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General Introduction

BACKGROUND

Cancer immunotherapy is one of the most recent successes in cancer drug development. Cancer immunotherapy improves patient survival across several tumor types. Most used are the immune checkpoint inhibitors directed at the programmed death-1 (PD-1) receptor/ programmed death-ligand 1 (PD-L1) axis. Tumors exploit this checkpoint by suppressing T cell activation and thereby preventing tumor cell death. Blocking this axis by PD-1/PD-L1 inhibitors reinvigorates T cell activity and subsequent tumor cell killing. Unfortunately, not all patients respond to immune checkpoint inhibitors, and other strategies are therefore needed. An alternative option to activate T cells might be to redirect T cells to the tumor. This can potentially be achieved by a category of bispecific antibodies, of which one arm can bind to a tumor antigen, and the other arm is directed at CD3ε on T cells. The simultaneous binding to the tumor and T cells leads to specific T cell activation and subsequent tumor cell killing.[1] This approach is used by bispecific T cell engagers (BiTEs), a new class of bispecific therapeutics. BiTEs are comprised of two single-chain variable fragments resulting in a 55 kilodalton (kDa) antibody constructs. The only BiTE that has been registered for clinical use is blinatumomab, targeting CD3ε and CD19. Blinatumomab is applied for the treatment of patients with relapsed or refractory B-cell precursor acute lymphoblastic leukemia. Next to BiTE constructs, also full-sized bispecific monoclonal antibodies (mAbs; ±150 kDa) are available, such as ERY974 targeting glypican 3 (GPC3) and CD3ε. Although many bispecific antibodies targeting a tumor antigen and T cells are in clinical development for both hematologic and solid malignancies, only blinatumomab has been approved for clinical use.[1]

T cell activation can also be achieved by eliminating immunosuppressive cell types in the tumor microenvironment. Tumor-associated macrophages (TAMs), which do suppress T cell activity, play a definite role.[2] Preclinically, depleting TAMs from the tumor microenvironment by TAM-directed antibodies synergizes with other immunotherapeutic strategies.[3] Both bispecific antibodies and TAM-directed therapeutics are currently in clinical trials.

A pharmacological audit trail has been proposed by trialists at The Institute of Cancer Research (London, United Kingdom) to support drug development. This trail comprises a set of critical questions about drug performance, for instance, target engagement and pharmacokinetic characteristics.[4] Molecular imaging can help understand some of those aspects as it allows non-invasive whole body information regarding biodistribution and tumor targeting.

THE AIM OF THE THESIS

This thesis aims to gain insight into the pharmacological behavior of novel antibody-based immunotherapeutics using molecular imaging to support cancer drug development.

OUTLINE OF THE THESIS

Molecular imaging with labeled cancer drugs is used in the preclinical and the clinical setting

to understand pharmacological drug behavior better. Directly labeling cancer drugs with either a radioactive nuclide to allow positron emission tomography (PET) or a fluorescent dye for optical imaging can study the distribution and targeting characteristics of these drugs. For radiolabeling, several isotopes can be employed. The PET isotope zirconium-89 (^{89}Zr) is well suited for larger biomolecules due to its matching physical half-life of 3.27 days. A chelator needs to be linked to the cancer drug to allow ^{89}Zr-labeling. *N*-succinyl (*N*-suc) desferrioxamine (DFO) is commonly used as a linker. For optical imaging, fluorescent dyes in the near-infrared spectrum are suitable, as these dyes have limited autofluorescence. Fluorescently labeled biomolecules allow higher spatial resolution at the microscopic level to visualize, for example, intratumoral drug distribution.

To better understand how molecular imaging could support cancer drug development, a literature study was performed, which is described in **chapter 2**. PubMed was searched for English literature with a focus on molecular imaging in the context of drug-target expression, drug pharmacokinetics, and pharmacodynamics in cancer. Molecular imaging with small molecules targeting tyrosine kinases are discussed, as well as imaging with mAbs targeting growth factor receptors or the immune system. Moreover, molecular imaging of hormone receptors as a pharmacodynamic marker is being described.

In **chapters 3**, **4**, and **5**, we focus on molecular imaging using BiTE antibody constructs. Following blinatumomab, other BiTE molecules have been developed. These include multiple BiTEs directed at solid tumor targets. To support their drug development, we radiolabeled two such BiTEs: AMG-110 and AMG-211, both apart from directed at CD3ε, target the epithelial cell adhesion molecule (EpCAM) and carcinoembryonic antigen (CEA), respectively. Labeled AMG-110 is preclinically studied in **chapter 3**. Molecular imaging with ^{89}Zr-labeled AMG211 was assessed preclinically in **chapter 4** and in patients with gastrointestinal adenocarcinomas in **chapter 5**.

The BiTE AMG-110 targets EpCAM on tumor cells and CD3ε on T cells. EpCAM is overexpressed by many solid tumors such as colon, gastric, prostate, ovarian, and lung cancer.[5] In a phase 1 clinical trial (n = 65; 54 with response assessment), 31% of patients showed stable disease as best response with a median duration of 84 days (range, 21-355 days).[6] To better understand AMG-110's tumor-targeting properties, we assessed in **chapter 3** the biodistribution of ^{89}Zr-labeled AMG-110 in mice using noninvasive small-animal PET imaging. We studied [^{89}Zr]Zr-DFO-*N*-suc-AMG110 (^{89}Zr-AMG110) uptake up to 144 hours after intravenous injection in mice bearing an EpCAM expressing HT-29 xenograft model using small-animal PET imaging as well as *ex vivo* biodistribution. Furthermore, we studied the effect of increased protein dose up to 500 µg on the biodistribution and tumor-targeting of ^{89}Zr-labeled AMG110. The non-EpCAM binding BiTE [^{89}Zr]Zr-DFO-*N*-suc-Mec14 (^{89}Zr-Mec14) was used as a control in HT-29 xenografts. ^{89}Zr-AMG110 uptake was also assessed in FaDu and HL-60 xenograft models with medium or no EpCAM expression, respectively. Subsequently, ^{89}Zr-AMG110 tumor uptake was correlated to EpCAM expression. Finally, we

visualized the intratumoral distribution of fluorescently labeled AMG-110. This was done in conjunction with Mec14 conjugated to a different fluorescent dye as a non-specific control.

Clinical development of AMG-110 was discontinued due to toxicity issues. These may be related to the expression of EpCAM in normal epithelial tissues.[6] To reduce dose-limiting toxicity, alternative targets that have limited physiological expression and sufficient overexpression on tumor cells might be a better target. This led to the development of AMG-211, targeting CEA.

In **chapter 4**, we performed a preclinical approach to study the behavior of [^{89}Zr]Zr-DFO-*N*-suc-AMG211 (^{89}Zr-AMG211). This included tumor targeting, tissue distribution, and *in vivo* tracer integrity in blood and tumor lysate. Using *ex vivo* biodistribution, we assessed dose-dependent tissue distribution and tumor targeting of ^{89}Zr-AMG211 in immunodeficient mice bearing a CEA expressing LS174T colorectal adenocarcinoma xenograft. Tumor uptake and biodistribution of 10 µg ^{89}Zr-AMG211 were visualized with small-animal PET imaging in LS174T xenograft bearing immunodeficient mice. To determine specific uptake, we visualized ^{89}Zr-Mec14 tumor targeting and tissue distribution in the same model. The influence of differential tumor CEA expression on ^{89}Zr-AMG211 tumor uptake was assessed in another CEA positive xenograft, namely human breast cancer BT474, and a CEA negative promyelocytic leukemia HL-60 xenograft, both in immunodeficient mice. Furthermore, we assessed the integrity of ^{89}Zr-AMG211 in both blood and tumor lysate. To study the intratumoral distribution of AMG-211, we co-injected AMG-211 and Mec14 labeled with different fluorescent dyes in LS174T xenograft bearing immunodeficient mice. The intratumoral distribution of AMG-211 and Mec14 was subsequently assessed *ex vivo*. Finally, we manufactured ^{89}Zr-AMG211 according to GMP guidelines to allow the exploration of ^{89}Zr-AMG211 in a clinical setting.

As the preclinical study was performed in an immunodeficient environment, it was unknown how the presence of CD3 target would affect the distribution of ^{89}Zr-AMG211 *in vivo*. The GMP-manufactured ^{89}Zr-AMG211 allowed assessment of its biodistribution in patients with gastrointestinal carcinomas, described in **chapter 5**. These tumors are known to overexpress CEA. In this feasibility study, we performed PET imaging at 3, 6, and 24 hours after tracer administration to study the time-dependent biodistribution. Uptake in organs or interest was quantified as standardized uptake values. Next to the impact of time, we investigated the effect of different tracer protein doses on the biodistribution of ^{89}Zr-AMG211. We used a radiolabeled dose of 200 µg ^{89}Zr-AMG211 and supplemented with 0, 1800, or 4800 µg unlabeled AMG-211. Furthermore, ^{89}Zr-AMG211 distribution was studied both before and on AMG-211 treatment. Integrity of ^{89}Zr-AMG211 in plasma and urine samples was studied with gel electrophoresis and autoradiography.

Due to the small size, BiTEs have a relatively short elimination half-life in the human circulation as kidneys excrete them. BiTEs like AMG-110 and AMG-211 are administered by continuous infusion to allow sufficient exposure to patients. Full-sized antibodies have

usually have elimination half-lives up to several days and therefore, do not require continuous infusion. An example of a full-sized bispecific antibody is ERY974 that targets CD3ε on T cells and glypican 3 on tumor cells. ERY974 demonstrated to be a potent inducer of T cell-mediated cytotoxicity in the preclinical setting.[7] However, limited information is available about its *in vivo* biodistribution and the impact of T cells on this distribution. In **chapter 6**, we studied with [^{89}Zr]Zr-DFO-*N*-suc-ERY974 (^{89}Zr-ERY974) the role of T cells on the biodistribution of ERY974 in tumor-bearing mice with a humanized immune system. Immunodeficient mice bearing a GPC3 expression hepatocellular carcinoma HepG2 xenograft received ^{89}Zr-ERY974 intravenously. ^{89}Zr-ERY974 distribution over time up to 7 days after administration was studied using small-animal PET. ^{89}Zr-ERY974 distribution was additionally studied in another GPC3 expressing xenograft, the ovarian clear cell carcinoma cell line TOV-21G, as well as in a GPC3 negative hepatocellular carcinoma SK-HEP-1. The influence of T cells on the biodistribution of ^{89}Zr-ERY974 was studied in HepG2 xenograft-bearing immunodeficient mice reconstituted with human CD3+ T cells via CD34+ hematopoietic stem cells. A ^{89}Zr-labeled bispecific antibody targeting CD3+ and a non-mammalian target keyhole limpet hemocyanin (KLH), served to determine the CD3 arm contribution. Besides, a ^{89}Zr-labeled antibody bivalent for KLH served as a negative control molecule. Intratumoral, intrasplenic, and intranodal distribution were studied *ex vivo* using autoradiography. Findings were correlated with CD3+ infiltration assessed immunohistochemically.

Besides T cells, also other immune cells impact cancer biology. These include cells of the myeloid lineages of which TAMs are an important player. A literature study was performed and described in **chapter 7** using breast cancer as a tumor model to better understand the role of TAMs in cancer biology. We searched PubMed with the following terms: "macrophage", "tumor-associated macrophage", "breast cancer", "prognosis", "molecular imaging", and "breast tumor" using different combinations. Besides, abstracts of annual oncology meetings of the American Society of Clinical Oncology, American Association of Cancer Research, European Society of Medical Oncology, and San Antonio Breast Cancer Symposium between 2014-2018 were reviewed using the same terms. Preclinical studies using models of human breast cancer, mammary tumor cell lines, or transgenic mammary tumor models were included. Concerning TAM targeting therapies, ClinicalTrials.gov and EudraCT databases were searched. This study discusses the rationale of targeting TAMs in breast cancer, including the prognostic value of TAMs, its role in tumor growth and metastasis and treatment resistance. In addition, the current evidence of TAM targeting is evaluated.

One of the therapeutic options for targeting TAMs is by inhibiting the colony-stimulating factor 1 (CSF1)/CSF1 receptor (CSF1R) axis. Several therapeutic options ranging from small molecules to mAbs are currently evaluated in the clinic. To better understand the behavior of CSF1R targeting drugs, we radiolabeled a mAb targeting murine CSF1R, with ^{89}Zr. We evaluated its distribution in immunocompetent non-tumor and tumor-bearing mice in **chapter 8**. Non-tumor-bearing mice received 0.4 mg/kg of [^{89}Zr]Zr-DFO-*N*-suc-CSF1R-mAb

(^{89}Zr-CSF1R-mAb) intravenously, followed by small-animal PET and *ex vivo* biodistribution at 24 and 72 hours. *Ex vivo* autoradiography and tissue morphology using hematoxylin and eosin staining were performed to study macroscopic tissue distribution. The impact of the tracer protein dose up to 10 mg/kg was analyzed using both small-animal PET and *ex vivo* biodistribution at 24 and 72 hours. Next, ^{89}Zr-CSF1R-mAb was evaluated in a tumor-bearing mouse model. A donor tumor from *K14cre;Cdh1$^{F/F}$;Trp53$^{F/F}$* mouse model for spontaneous mammary tumorigenesis served as a tumor model.[8,9] This murine mammary tumor resembles human invasive lobular carcinomas and is strongly infiltrated with macrophages.[8,9] Smaller pieces of a donor tumor were orthotopically transplanted in wild type mice. In tumor-bearing mice, [^{89}Zr]Zr-DFO-*N*-suc-IgG_{2a} was the control molecule to determine CSF1R specific uptake. Tumoral infiltration of macrophages was determined with immunohistochemistry using the murine pan-macrophage marker F4/80.

Finally, in **chapter 9,** we summarized the experimental results of this thesis and discussed the implications and perspectives for future studies.

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