**Zr-radiopharmaceuticals to study whole-body distribution and response to antibody-based cancer immunotherapies**

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Chapter 1

General introduction
Background

Immunotherapy has become an essential pillar of cancer treatment and obtained a clear role in clinical cancer care. This type of therapy has shown impressive results by increasing the survival of patients with advanced stages of several tumor types. Immunotherapeutic drugs are characterized by using components of the immune system to induce an effective antitumor immune response (1). Some cancer immunotherapies consist of (parts of) antibodies that inhibit the function of proteins expressed by cancer cells, mostly immune checkpoints. Other cancer immunotherapies include small immunomodulating molecules, cell-based immunotherapies, vaccines and oncolytic viruses. Currently, the European Medicines Agency (EMA) and Food and Drug Administration (FDA) have approved 8 different immune checkpoint-inhibiting antibodies to treat 19 tumor types. These immune checkpoint-inhibiting antibodies are directed against the programmed cell death-1 (PD-1) receptor and its ligand, programmed cell death-ligand 1 (PD-L1), as well as cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) and lymphocyte activation gene-3 (LAG-3).

As of December 2019, the number of immunotherapeutic anticancer agents in the global drug development pipeline showed a 91% increase during the past 2 years: From 2,030 to 3,876 (2). The clinical trial landscape for cancer immunotherapies involves numerous studies with PD-1/PD-L1 immune checkpoint-inhibiting antibodies as a single agent for multiple indications and in combination regimens. CTLA-4 combined with PD-1/PD-L1 improves response rates and overall survival for specific cancers (3–6), but also demonstrates increased side-effects, mostly related to the immune system (7). With many new immunotherapeutic drugs being developed, companion diagnostics to tailor treatment to individual patients are urgently needed.

PD-L1 expression, microsatellite-instability (MSI)/defective mismatch repair (dMMR) and tumor mutational burden (TMB) are clinically used predictive biomarkers for immune checkpoint inhibitors in cancer patients (8). These biomarkers play an important role in assisting patient selection. However, different tumor types require distinct assays with their corresponding limitations. Treatment decisions are often based on immunohistochemistry analysis in a single tumor biopsy and dosing schedules are determined using blood-based pharmacokinetic analyses. Heterogeneity in target expression and variable drug uptake between tumor lesions within one patient are thereby not considered. Molecular imaging with positron emission tomography (PET) can serve to gain real-time information on all tumor lesions within the patient’s body. Radiolabeling immune-targeting antibodies with a PET isotope allows for non-invasive evaluation of drug pharmacokinetics, immune target expression, and immunological responses. When developing these radiopharmaceuticals, the half-life of the applied radioisotope ideally matches the time that antibodies need to distribute and accumulate in
target-expressing tissues. The PET isotope zirconium-89 ($^{89}$Zr) has a half-life of 3.3 days and is compatible with the serum half-life of most immunotherapeutic antibodies.

The aim of the research described in this thesis is to develop $^{89}$Zr-radiopharmaceuticals to advance the development of novel cancer immunotherapies and explore their use as a biomarker of response.

**Thesis outline**

Radiopharmaceuticals with antitumor activity can be used for therapy and diagnostics, and represent a rapidly expanding group of cancer medicines. In **chapter 2**, we aim to provide an overview of current research on radiolabeled antibodies and antibody-related therapeutics that may be used for both therapy and diagnostics using PET imaging. We perform a PubMed search using the terms “PET” AND “Cancer” AND “Antibody” OR “ADC” OR “Bispecific” in combination with the most commonly used PET radionuclides: $^{64}$Cu, $^{68}$Ga, $^{86}$Y, $^{89}$Zr, and $^{124}$I. In addition, we search ClinicalTrials.gov for ongoing studies using the terms “Cancer” AND “PET” NOT “FDG.” A total of 1,448 (pre)clinical studies are reviewed to provide an up-to-date overview. Also, we identify several challenges for translating the use of radiopharmaceuticals to standardized and, ultimately, daily routine patient care.

Radioimmunotherapeutic (RIT) agents are a subclass of radio-pharmaceuticals, which employ antibodies as targeted delivery vehicles for therapeutic $\alpha$- or $\beta$-emitting radionuclides to selectively eradicate tumor cells. $^{177}$Lu-NNV003, a RIT agent targeting leukocyte antigen CD37, is developed to potentially treat patients with B cell non-Hodgkin’s lymphoma (NHL) refractory to or relapsed during anti-CD20 radioimmunotherapy (RIT). As CD37 is expressed on malignant and normal B cells, patients are at risk for developing hematological toxicities. Therefore, a tool to non-invasively assess CD37–targeting by $^{177}$Lu-NNV003 RIT is of utmost interest and could assist its clinical development and use. In **chapter 3**, we evaluate the utility of $^{89}$Zr–labeled NNV003 PET imaging in predicting whole-body distribution and tumor uptake of $^{177}$Lu-NNV003 RIT. NNV003 is radiolabeled with $^{89}$Zr and its in vivo distribution is evaluated in immune-compromised mice bearing human CD37–expressing REC1 B cell NHL or RAMOS Burkitt’s lymphoma xenograft tumors. Indium-111 ($^{111}$In)–labeled IgG served as control antibody. PET imaging is performed at day 5 post $^{89}$Zr–NNV003 administration, followed by ex vivo quantification of uptake per organ. Whole-body distribution and tumor-targeting properties of $^{89}$Zr–NNV003 are compared to $^{177}$Lu-NNV003 in the same mouse model.

To better predict response to immune checkpoint therapy, $^{89}$Zr–radiopharmaceuticals can be applied to gain insight in the in vivo behavior of immune checkpoint–targeting antibodies. The PD–I immune checkpoint its primarily expressed by T cells, while its ligand PD–L1 is expressed...
by tumor cells, B-cells, NK-cells, dendritic cells and macrophages. In **chapter 4**, we study the whole-body distribution of $^{89}$Zr-labeled anti-PD-1 antibody pembrolizumab and $^{89}$Zr-IgG$_4$ control antibody in humanized mice compared to non-humanized mice, xenografted with A375M human melanoma tumors. We perform PET imaging at day 7 post $^{89}$Zr-pembrolizumab administration to visualize distribution to human peripheral mononuclear blood cells (PBMCs) present in the tumor, spleen, lymph nodes, thymus and bone marrow, quantified by ex vivo analysis. Tumor and spleen tissues are analyzed by immunohistochemistry for PD-1, CD3 and CD8 expression and autoradiography.

While PD-L1 expression and TMB are EMA-approved biomarkers for non-small cell lung cancer (NSCLC) and solid tumors respectively, not all patients with high tumor PD-L1 expression and high TMB respond to pembrolizumab treatment. In **chapter 5**, we used PET imaging to assess $^{89}$Zr-pembrolizumab tumor uptake and whole-body distribution before immune checkpoint inhibitor therapy and explored its relationship with patient response. First, $^{89}$Zr-pembrolizumab analytical methods and manufacturing procedures are validated under good manufacturing practice (GMP) conditions to enable administration to patients. Eighteen patients, 11 with melanoma and 7 with NSCLC, received 37 MBq $^{89}$Zr-pembrolizumab (~2.5 mg) intravenously plus 2.5 or 7.5 mg unlabeled pembrolizumab followed by PET imaging on days 2, 4, and 7. Thereafter, PD-1-targeting antibody treatment per standard of care (pembrolizumab or nivolumab ± ipilimumab) is initiated. $^{89}$Zr-pembrolizumab tumor uptake is determined as maximum standardized uptake value (SUV$_{\text{max}}$), normal organ uptake is determined as mean standardized uptake value (SUV$_{\text{mean}}$). Tumor response is assessed according to (i)RECIST v1.1. Archival tumor tissue or fresh biopsies obtained after the last PET scan were stained immunohistochemically for PD-1, PD-L1, and CD8.

Immune-related adverse events are often observed during treatment with immune checkpoint-inhibiting antibodies. Side-effects experienced by patients treated with single-agent anti-PD-1 or anti-CTLA4 antibody increase by 34% when they are combined. CX-072, a protease-activatable antibody targeting both human and murine PD-L1, is designed to be specifically activated in tumor tissues, thus potentially reducing immune-related toxicities in normal tissues. The in vivo behavior of such a protease-activatable antibody is unknown. In **chapter 6**, we perform $^{89}$Zr-CX072 PET imaging in immune-competent and immune-compromised mice bearing PD-L1-expressing tumors. $^{89}$Zr-CX-072 uptake in tumors and immune tissues at day 7 post $^{89}$Zr-CX072 administration is quantified ex vivo and compared to $^{89}$Zr-labeled normal anti-PD-1 antibody (not protease-activatable) and $^{89}$Zr-labeled control Probody. Tumor, spleen, lymph nodes, bone marrow and thymus are analyzed for PD-L1 expression by immunohistochemistry and flow cytometry. We use autoradiography to correlate $^{89}$Zr-CX-072 distribution to PD-L1-expressing tumor areas. Levels of activated CX-072
are measured by Western blot analysis in the tumor and spleen to further explore tumor-specific activation.

To enable administration in patients, a GMP-compliant manufacturing process for $^{89}$Zr-CX-072 was developed and validated. In chapter 7, we investigate how CX-072’s Probody therapeutic design affects its whole-body distribution in eight patients with variable types of solid tumors. Patients received 37 MBq $^{89}$Zr-CX-072 (~1 mg) plus 0, 4, or 9 mg unlabeled CX-072, followed by PET imaging on days 2, 4, and 7. Thereafter, treatment with CX-072 or CX-072 + ipilimumab is initiated. $^{89}$Zr-CX-072 uptake in normal tissues is expressed as SUV$_{\text{mean}}$ and tumor uptake as SUV$_{\text{max}}$. Tumor response is determined according to (ir)RECIST v1.1. We measure PD-L1 expression immunohistochemically in archival tumor tissue. In the blood pool, presence of intact (inactive) $^{89}$Zr-CX-72 was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Bispecific antibodies may represent an alternative strategy to increase tumor immunogenicity if they bind T cells with one arm and tumor cells with the other arm, thereby redirecting the T cell response to the tumor. ERY974 is a bispecific antibody that engages CD3 on T cells and glypican 3 (GPC3) on tumors. ERY974 has different binding affinities for CD3 and GPC3, with dissociation constants ($K_d$) of 207 nM and 1.5 nM respectively, and therefore its in vivo pharmacokinetics are not easily predicted. In chapter 8, PET imaging is used to reveal the whole-body distribution of $^{89}$Zr-ERY974, followed by ex vivo quantification of organ uptake in mice bearing xenograft tumors with different levels of GPC3 expression. We use immune-compromised mice and mice reconstituted with human PBMCs, as well as $^{89}$Zr-labeled control antibodies targeting CD3/non-mammalian protein keyhole limpet hemocyanin (KLH) or KLH only. The redirection of T cells in ex vivo tumor tissues is evaluated by autoradiography and CD3 immunohistochemistry.

Immune checkpoint-inhibiting antibodies stimulate the distribution of CD8$^+$ T cells from lymph nodes to normal immune tissues and tumors (9–11). Immune checkpoint inhibitor-induced tumor-infiltrating CD8$^+$ T cells are associated with response across multiple tumor types, including melanoma. In chapter 9, we study the whole-body distribution of CD8$^+$ T cells by $^{89}$ZED88082A PET imaging in patients with solid tumors before and after immune checkpoint inhibitor therapy. Thirty-eight eligible patients with locally advanced or metastatic solid tumors receive 37 MBq (~1.7 mg) $^{89}$ZED88082A followed by PET imaging after 1 hour and on days 2, 4, 7. After selecting the optimal dose, patients receive $^{89}$ZED88082A before and after two cycles of atezolizumab treatment and PET scans on days 2 and 4. Normal organ tracer uptake was calculated as SUV$_{\text{mean}}$, tumor lesion and lymph node uptake as SUV$_{\text{max}}$. Tumor response is determined based on (i)RECIST1.1. Tumor biopsies are collected after the last PET scan and
analyzed by CD8 immunohistochemistry and autoradiography. Blood samples are drawn for 
$^{89}$ZED88082A pharmacokinetic and anti-drug antibody (ADA) analyses.

Finally, a summary of thesis results and future perspectives are described in chapter 10. A 
Dutch thesis summary is provided in chapter 11.

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