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## Molecular imaging applications of antibody-based immunotherapeutics to understand cancer drug distribution

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## Summary, General Discussion and Future Perspectives

## SUMMARY

Cancer is a significant cause of death worldwide.<sup>1,2</sup> Treatment consists of surgery, radiation therapy, and systemic therapy. Monoclonal antibodies (mAb) have become an increasing part of the systemic treatment of cancer.<sup>3</sup> With the landscape-transforming arrival of cancer immunotherapy, such as drugs that block immune checkpoints, durable responses are observed for several different cancer types, including melanoma and non-small-cell lung cancer. Regrettably, only part of the patients initially respond, and once responded, resistance to immune checkpoint blockade may occur.<sup>4</sup> Therefore, new treatment options are explored to enhance the immune system. These approaches include engaging T cells or inhibit immunosuppressive cell types like tumor-associated macrophages (TAMs).

By redirecting T cells to infiltrate the tumor, T cells might release their cytotoxic potential.<sup>5</sup> Bispecific T cell engagers (BiTEs) or T cell-directed bispecific antibodies redirect T cells to a predefined tumor target. BiTEs redirect T cells by the CD3 $\epsilon$  binding arm and the other arm directed at the tumor target. Tumor targets include epithelial cell adhesion molecule (EpCAM), carcinoembryonic antigen (CEA) and glypican 3 (GPC3). Upon the simultaneous binding of a T cell to CD3 $\epsilon$  and its tumor target, T cells become activated and can kill tumor cells in an antigen-specific manner. The CD19 BiTE blinatumomab is the only bispecific anticancer drug approved.<sup>5</sup> For solid malignancies, multiple T cell-directed bispecific antibody-based immunotherapeutics are in development.<sup>6</sup>

TAMs in the tumor microenvironment can act as an immunosuppressive cell type promoting tumor progression.<sup>7</sup> Targeting TAMs by for example targeting the survival pathway colony-stimulating factor 1 (CSF1)/CSF1 receptor (CSF1R) using mAbs, promotes the anti-tumor effect of other treatment strategies in preclinical cancer models. Multiple TAM targeting approaches are being evaluated in clinical trials.<sup>7</sup>

Limited information is available regarding the pharmacological behavior of these new molecular entities. Radiolabeling these types of drugs with positron emission tomography (PET) isotopes allows molecular imaging using PET to assess whole-body drug distribution and tumor targeting. *Ex vivo*, techniques like tissue autoradiography, radioactive gel electrophoresis of plasma or tissue lysate, and *ex vivo* biodistribution complement PET imaging. Thus, information is obtained on respectively intratumoral drug distribution, tracer integrity, and quantitative organ distribution. Overall, molecular imaging of radiolabeled drugs could provide information to support drug development.

The research described in this thesis aims to gain insight in the pharmacological behavior of antibody-based immunotherapeutics using molecular imaging.

In **chapter 1**, the background and outline of this thesis are described. In **chapter 2**, we aimed to define the role of molecular imaging in cancer drug development. We searched the literature with a focus on molecular imaging in the context of target expression, pharmacokinetics, and pharmacodynamics in cancer. We provide applications of molecular imaging regarding small-molecule cancer drugs, including inhibitors of epidermal growth

factor receptor, anaplastic lymphoma kinase, and poly(adenosine diphosphate ribose) polymerase. Also, molecular imaging applications of monoclonal antibodies are highlighted for growth factor receptors, immuno-oncology, and antibody-drug conjugates.

Furthermore, examples of monitoring pharmacodynamic responses upon anti-hormonal treatment using molecular imaging have been reported. Molecular imaging can answer multiple questions regarding *in vivo* drug behavior. Together with complementary techniques such as genomics, transcriptomics or proteomics, molecular imaging can serve as a tool to improve biomarker discovery, patient selection, and gain insight into drug mechanism of action and target engagement.

Radiolabeling new cancer therapeutics allows us to study tumor targeting and whole-body biodistribution. A new class of cancer therapeutics is BiTEs, a 55-kDa drug comprised of two single-chain Fv binding CD3 $\epsilon$  and a tumor-associated antigen. AMG 110 is such a BiTE, directed at CD3 $\epsilon$  on T cells and EpCAM on tumor cells, which is often overexpressed in epithelial malignancies. In **chapter 3**, we aimed to assess the tumor-targeting properties of zirconium-89 ( $^{89}\text{Zr}$ ) labeled and fluorescently labeled AMG 110 in xenograft bearing mice. Tumor uptake of  $^{89}\text{Zr}$ -AMG110 in an EpCAM positive xenograft was clearly visualized by PET imaging up to 72 hours after intravenous administration. Tumor uptake peaked at 6 and 24 hours after  $^{89}\text{Zr}$ -AMG110 administration, reaching around 5% injected dose per gram of tissue. EpCAM negative xenografts were barely visible on PET images. Fluorescently labeled AMG 110 showed intratumoral distribution associated with viable tumor tissue. A non-tumor targeting BiTE predominantly localized to necrotic tumor tissue. Together the data in this chapter showed proof-of-concept ability of BiTEs to distribute to tumor tissue in an antigen-dependent fashion.

AMG 110 showed limited anti-tumor efficacy and dose-limiting toxicity associated with physiological gastrointestinal EpCAM expression.<sup>8</sup> The development of AMG 110 was subsequently discontinued. To improve the anti-tumor efficacy of the BiTE platform for solid tumors, a more restrictive tumor antigen is required. This led to the development of AMG 211, targeting human CEA, a pronounced tumor-associated antigen in gastrointestinal malignancies. We aimed to determine its tumor-targeting properties and biodistribution. For this,  $^{89}\text{Zr}$ -labeled and fluorescently labeled AMG 211 were studied in preclinical xenograft models in **chapter 4**. On top of *in vivo* distribution, we used *ex vivo* techniques to study AMG 211 integrity and intratumoral distribution. Finally, we manufactured  $^{89}\text{Zr}$ -AMG211, according to Good Manufacturing Practice (GMP), for a future clinical trial.  $^{89}\text{Zr}$ -AMG211 showed dose-dependent tumor uptake at 6 hours after intravenous administration. The highest tumor uptake was observed with 2  $\mu\text{g}$  and lowest tumor uptake with 500  $\mu\text{g}$  of  $^{89}\text{Zr}$ -AMG211. Also, PET visualized only CEA positive xenografts after 10  $\mu\text{g}$  administration at 24 hours after  $^{89}\text{Zr}$ -AMG211 administration. Despite an elimination half-life of approximately 1 hour, the tumor retained tracer uptake for at least 24 hours.  $^{89}\text{Zr}$ -AMG211 showed a time-dependent and tumor-specific disintegration, resulting in low molecular weight species over 50% at 24

hours. Fluorescently labeled AMG211 localized predominantly to viable CEA-positive tumor tissue. Lastly,  $^{89}\text{Zr}$ -AMG211 was successfully manufactured according to GMP guidelines, fulfilling all predefined release specifications. This study illustrated the feasibility for assessing the *in vivo* pharmacological behavior and tumor-targeting properties of  $^{89}\text{Zr}$ -AMG211 in a preclinical setting, and GMP-compliance allowed for a subsequent clinical study.

In the preclinical setting, as described in **chapter 4**, physiological tissue expression of human CEA and human CD3 $\epsilon$  were absent. Therefore in **chapter 5**, we aimed to characterize the biodistribution and tumor uptake of  $^{89}\text{Zr}$ -AMG211 in a first-in-human study. This two-center, molecular PET imaging phase I study was performed in patients with advanced gastrointestinal adenocarcinomas. We studied the biodistribution of  $^{89}\text{Zr}$ -AMG211 in healthy tissues and tumor lesions before and/or directly succeeding AMG 211 treatment. Patients received 37 MBq  $^{89}\text{Zr}$ -AMG211 intravenously with or without unlabeled AMG 211. Following tracer infusion, adverse events were monitored and graded according to NCI CTCAE v 4.03.<sup>9</sup> Before AMG 211 treatment, optimal imaging dose was 200  $\mu\text{g}$   $^{89}\text{Zr}$ -AMG211 and 1,800  $\mu\text{g}$  unlabeled AMG 211. This dose resulted in a mean standardized uptake value ( $\text{SUV}_{\text{mean}}$ ) of 4.0 in the blood pool 3 hours after intravenous tracer administration. PET imaging revealed CD3 $\epsilon$ -mediated uptake in spleen and bone marrow, with corresponding  $\text{SUV}_{\text{mean}}$  of 3.2 and 1.8, respectively. Of 43 visible tumor lesions, 37 were quantifiable with PET with a median maximum SUV of 4.0 (interquartile range 2.7 - 4.4). Within and between patients, heterogeneity in tumor uptake was reflected by a 5-fold and 9-fold difference, respectively. *Ex vivo* analysis showed intact  $^{89}\text{Zr}$ -AMG211 in the blood plasma and disintegrated species in the urine. After AMG 211 treatment,  $^{89}\text{Zr}$ -AMG211 was present in the circulation but was unable to visualize tumor lesions. The data presented in this chapter showed an accumulation of  $^{89}\text{Zr}$ -AMG211 in CD3 $\epsilon$ -rich lymphoid tissues, as well as a clear, inter- and intra-individual heterogeneous tumor uptake.

BiTEs are relatively small antibody-based therapeutics with serum half-lives of only several hours.<sup>5</sup> They are prone to be eliminated from the circulation by kidneys due to the 55-kDa size. Consequently, BiTEs are administered through continuous intravenous infusion to achieve stable serum levels and, thereby, sufficient drug exposure.<sup>5</sup> Using a full-sized bispecific antibody format of around 150 kDa, circulating half-lives usually range from days to weeks in human, allowing a more patient-friendly dosing scheme. An example of such a full-sized T cell-redirecting antibody is ERY974, targeting CD3 $\epsilon$  on T cells and glypican 3 on tumor cells. Glypican 3 is overexpressed by several solid tumors, including a majority of hepatocellular carcinoma and a subset of breast cancers.<sup>10</sup> In **chapter 6**, we radiolabeled ERY974 with  $^{89}\text{Zr}$  and studied its biodistribution by PET imaging in both xenograft-bearing immunodeficient as immunoprecient mouse models reconstituted with human immune cells.  $^{89}\text{Zr}$ -labeled control antibodies targeting CD3 $\epsilon$  and non-mammalian protein keyhole limpet hemocyanin (KLH) or KLH only served to determine the impact of each arm on its biodistribution. Information on deep tissue distribution was obtained by *ex vivo* tissue

autoradiography. In immunodeficient mice,  $^{89}\text{Zr}$ -ERY974 tumor uptake was dependent on tumoral GPC3 expression. In mice engrafted with human immune cells,  $^{89}\text{Zr}$ -ERY974 tumor uptake was higher than for the same xenograft in immunodeficient mice. *Ex vivo* tissue autoradiography demonstrated preferential accumulation of  $^{89}\text{Zr}$ -ERY974 in stromal T-cell rich infiltrate. Next to the tumor, the highest uptake for  $^{89}\text{Zr}$ -ERY974 was observed in the spleen and lymph nodes. This study allows for a future clinical trial with  $^{89}\text{Zr}$ -ERY974 to study its pharmacological behavior in patients with cancer.

In contrast to cytotoxic T cells, TAMs play an important role in creating an immunosuppressive tumor microenvironment, thereby promoting cancer progression. TAMs are known to be involved in breast cancer progression.<sup>11</sup> In a meta-analysis including over 2,000 patients with all-stage breast cancer, high TAM infiltration in the primary tumor predicted worse patient prognosis.<sup>12</sup> In **chapter 7**, we aimed to define the landscape of the role of TAMs in breast cancer. We reviewed the available literature and clinical trials to identify the influence of TAMs on tumor progression and potential targets to alter TAM biology. TAMs are associated with poor prognosis in patients with breast cancer. In the preclinical setting, TAMs were found to promote breast cancer growth, invasion, and metastasis. In addition, we pointed out that TAMs mediate resistance to chemotherapy, radiotherapy, targeted therapy, and immunotherapy in mouse models of mammary carcinoma. Furthermore, we provided an overview of clinical trials with therapeutics targeting TAMs. Based on this data, targeting TAMs is a potential therapeutic strategy for breast cancer.

Targeting TAMs by inhibiting the pro-survival axis CSF1/CSF1R with mAbs is currently evaluated in clinical trials, as described in **chapter 7**. However, limited information is available regarding the biodistribution and tumor-targeting of such mAbs. Therefore in **chapter 8**, we radiolabeled an anti-murine CSF1R mAb to evaluate its biodistribution. For this, we used an immunocompetent mouse model of mammary carcinoma. First, the distribution of  $^{89}\text{Zr}$ -CSF1R-mAb to healthy tissues was determined in non-tumor-bearing mice in a dose-escalation study. *Ex vivo* autoradiography and immunohistochemistry were served to study the intratumoral distribution of  $^{89}\text{Zr}$ -CSF1R-mAb and the presence of TAMs. Next in tumor-bearing mice, the biodistribution of  $^{89}\text{Zr}$ -CSF1R-mAb was compared to a  $^{89}\text{Zr}$ -labeled isotope control. In non-tumor-bearing mice, 10 mg/kg resulted in circulating levels of  $^{89}\text{Zr}$ -CSF1R-mAb for up to 72 hours. In contrast, 0.4 mg/kg  $^{89}\text{Zr}$ -CSF1R-mAb distributed mainly to spleen and liver, resulting in no tracer in the circulation at 24 hours after administration. In a mammary tumor model, 10 mg/kg  $^{89}\text{Zr}$ -CSF1R-mAb resulted in higher uptake in liver, lymphoid tissues, duodenum, and ileum, but not in tumor compared to  $^{89}\text{Zr}$ -labeled isotope control at 72 hours. Tissue autoradiography demonstrated CSF1R-specific localization of  $^{89}\text{Zr}$  in lymphoid tissues. Following  $^{89}\text{Zr}$ -CSF1R-mAb administration, TAMs were near absent as assessed by immunohistochemistry, whereas over 500 TAMs per  $\text{mm}^2$  were observed after  $^{89}\text{Zr}$ -labeled control. We hypothesize that the depletion of TAMs resulted in lower tumor uptake of  $^{89}\text{Zr}$ -CSF1R-mAb compared to  $^{89}\text{Zr}$ -labeled isotope control. In this study,

we provided data that show the potential of evaluating molecular imaging of macrophage-targeting therapeutics in clinical trials to understand their pharmacological behavior.

In conclusion, this thesis describes the development, characterization, and *in vivo* evaluation of radiolabeled antibody or antibody constructs to study its biodistribution and tumor targeting properties.

## GENERAL DISCUSSION AND FUTURE PERSPECTIVES

### Pharmacological behavior of T cell-directed therapeutics and new drug classes

Not all patients benefit from current cancer therapies. Therefore, new treatment modalities are explored. The drug distribution of such novel modalities is often poorly understood. As summarized in **chapter 2**, molecular imaging allows studying whole body drug distribution, target visualization, and heterogeneity in drug target expression, thereby supporting drug development. In **chapters 3, 4, 5, and 6**, we studied the biodistribution of a new class of drugs, namely T cell-directed bispecific antibody-based therapeutics. We showed distribution to the tumor in both the preclinical and the clinical setting. In an environment with CD3 $\epsilon$ , the CD3 $\epsilon$  binding arm also directs the bispecific drug to lymphoid tissues such as spleen and lymph nodes. So far, there are no approved T cell-directed bispecific therapeutic in the solid tumor setting. However, different novel formats are being developed, such as half-life extended versions or a full-sized antibody with a 2:1 format, creating bivalent tumor binding and monovalent T cell binding.<sup>6</sup> Molecular imaging with these new compounds might gain additional insight into solid tumor targeting of T cell-directed bispecific antibody therapeutics.

Besides the bispecific antibody class, other new approaches, such as gene therapy using oligonucleotides or cell therapy using chimeric antigen receptor (CAR) engineered T cells, are being developed. However, information about the pharmacological behavior of these therapeutics is scarce. By incorporating a PET reporter gene into a CAR T cell construct, PET imaging allows longitudinal tracking of CAR T cells. Several studies have demonstrated the potential of this approach, including a small clinical trial in patients with recurrent glioma.<sup>13-16</sup> Monitoring the persistence of CAR T cells in the tumor might provide additional insight next to the persistence in the systemic circulation by flow cytometry approaches.

Another emerging drug class is oligonucleotides.<sup>17,18</sup> These are synthetic therapeutics comprised of a single strand of deoxyribonucleic acid or ribonucleic acid. Oligonucleotide therapies can explicitly target genetic aberrations. Although in oncology, there are no clinically approved drugs available, other areas like in the case of patients with rare diseases and neurological disorders have shown encouraging results.<sup>17</sup> Single-photon emission computed tomography imaging of a radiolabeled antisense oligonucleotide (ASO) was studied after lumbar intrathecal administration in rats.<sup>19</sup> The radiolabeled ASO distributed to the cranium, associated with the meningeal lymphatics, egressed through peripheral lymph nodes, and was eliminated from the systemic circulation by the kidneys. Molecular imaging in future small-scale clinical trials using PET imaging may help in better understanding the pharmacological

behavior of these novel therapeutics and support their development. However, for all indications, the radiation burden has to be taken into account, especially in the non-oncology setting. Nevertheless, the development of the total-body PET scanner has led to improved sensitivity and allows lower radiation exposure with a similar resolution.<sup>20,21</sup> Alternatively, non-radioactive labeling with near-infrared fluorophores allows assessing tissue distribution in the intraoperative setting.<sup>22,23</sup>

### **Molecular imaging of immune cells to support cancer drug development**

Directly radiolabeling a drug of interest to study its biodistribution helps to understand its pharmacological behavior. Nevertheless, visualizing pharmacodynamic changes in cell populations upon treatment might provide additional information for drug development. The field of immunotherapy is rapidly expanding, and many cells of the tumor microenvironment are involved. Therefore, several cell populations might be a candidate for pharmacodynamics assessment by molecular imaging. Many immunotherapeutics, including T cell-directed bispecific antibody-based therapeutics, rely on the cytotoxic potential CD8 T cells to kill tumor cells. Imaging CD8 T cells could potentially identify patients likely to respond to immunotherapy. Moreover, it might allow to monitor changes in CD8 T cells in the tumor upon immunotherapy treatment and thereby identify early responders.<sup>24</sup> Multiple clinical trials are studying CD8 populations using molecular imaging (e.g., NCT03802123, NCT04029181), and first-in-human data ( $n = 6$ ) has recently been described.<sup>25</sup>

Besides cytotoxic T cells, TAMs play an important role in cancer, particularly in breast cancer, as summarized in **chapter 7**. Strategies include the depletion of TAMs but also reprogramming macrophages to a more anti-tumoral phenotype. An example of a TAM depleting strategy is by targeting CSF1R, a crucial receptor for macrophage survival. In **chapter 8**, we describe the distribution of a CSF1R mAb by PET imaging and *ex vivo* biodistribution. CSF1R mAb distributed mainly to the liver and spleen, showing limited tumor selectivity. However with a high tracer dose, tumoral macrophages were depleted. Instead of macrophage depletion, the anti-tumoral role of macrophages has gained interest. Activation of the signal regulatory protein  $\alpha$  (SIRP $\alpha$ )-CD47 axis, of which SIRP $\alpha$  is expressed by macrophages and CD47 by tumor cells, inhibits phagocytosis by macrophages.<sup>26</sup> Another interesting approach is the use of macrophages as a cellular therapy. Recently, macrophages with a CAR were found to enhance tumor-antigen specific phagocytosis.<sup>27</sup> Administration of CAR-macrophages resulted in decreased tumor burden and prolonged overall survival in mice bearing a solid tumor xenograft.<sup>27</sup>

Preclinical models usually do not provide a full context to study the biodistribution of immunotherapeutics or specific immune cells with molecular imaging.<sup>28</sup> Although immunocompetent mouse models can serve to study the interaction between murine tumors and the murine immune system, mice are still inherently different from humans. Therefore, early phase clinical trials with novel imaging tracers are ultimately warranted.



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