) Representative images of UM-SCC-74B spheroids shown in A, as well as 100 kBq of radiolabeled rituximab. C) Growth-response of HCT116 spheroids normalized to % size of untreated controls at each time point. D) Representative images of HCT116 spheroids shown in C, as well as 100 kBq of radiolabeled rituximab. n≥5, error bars represent 95% confidence intervals.

Discussion

The use of RIT is usually restricted to high antigen-expressing cancers. Naturally, the cumulative absorbed dose to the tumor increases with increasing antigen density. However, HCT116, a moderate CD44v6-expressing cell line, demonstrated a clear activity-dependent response to RIT in an in vitro 3D setting. The highest activity treatment, 100 kBq of either $^{177}$Lu- or $^{131}$I-AbN44v6, resulted in a decreased size by approximately 50%. Additionally, the majority of the growth inhibition was a direct result of targeted as opposed to non-targeted radiation. In contrast, the majority of the growth inhibition detected in the UM-SCC-74B spheroids was caused by the mere presence of therapeutic nuclides in close proximity to the spheroid. These results verify that the
The cytotoxic effect of RIT was greater in the cell line with a greater antigen-density while also indicating that this therapeutic strategy may be feasible for both high and moderate antigen-expressing cancers.

The dual-nuclide normal tissue biodistribution results were similar for both radioconjugates. However, the $^{177}\text{Lu}$-labeled conjugate demonstrated a tendency to accumulate more within organs (e.g., liver and spleen) but was cleared faster from circulation. This finding indicates possible tendencies toward aggregation in vivo that would result in elimination from circulation via a size-dependent mechanism, transchelation to metal-binding proteins or a lowered stability of the conjugate in vivo (107). The $^{125}\text{I}$-conjugate was more rapidly cleared from all organs, but remained in the bloodstream for an extended period compared to the $^{177}\text{Lu}$-conjugate. This increased the absorbed dose to the red marrow, making the red marrow the primary dose-limiting organ of the iodine-conjugate. The differences in biodistribution profiles of each radioconjugate offer valuable information. One of the limiting factors of any radiopharmaceutical, is the absorbed dose to healthy tissues, usually accumulated during elimination (108,109). Previous studies on the radiolabeled Fab AbD15179, which contains an identical binding sequence to the full-length antibody, highlighted the liver and spleen ($^{111}\text{In}$-AbD15179), and kidneys ($^{125}\text{I}$-AbD15179) as possible dose-limiting organs.

The biodistribution of the radiolabeled AbN44v6 conjugates demonstrated an increase in uptake in the liver, spleen and kidneys compared to the biodistribution of the smaller AbD15179 and AbD19384. Additionally, the full-length antibody demonstrated an increased time in circulation compared to AbD15179 and AbD19384. This was expected, as studies have shown that full-length antibodies are present in the circulation for an extended period compared to smaller moieties (86,91). The extended time in the blood of both radiolabeled AbN44v6 conjugates is likely the reason for the increased uptake in % ID/g in high-perfusion organs (e.g., the liver, spleen and kidneys).

The relatively low uptake of $^{125}\text{I}$-AbN44v6 by organs of elimination such as the liver indicates that the full-length antibody is possibly suitable as a targeting moiety. Furthermore, the differences in dose-limiting organs, depending on radionlabel, make it possible to customize therapy for individual patients or patient groups, depending on their medical profile to avoid toxicity issues. The indication of decreased in vivo stability or possible aggregation of the $^{177}\text{Lu}$-labeled conjugate, as demonstrated in the rapid elimination from the blood, is of concern and should be further addressed. Moreover, other potential chelators and labeling techniques for radiometal labeling should be assessed.
Notably, AbN44v6 does not cross-react with the murine CD44v6-receptor, and the results of the xenograft study are not completely extrapolatable to humans without further studies. However, previous studies using anti-CD44v6 antibodies in humans have demonstrated very limited uptake in normal tissues, which is promising for future studies with AbN44v6. However, size, aggregation, charge, lipophilicity and choice of radiolabel are crucial to determine both the clearance mechanisms and potential off-target effects. Therefore, this study still offers important information about the future prospects of utilizing AbN44v6 as a radio-immunotherapeutic agent. However, as tumors were not included in the biodistribution and dosimetry studies, the tumor-to-organ ratios as well as absorbed dose to the tumors have yet to be defined. As both AbD15179 and AbD19384 demonstrated excellent specific binding in vivo, we expect greater uptake by the full-length antibody in future xenograft studies.

Figure 7. Biodistribution presented as % ID/g of $^{177}$Lu-AbN44v6. n=5, error bars represent SD.
In the present thesis perspective, paper II further evaluated AbD15179-based CD44v6-constructs by moving from the F(ab')2-construct to a full-length antibody for potential use in RIT applications. The full-length antibody was successfully radiolabeled with both a radiometal and radio-iodine and retained antigen-binding. RIT using radiolabeled AbN44v6 resulted in activity-dependent growth inhibition in vitro and demonstrated promising normal tissue clearance and dosimetry in vivo, indicating that AbN44v6 could be a promising future candidate for RIT in CD44v6-expressing cancers.

Figure 8. Biodistribution presented as % ID/g of $^{125}$I-AbN44v6. n=5, error bars represent SD.
The potency of PM2 in combination with external beam radiotherapy (papers III & IV)

Due to its role as a crucial tumor suppressor and high mutation rate in cancer, p53 remains an interesting target for precision medicine. Small-molecule antagonists of the p53-MDM2 protein-protein interaction are generating promising results in clinical trials; however, so far without exploring the combination with radiotherapy despite the central role played by wt p53 in radiation response and DNA damage mechanisms. The aim of papers III and IV was therefore to investigate the potential of PM2-based therapy and evaluate the combination of PM2 treatment and EBRT in a proof-of-principle setting.

Paper III determined the potency of PM2 monotherapy and the combination therapy in vitro. The paper sheds light on the mechanisms behind the observed effects via e.g., Western blotting and flow cytometry. Paper IV further established the potency of PM2 treatment in combination with EBRT in an in vivo setting using wt p53 (HCT116) and p53-knockout (HCT116 -/-) xenografts.

While PM2 treatment demonstrated anti-tumorigenic effects on wt p53, Human papillomavirus (HPV)-negative cell lines, the combination of PM2 and EBRT proved a more potent cocktail. Upregulated expression levels of wt p53, cleaved caspase-3, and Noxa were measured following PM2 exposure (Figure 9). The expression levels were further elevated in the combination-treated samples (i.e., PM2 with EBRT), exemplifying the potential of merging the two therapies. The chosen in vitro therapy setting, 3D multicellular tumor spheroids, confirmed dose-dependent growth inhibition for both monotherapies. Five repeated doses of PM2 or EBRT at 48 h intervals resulted in significantly decreased spheroid sizes compared to untreated controls (Figure 10A, 10B, 10D, 10E). The effects of the combination therapies were however superior to each monotherapy. Three repeated combination doses resulted in near-complete stagnation in growth and five repeated combination doses led to complete spheroid disintegration and cell death (Figure 10C and 10F). The selectivity of PM2 towards wt p53 cancers was evident following the evaluation of the effects of the peptide on a p53-knockout cell line (HCT116 -/-). The peptide had no detectable anti-tumorigenic effects on the HCT116 -/- samples in either 2D or 3D cultures, regardless of combination with radiotherapy (Figure 2, paper IV). This was confirmed in vivo, where no significant effects on the growth of HCT116 -/- xenografts were observed following PM2-based therapy (Figure 11G-11I). In contrast, the wt p53 xenografts were greatly affected by both monotherapies and the combination therapy, ultimately resulting in a prolonged survival of 50% in the combination-treated group compared to the controls (Figure 11A-11F). Lastly, 125I-labeled PM2 biodistribution demonstrated clear tumor uptake and retention with rapid elimination from normal tissues and even distribution of the compound throughout the tumor, as visualized by autoradiography (Figure 3, paper IV).
Figure 9. Western blot analyses of (A) p53 expression at 24 h and (B) cleaved caspase-3 expression at 72 h and 96 h of UM-SCC-74B and HCT116 samples post treatment with either 2 Gy, PM2 (20 µM), or the combination. Samples (n≥3) were normalized to controls, represented as a dotted line at y=1. Error bars presented as SD. (C) Representative Western blot images of p53 and cleaved caspase-3 expression of UM-SCC-74B samples at 24 h (p53) and 72 h (cleaved caspase-3) post treatment, and HCT116 samples (D) at 24 h (p53) and 96 h (cleaved caspase-3) post treatment. Flow cytometric analyses of UM-SCC-74B and HCT116 samples at 24 h, 48 h, 72 h, and 96 h post treatment of (E) cleaved caspase-3 expression and (F) Noxa expression. n≥3, error bars presented as SD. Significance was determined using one-way ANOVA: p≤0.05 (*), p≤0.01 (**), p≤0.001 (***) , p≤0.0001 (****).

Discussion

Two of the main cellular responses to p53 activation are cell cycle arrest and apoptosis. While cell cycle arrest is relevant for cancer therapy, apoptosis is of far greater interest as a therapeutic response to p53 activation. It has been suggested that the abovementioned responses are dependent on the level of p53 protein accumulation, with lower levels resulting in cell cycle arrest and higher levels in p53-mediated apoptosis (110). The findings presented in paper III support this hypothesis, as PM2 monotherapy indicated p53-mediated apoptosis, as exemplified by the upregulation of both cleaved caspase-3 and Noxa (Figure 9). The detected apoptosis in the PM2 monotherapy samples is an example of the potency of a therapeutic strategy that targets the negative regulators of the p53 protein. By inhibiting MDM2 and MDMX and thus restoring expression levels, wt p53 is able to assert its innate capacity as a tumor suppressor.
However, in order to potentiate the p53-mediated tumor suppressive mechanisms, a stimulant of p53 activation pathways, such as ionizing radiation, could be of great beneficial use. Induction of a wt p53 response via exposure to ionizing radiation should potentiate the effects of both radiation and p53-based therapy. In paper III, this hypothesis was supported as the levels of pro-apoptotic proteins (cleaved caspase-3 and Noxa) were amplified in all wt p53 combination-treated samples compared to monotherapy samples (Figure 9). In the 3D in vitro therapeutic setting, this result was even more evident. Repeated monotherapies resulted in growth inhibition, whereas repeated combination treatments resulted in complete sample disintegration of the UM-SCC-74B spheroids (Figure 10).

Modest clinical successes of current anti-MDM2 therapies have been attributed to overexpression of MDMX as well as mutations affecting the binding properties of MDM2 as a consequence of MDM2-targeted therapy (81). As both MDM2 and MDMX are in an autoregulatory loop with p53, induction of p53 accumulation will naturally result in a similar upregulation of the negative regulators. Unlike most antagonists of the p53-MDM2 protein-protein interaction, PM2 targets and inhibits both MDM2 and MDMX. Therefore, PM2 could potentially negate these limitations by blocking the interaction between wt p53 and both MDM2 and MDMX. Additionally, directly inducing a p53 response via EBRT in combination with a potent MDM2/X inhibitor could further increase the therapeutic success rates. In paper IV, this strategy was assessed in vivo. Using HCT116 in parallel with HCT116 -/- xenografts, combination therapy using PM2 and EBRT was tested. The results obtained...
in vitro in an identical experimental setting correlated well with the outcome of the in vivo xenograft trial. Both monotherapies resulted in growth inhibition of the wt p53 xenografts, whereas only EBRT had an inhibitory effect on the growth of HCT116 -/- xenografts (Figure 11).

Figure 11. HCT116 wt p53 and p53 -/- xenografts treated with PM2 and EBRT. A) Growth of wt p53 tumors until time of first termination of animals from each group (controls, 10xPM2 (daily injections), 3xEBRT (48 h intervals) or the combination of the two monotherapies. n≥6, error bars presented as 95% confidence intervals. B) Tumor size of wt p53 xenografts shown in (A) at day 10 post start of treatment. n≥6, error bars presented as SD. p<0.05 defined as *. C) Survival proportions of wt p53 xenografts. D) Growth of wt p53 tumor size until time of first termination of animals from each group (controls, 3xPM2 (48 h intervals), 3xEBRT (48 h intervals) or the combination. n=6, error bars presented as 95% confidence intervals. E) Tumor size of wt p53 xenografts shown in (D) at day 10 post start of treatment. n=6, error bars presented as SD. p<0.05 defined as *. F) Combination index plot of synergistic effects of 3xPM2 and 10xPM2 and 3xEBRT. G) Growth of p53 -/- tumors until time of first termination of animals from each group with identical treatment regimens as animals presented in (A). n=5, error bars presented as 95% confidence intervals. H) Tumor size of -/- p53 xenografts shown in (G) at day 10 post start of treatment. n=5, error bars presented as SD. I) Survival proportions of -/- p53 xenografts.
Additionally, combination treatments yielded synergistic results, and an increased median survival by 50% between combination-treated and control groups in HCT116 xenografts was observed. No such effects were detected in the p53-knockout samples, *in vitro* or *in vivo* as only EBRT resulted in growth inhibition. These results confirm the specificity of PM2 toward wt p53 cancers as well as the potency of combining PM2-based therapy with EBRT in wt p53, HPV-negative cancers. Furthermore, radiolabeled PM2 (\(^{125}\text{I}-\text{PM2}\)), injected subcutaneously above the tumor, demonstrated excellent tumor penetration with an even distribution throughout the tumor, which was retained for at least 48 h post injection.

A rapid elimination rate and lack of accumulation of PM2 in normal tissues could possibly circumvent the familiar toxicities observed with current MDM2 inhibitors. However, as PM2 was injected subcutaneously, conclusions from the biodistribution results are merely indicative of the distribution of \(^{125}\text{I}-\text{PM2}\) following localized subcutaneous injection. Additional studies are needed to assess the toxicity profile of PM2, preferably in the intended means of future clinical administration. The potency of PM2, despite the subcutaneous injections at low doses, is exemplified in the success of the proof-of-principle *in vivo* combination study presented in paper IV. Granted, the need for further optimization concerning for example delivery, doses, and treatment-intervals as well as toxicity studies of PM2-based therapy exists. However, there is no denying the anti-tumorigenic effects of PM2 and its potential as a future cancer therapy of wt p53 cancers. Furthermore, the greatly increased therapeutic response to combination therapy with EBRT illustrates the promise of utilizing PM2 as a future radiosensitizer.

An indication of the benefit of combining two precision medicines (paper V)

Merging a radiosensitizing compound such as PM2 with a novel RIT agent (AbN44v6) is a unique strategy. The combination of two precision therapeutics is a promising concept that could exceed the success rate of traditional therapies, particularly in cases where EBRT is less likely to have a therapeutic effect (e.g., disseminated disease or infiltrating tumors). Earlier studies in this thesis focused on potentiating EBRT with PM2-based therapy on wt p53 cancer cell lines. The final study (paper V) combines the novel combination of wt p53-targeted therapy with RIT via radiolabeled AbN44v6 targeting CD44v6.

The evaluation was conducted in the same *in vitro* setting as Paper II, using a moderate CD44v6-expressing cell line (HCT116) and a low CD44v6-expressing cell line (UM-SCC-74B), both wt p53 cell lines. The therapy was assessed in a 3D multicellular tumor spheroid setting using previously determined doses of PM2 and activities of \(^{177}\text{Lu}-\text{AbN44v6}\) (papers II-IV).
Repeted treatments with PM2 (20 µM) at 48 h intervals failed to increase the level of growth inhibition in both HCT116 and UM-SCC-74B spheroids compared to a single dose incubated for six-to-seven days (Figure 2E and 2F, paper V). RIT using $^{177}$Lu-AbN44v6 resulted in activity-dependent growth inhibition of the HCT116 spheroids, which differed significantly from the growth inhibition observed using identical activities of the negative control $^{177}$Lu-rituximab (Figure 12). In contrast, RIT using $^{177}$Lu-AbN44v6 did not differ significantly from the effects of $^{177}$Lu-rituximab on the UM-SCC-74B spheroids (Figure 13). The combination of RIT and PM2-based therapy greatly improved the growth inhibition of both cell lines. The HCT116 spheroids treated with 100 kBq of $^{177}$Lu-AbN44v6 and a single dose of PM2 diminished two-fold in size compared to spheroids treated with RIT alone, and three-fold compared to PM2 monotherapy. Similar effects were observed in the UM-SCC-74B spheroids, where the highest activity treatment (100 kBq) had little effect on spheroid growth in itself, and yet, the addition of PM2 resulted in spheroid regression.
Discussion

Previous work presented in this thesis on the effects of PM2 treatment in a 3D in vitro model system (paper III), has illustrated the potency of combining radiotherapy with PM2-based therapy. However, as EBRT is rarely applicable in cases of disseminated disease, the combination therapy of PM2-based therapy and EBRT has certain limitations. Changing the nature of the applied radiotherapy by substituting EBRT with RIT may help overcome these limitations.

As seen in paper III, repeated treatments with PM2 resulted in partial growth inhibition, whereas the combination with repeated doses of radiotherapy resulted in complete growth inhibition and even in spheroid regression and disintegration (Figure 4, paper III). However, the repeated treatments with PM2, at 48 h intervals, only resulted in momentary growth inhibition, which ended nearly directly after the peptide was removed from the cell medium. A direct comparison between three repeated PM2 treatments at 48 h intervals and one PM2 treatment incubated for the same period was executed in paper V (Figure 2E and 2F, paper V). The two different treatment schedules resulted in near identical growth inhibition of both HCT116 and UM-SCC-74B spheroids, establishing that PM2 remains active in cell culture medium for at least six days. Additionally, as repeated treatments failed to improve the therapeutic response in the in vitro setting further, there was little interest in assessing repeated treatments in combination with RIT in the in vitro spheroids. Excluding the need for repeated PM2 treatments prolonged the incubation time of $^{177}$Lu-AbN44v6 to six days. The six-day incubation of both therapies resulted in remarkable amplifications in terms of growth inhibition on both cell lines. The combination of a single PM2 treatment with $\geq 30$ kBq of $^{177}$Lu-AbN44v6 induced a significant size reduction of both HCT116 and UM-SCC-74B spheroids compared to monotherapies as well as repeated PM2 treatments (Figures 12 and 13). Furthermore, combination therapy of PM2 with 100 kBq of $^{177}$Lu-AbN44v6 resulted in complete growth inhibition of the UM-SCC-74B spheroids, which toward the end of the assay demonstrated signs of disintegration (Figure 13).

While the potency of the combination cocktail is by far more striking in the UM-SCC-74B samples, it is imperative to remember that the primary radiation damage in UM-SCC-74B was caused by non-targeted radiation. In all studies presented in this thesis, UM-SCC-74B has proven highly sensitive to the combination of PM2 with radiotherapy. Therefore, although impressive, the spheroid results of combined PM2 treatment and RIT are slightly misleading when using this cell line as these results would likely not be translatable to an in vivo setting. Additionally, attempting RIT on a low antigen-expressing cell line is likely not a plausible strategy in a clinical setting. Furthermore, although all wt p53, HPV-negative cell lines responded to PM2-based therapy (Table 1, paper III), there was a difference the sensitivity of each cell line to
the treatment, particularly in combination with radiotherapy. The difference in sensitivity should be investigated in future studies in order to ensure the applicability for wide-scope use of PM2-based therapy of wt p53 cancers. The effects of combined RIT and PM2-based therapy on HCT116 spheroids were significant and indicate that this may be a feasible strategy for CD44v6-expressing cancers. This finding further elucidates the promise of combining two targeted compounds and could translate well to an in vivo setting using high or moderate CD44v6-expressing xenografts.

![Figure 13. A) UM-SCC-74B 3D in vitro RIT treated with 30 kBq and 100 kBq of $^{177}$Lu-AbN44v6 and 100 kBq of $^{177}$Lu-rituximab. Also shown are representative images of the same treatments, B) UM-SCC-74B 3D in vitro therapy of spheroids treated with 20 µM of PM2 and the combination of PM2 and RIT. Also shown are representative images of the same treatments. n≥4, error bars presented as 95% confidence intervals.](image)

The radiosensitizing or potentiating effects of PM2 were greater when the peptide was administered after irradiation (data not shown). This observation is attributed in part to the rapid elimination of the peptide and to the peak expression levels of MDM2/X following irradiation. Barring an excretion process, PM2 is active in vitro for an extended period and remains active for at least six days in cell culture medium, as demonstrated by the spheroid data.
presented in paper V. The therapeutic window of PM2 is thus wider in the *in vitro* assays, and the timing and method of drug administration is of lesser importance. However, the *in vitro* therapeutic effects measured as a result of the widened therapeutic window can be falsely amplified and should be taken into consideration when translating the *in vitro* data into an *in vivo* setting.

The findings presented in paper V illustrate the potency of combining PM2-based therapy with RIT using radiolabeled AbN44v6. Through combining PM2-based therapy with a “magic bullet” (RIT), this potent cocktail will seek out and interact with tumor tissues. Furthermore, barring no notable expression of the molecular target in healthy tissues, the combination therapy will likely only affect organs involved in the excretion pathways. Overall, these factors highlight the potency of this combination therapy and offer a promising and exciting future for radiosensitization strategies.
Conclusions

Due to rapid technological advances, a deeper understanding of the molecular characteristics of cancer has warranted a revolution within the field of precision medicine. Targeted compounds for both diagnosis and therapy have increased the success rates of cancer therapies and improved the means of early detection. However, the field is complex and far from complete and new targets, tracers and therapies are essential for ultimately defeating cancer.

The work presented here is comprised of two main components, namely, targeting the CD44v6-receptor and combining MDM2/X inhibition with radiotherapy. In particular, this work has focused on combination therapies, primarily of PM2 and radiotherapy, which we believe is a potent and promising strategy for treatment of wt p53 cancers.

The main findings are listed below:

- AbD19384 labeled with $^{124}$I can detect CD44v6-positive xenografts and can selectively distinguish between high- and low-expressing tumors.
- CD44v6 was not downregulated following HSP90 inhibition and can be used as a target for radio-immunotherapy in combination with HSP90 inhibition.
- *In vitro* 3D RIT using radiolabeled AbN44v6 resulted in activity-dependent targeted growth inhibition of a moderate CD44v6-expressing cell line.
- Normal tissue biodistribution and dosimetry of radiolabeled AbN44v6 resulted in a total effective dose of approximately 0.1 mSv/MBq for both radioconjugates.
- PM2-based therapy indicated increased p53-mediated apoptotic activity in wt p53, HPV-negative cancer cell lines *in vitro*.
- The effects of PM2-based therapy are potentiated by external beam radiotherapy, resulting in increased levels of pro-apoptotic proteins *in vitro*.
- Repeated PM2 treatment and external beam radiotherapy results in wt p53 spheroid disintegration *in vitro* and growth inhibition of wt p53 xenografts *in vivo*. 
• The combination of radio-immunotherapy using $^{177}$Lu-AbN44v6 and PM2-based therapy is a potent strategy for future therapeutic treatment of wt p53, HPV-negative CD44v6-expressing cancers.

The work presented here has illuminated the possibility of utilizing CD44v6 as a target for both radiodiagnostics and therapy. Furthermore, we have established the potency of combining external beam radiotherapy with MDM2/MDMX inhibition using PM2 both \textit{in vitro} and \textit{in vivo}. Lastly, a proof-of-principle \textit{in vitro} study confirmed the promising and unique concept of combining radio-immunotherapy targeting CD44v6 with PM2-based therapy. It remains our hope that utilizing not one but several of the molecular characteristics of a specific cancer will help turn the tables on cancer and improve patient outcomes in the future.
The future at a glance

Efficacy of PM2

Although the effects of PM2-based therapy and combination therapy with EBRT were evaluated in vivo, the doses and particularly the delivery of PM2, (i.e., testing intravenous or intraperitoneal injections) would benefit from further investigation. Furthermore, while PM2-based therapy had a growth suppressive effect on all wt p53, HPV-negative cell lines, the responses varied in strength. This finding is exemplified by the differences in growth inhibition and spheroid disintegration between HCT116 and UM-SCC-74B. The reasons behind the differences in efficacy observed between cell lines have not yet been investigated. However, HCT116 has a KRAS mutation, which is upstream of p53, and could potentially influence the level of p53 activation following irradiation and explain the lack of potency of PM2-based therapy. A thorough investigation into the mechanisms behind these differences in efficacy could pinpoint new and interesting combination therapies using PM2.

RIT and PM2 in vivo

The original goal of the work presented in this thesis was to assess the combination of RIT and PM2-based therapy. However, for proof-of-principle, EBRT replaced RIT for simplicity as well as practicality. Paper V evaluated the potency of combining RIT targeting CD44v6 with PM2-based therapy in vitro and paper II assessed the biodistribution and dosimetry of AbN44v6. The next logical step of evaluation is to not only examine RIT in vivo but also test the combination of RIT with PM2. The use of a high CD44v6-expressing, wt p53, HPV-negative xenograft would be ideal in this experimental setup. Additionally, for future therapy with radiometals (i.e., $^{177}$Lu), a different chelator should be considered. Although the results from chelating AbN44v6 with CHX-A”-DTPA were promising, concerns of aggregation or stability issues in vivo were observed in the biodistribution study in paper II. Moreover, the specific activities of $^{177}$Lu-AbN44v6 were generally lower than those following direct radio-iodination. These issues could potentially be overcome by using a different chelator.
Targeted drug delivery of PM2 and newer generations of the peptide

Studies have shown that PM2 enters the cytoplasm and further localizes to the nucleus, but the mechanism by which the stapled peptide makes its way both into the cell and the nucleus remains unclear. Furthermore, PM2 does not target cancer cells specifically. To ensure delivery of PM2 or newer generations of the peptide specifically to cancer cells, targeted drug delivery of PM2 could be highly beneficial. The use of targeted lipo-discs or nanoparticles loaded with the stapled peptide could ensure a higher delivery rate to cancer cells while lowering uptake in normal tissues. This is an interesting concept, which in the future could lower the toxicity following therapy with MDM2/X inhibitors. Furthermore, by labeling PM2 with $^{125}$I, the need for combining PM2-based therapy with EBRT or RIT as well as the need for lipo-discs may become redundant. The Auger-electrons emitted by $^{125}$I would cause extensive damage to the DNA of cancer cells when in close proximity to the nucleus.

Combining RIT with HSP90 inhibition

Although featured and discussed in paper I, the combination of RIT and HSP90 inhibition was not the main focus of this thesis. A substantial decrease in EGFR expression was observed following treatment with AT13387, indicating that EGFR would be a poor target for combination therapy. However, the unchanged expression levels of CD44v6 following AT13387 therapy were highly promising for future combination studies. In vivo therapy with AT13387 in combination with EBRT has been assessed by our group and it would be easy to repeat the study, albeit this time in combination with RIT targeting CD44v6.
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