Molecular imaging of immunotherapy biodistribution and the tumor immune environment

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Summary and future perspectives

SUMMARY

The major anticancer effects of cancer immune checkpoint inhibitors changed treatment options for patients with several tumor types. Durable responses and cures have been observed in the metastatic setting. However, not all patients respond to currently available immunotherapies. To optimally exploit the effects of cancer-immunotherapy it is paramount to identify as early as possible the patients that will and will not respond, and to understand why patients respond. Furthermore, developing novel strategies beneficial to patients that previously did not respond is critical to further advance cancer care. A novel approach is the application of bispecific T-cell engager molecules. They, in contrast to other cancer drugs, actively involve and bind immune cells to induce T-cell mediated tumor cell killing. Understanding their biodistribution will provide insight into their application in the clinic.

Molecular imaging can be used to non-invasively visualize the tumor immune microenvironment and show how it changes in response to cancer-immunotherapy. In addition, molecular imaging can facilitate drug development by providing information on drug biodistribution and tumor targeting.

The research performed in this thesis uses molecular imaging and aims to evaluate the biodistribution of novel immunotherapies, with a focus on bispecific T-cell engager molecules, and to visualize the tumor immune microenvironment.

Chapter 1 introduces and outlines the thesis. In **chapter 2**, we reviewed the available literature in PubMed and the clinical trial database of ClinicalTrials.gov to establish an overview of bispecific antibody constructs in oncology and their current state in clinical development. We describe the different bispecific antibody constructs and their mechanism of action. We identified 57 bispecific antibody constructs currently undergoing clinical evaluation. One of them, blinatumomab, is registered already for the treatment of patients with B-cell acute lymphoblastic leukemia. Of these 57 bispecific antibody constructs, 38 engage immune cells to the tumor, 5 deliver a payload and 14 block signaling in the tumor environment. But despite a large number of bispecific antibodies in clinical trials, not one was being evaluated already in a phase 3 clinical trial, apart from blinatumomab. We identified several hurdles that help explain the gap in the development of bispecific antibody constructs in oncology, including toxicity and limited efficacy in solid tumors. Molecular imaging can help to identify these hurdles early in preclinical and phase 1 trials and thereby support drug development.

Little is known about the biodistribution in patients of bispecific T-cell engager (BiTE) molecules targeting solid tumors. This has only been preclinically studied in immunodeficient mice. In **chapter 3** the results are described of our study which aimed to better understand the biodistribution of BiTE molecules and the influence of each targeting arm. The positron emission tomography (PET) isotope zirconium-89 (^{89}Zr) was labeled to three

different BiTE molecules. BiTE molecule muS110, targeting murine epithelial cell-adhesion molecule (EpCAM; K_d = 21 nM) on tumor cells and targeting murine CD3 (K_d = 2.9 nM) on T-cells, hyS110 targets human EpCAM, absent in mice, and murine CD3 (K_d = 2.9 nM) and a non-targeting human-specific control BiTE AMG 110. Their biodistribution was evaluated in immunocompetent and immunodeficient solid tumor-bearing mouse models. ^{89}Zr-muS110 showed rapid renal clearance and a distribution blood half-life of 0.4 hours and elimination half-life of 12.8 hours in immunocompetent mice bearing an EpCAM-positive murine mammary carcinoma. PET imaging and *ex vivo* biodistribution in immunocompetent mice with ^{89}Zr-muS110 and ^{89}Zr-hys100 showed uptake in tumor, spleen, and other lymphoid tissues, while the control BiTE ^{89}Zr-AMG 110 showed similar tumor uptake but lacked spleen uptake. ^{89}Zr-muS110 spleen uptake, expressed as percentage injected dose per gram (%ID/g) was lower in immunodeficient (3.37 [2.62 to 3.76] %ID/g) than in immunocompetent mice (6.89 [6.74 to 8.29] %ID/g). *Ex vivo* biodistribution, after repeated administration of non-radiolabeled muS110 to immunocompetent mice, showed decreased ^{89}Zr-muS110 uptake in spleen and other lymphoid tissues compared to mice without repeated administration. The uptake after repeated administration of muS110 was comparable to the uptake in immunodeficient mice, indicating saturation of CD3 binding sites by repeated administration of muS110. Autoradiography and immunohistochemistry demonstrated colocalization of ^{89}Zr-muS110 and ^{89}Zr-hyS110 with CD3-positive T-cells in the tumor and spleen but not with EpCAM expression. This study showed that in immunocompetent mice, the BiTE ^{89}Zr-muS110 distribution is predominantly based on its high affinity CD3 binding arm with a limited contribution of its second arm, targeting EpCAM.

To increase their half-life, BiTE molecules were genetically fused to an Fc-domain resulting in half-life extended BiTE (HLE BiTE) molecules. In **chapter 4**, we describe the evaluation of the biodistribution of a novel HLE BiTE molecule compared to a non-targeting HLE BiTE in solid tumor-bearing mice. The MSLN HLE BiTE molecule targets murine mesothelin (K_d = 3.0 nM), predominantly expressed on the tumor cells, and targets murine CD3 (K_d = 26.8 nM) on T-cells. The MSLN HLE BiTE was labeled with ^{89}Zr enabling molecular imaging. PET imaging with 50 µg ^{89}Zr-MSLN HLE BiTE revealed a long blood half-life of 63.4 hours in immunocompetent mice bearing a MSLN-positive murine mammary carcinoma. Five days after administration, ^{89}Zr-MSLN HLE BiTE showed higher uptake, expressed as mean standardized uptake value (SUV_{mean}), compared to the control HLE BiTE in tumor (SUV_{mean} = 1.5 ± 0.2 vs 0.8 ± 0.1) and spleen (SUV_{mean} = 1.3 ± 0.1 vs 0.5 ± 0.1). Quantification of PET data obtained 5 days after tracer injection revealed that a lower dose of 10 µg ^{89}Zr-MSLN HLE BiTE cleared faster from the blood than the 50 µg and the 200 µg dose. Tumor SUV_{mean} was lower in mice that received 10 µg (1.2 ± 0.1) than those receiving 50 µg (1.5 ± 0.2). Moreover, spleen uptake was dose-dependent (SUV_{mean}: 10 µg = 1.6 ± 0.2; 50 µg = 1.3 ± 0.1; 200 µg = 0.8 ± 0.1). Uptake of ^{89}Zr-MSLN HLE BiTE in the gastrointestinal tract overlapped with the presence of lymph nodes. Autoradiography and immunohistochem-

istry showed that ^{89}Zr-MSLN HLE BiTE in the spleen colocalized with positive staining for CD3. In the tumor, positive MSLN staining colocalized with increased accumulation of ^{89}Zr-MSLN HLE BiTE. This shows the involvement of both targeting arms in the biodistribution. In conclusion, the extended half-life allowed for specific tumor uptake and supported the potential for clinical translation of HLE BiTE molecules.

The biodistribution of bispecific antibodies constructs, including BiTE molecules, is largely unknown in patients. To reveal this, the first-in-human PET imaging study with a BiTE molecule, namely AMG 211, is reported in **chapter 5**. A dose of 200 µg ^{89}Zr-AMG 211, targeting carcinoembryonic antigen (CEA) on tumor cells and CD3 on T-cells, was administered to nine patients with advanced gastrointestinal adenocarcinomas with or without a cold (unlabeled) dose of AMG 211. The optimal imaging dose before AMG 211 treatment was 200 µg ^{89}Zr-AMG 211 plus 1800 µg cold AMG 211. At 3 hours, the highest blood pool SUV_{mean} was 4.0, and the tracer serum half-life was 3.3 hours. Uptake was observed in CD3-rich lymphoid tissues, including spleen and bone marrow (SUV_{mean} = 3.2 and 1.8, respectively). ^{89}Zr-AMG 211 remained intact in plasma and was excreted predominantly via the kidneys in degraded forms. Thirty-seven of 43 visible tumor lesions were PET quantifiable, with a SUV_{max} of 4.0 (2.7 to 4.4) at 3 hours using the optimal imaging dose. Tracer uptake differed between tumor lesions 5-fold within and 9-fold between patients. During AMG 211 treatment, the tracer was present in the blood pool, whereas tumor lesions were not visualized, possibly reflecting target saturation. This first-in-human study showed high, specific ^{89}Zr-AMG 211 accumulation in CD3-rich lymphoid tissues, as well as a clear, heterogeneous tumor uptake both within and between patients.

Tools to select patients for immunotherapy and to predict response early during immunotherapy would be very helpful in the clinic. The PET tracer N-(4-[^{18}F]fluorobenzoyl)-interleukin-2 (^{18}F-FB-IL2) is being explored in clinical studies to visualize T-cell status and might be used to evaluate the immune status of patients. However, the production of [^{18}F]FB-IL2 is cumbersome. Therefore, in **chapter 6**, we describe the results of a comparison study of two newly developed IL2-tracers, namely ^{18}F-AlF-RESCA-IL2 and ^{68}Ga-Ga-NODAGA-IL2, in mice. ^{18}F-AlF-RESCA-IL2 and ^{68}Ga-Ga-NODAGA-IL2 were produced with a radiochemical purity >95% and high radiochemical yield within 60 and 90 minutes, respectively. Both tracers were stable in human serum and bound to activated human peripheral blood mononuclear cells (PBMCs) *in vitro*. *Ex vivo* biodistribution 60 minutes post-injection in BALB/c mice showed higher uptake of ^{18}F-AlF-RESCA-IL2 than ^{18}F-FB-IL2 in liver, kidney, spleen, bone, and bone marrow. ^{68}Ga-Ga-NODAGA-IL2 uptake in liver and kidney was higher than ^{18}F-FB-IL2 uptake. *In vivo*, all tracers revealed uptake in activated human PBMCs in SCID mice, and with a high cold dose, this uptake could be blocked. The fast and straightforward production of ^{18}F-AlF-RESCA-IL2 and ^{68}Ga-Ga-NODAGA-IL2 and their good *in vitro* and *in vivo* characteristics support clinical translation of these tracers.

Macrophages are an important player in the tumor immune microenvironment

and may stimulate or inhibit tumor growth. Tumor-growth stimulating macrophages express colony-stimulating factor 1 receptor (CSF1R), and many drugs are being developed that target CSF1R. To facilitate drug development of CSF1R targeting antibodies, information about their biodistribution is of interest. In **chapter 7** the results are described of an exploratory biodistribution study of an antibody targeting murine CSF1R in a mouse model with spontaneous breast cancer. The 10 µg ^{89}Zr-anti-CSF1R antibody dose was eliminated from the circulation within 24 hours. The highest uptake, 24 hours after injection, was observed in the spleen (126 ± 44 %ID/g) and liver (34 ± 7 %ID/g). Increasing the tracer dose to 250 µg resulted in increased blood pool levels at 72 hours of 10 ± 2 %ID/g, whereas spleen and liver uptake reduced to 17 ± 4 %ID/g and 11 ± 4 %ID/g, respectively. Compared to a ^{89}Zr-isotype control antibody, ^{89}Zr-anti-CSF1R antibody showed specific uptake in the liver, spleen, lymph nodes, duodenum, and ileum, but not in the tumor. In the tumors of the mice that received 250 µg ^{89}Zr-anti-CSF1R antibody, no macrophages were present. This might be due to macrophage depletion by the high tracer protein dose. Autoradiography and immunohistochemistry showed colocalization of ^{89}Zr-anti-CSF1R antibody with macrophages in mesenteric lymph nodes and spleen. These results show that the biodistribution of ^{89}Zr-anti-CSF1R antibody is protein dose-dependent. Low doses of this antibody are quickly eliminated from the circulation due to high uptake in the spleen and liver.

To reduce the reoperation rate after surgery for a positive tumor margin, better tools are needed to delineate the tumor in real-time. An interesting option is using the discriminating factors in the tumor microenvironment to create and enhance the contrast between tumor and healthy tissue. In **chapter 8** we describe the study in which we preclinically evaluate if a quenched fluorescent activity-based probe can potentially be used to visualize residual tumor tissue during surgery, allowing so-called image-guided surgery. This probe, called VGT-309, is only activated when cleaved by cathepsins. Cathepsins are predominantly found in the tumor. Therefore, background signal due to activation of VGT-309 outside of the tumor is minimized. Within 24 hours after intravenous administration to immunocompetent tumor-bearing mice, tumor-specific accumulation of the activated probe was seen. The tumor-to-background contrast increased over time up to 24 hours post probe injection. Also, VGT-309 could be used in combination with different, clinically used, optical fluorescent imaging camera systems to guide surgical resection of murine tumors. These results indicate that optical fluorescent molecular imaging using this cathepsin-targeted probe may improve intraoperative tumor detection.

DISCUSSION AND FUTURE PERSPECTIVES

T-cell engaging bispecific antibody constructs in oncology

Until now one T-cell bispecific antibody construct is registered for the treatment of cancer patients. Blinatumomab is approved for B-cell acute lymphoblastic leukemia (ALL). Solid tumors lack clean tumor-specific antigens, can have low perfusion, and a suppressive tu-

mor immune microenvironment.[1] This affects the development of T-cell bispecific antibody constructs. Current research is focusing on overcoming these hurdles. With the exponentially increasing amount of DNA, RNA, and cell-surface protein expression data available together with improved methods for data analysis, novel and specific tumor-associated antigens may be found for certain solid tumors. In this thesis we showed that molecular imaging can help evaluate whether a BiTE molecule, or any other T-cell engaging bispecific antibody construct, will reach the tumor. Thus, molecular imaging can potentially aid in selecting constructs from the large pool of possible formats for T-cell engaging bispecific antibodies. Also, visualization of their normal tissue accumulation may be used to understand the pharmacokinetics and explain pharmacodynamics. Notably, accumulation in the spleen and mesenteric lymph nodes or the tumor of mice can be modulated by altering the affinities of each targeting arm.[2] Therefore, a better understanding of the influence of the affinity ratio between the targeting arms on the biodistribution might contribute to their optimal design, and improve tumor targeting.

The tumor immune microenvironment can play a role in dampening responses with T-cell engaging bispecific antibody constructs (pre)clinically.[3-5] Preliminary results of clinical trials reported signs of enhanced anti-tumor activity of T-cell engaging bispecific antibody constructs when they were combined with immune checkpoint inhibitors.[6,7] Moreover, the high number of ongoing clinical trials combining immune checkpoint inhibitors and T-cell engaging bispecific constructs (20 trials, chapter 2) demonstrates that there is great interest in this synergy. Results from these trials are eagerly awaited. Besides, the results of these trials may show if these bispecific T-cell engaging constructs targeting solid tumors will have a future role as a single agent and / or in combination with immune checkpoint inhibitors.

Molecular imaging of the tumor immune microenvironment and the anti-cancer immune response

More cancer-immunotherapies are likely to be approved. Currently there are over 5000 active clinical trials evaluating immunotherapies and combinations of immunotherapies.[8,9] Tools that could select patients who might benefit or that predict tumor response early during treatment might help to provide the most optimal treatment for the patient.

To successfully develop such tools, a deep understanding of the underlying immunology is a prerequisite. Preclinical molecular imaging can contribute to enhance our knowledge by visualizing the tumor immune microenvironment in real-time and show how it changes in response to cancer-immunotherapies. This understanding can serve to develop tracers that might predict or evaluate early induced effects of the therapy in patients. For example, gene expression of markers for B-cells and tertiary lymphoid structures are associated with increased survival following immunotherapy of patients with melanoma and sarcoma.[10,11] This finding could be back-translated to the laboratory. Thus, preclinical

molecular imaging can provide a proof-of-concept for imaging cell-surface B-cell markers and whether their expression has predictive value for tumor response to immunotherapy in mouse models. This type of information can support selecting a target and tracer, and facilitate translation to the clinic.

To visualize the tumor immune microenvironment in patients with cancer multiple tracers targeting T-cells, T-cell subsets, or other immune cells are developed. Tracers for visualization of IL-2R, CD8, programmed cell death protein 1 (PD-1), and PD ligand 1 (PD-L1) are examples that are being evaluated in patients.[12-14] Together with tracers for other T-cell markers such as lymphocyte-activation gene 3 (LAG3) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), and possibly B-cell tracers, valuable data will become available. This data might aid in future patient selection, response evaluation and prediction, and understanding cancer-immunity. Consequently, an enhanced understanding of the cancer-immunity cycle may help to develop optimal combination and sequence strategies of immunotherapies.

Integrating molecular imaging with pharmacokinetic modeling

The biodistribution of immunotherapeutic drugs involves specific accumulation in lymphoid tissues. This is different from conventional tumor-targeting monoclonal antibody therapies (this thesis). In classical pharmacokinetic models, the dynamics of the drug amount per organ or compartment is mathematically fitted from blood pharmacokinetic data. Molecular imaging can visualize what is happening in each organ over time and this data may be used to determine physiological parameters involved in the biodistribution. Integrating molecular imaging with pharmacokinetic modeling is therefore of interest. It might accelerate the development of physiology-based pharmacokinetic models for immunotherapies. These models might support the a priori simulation of the biodistribution of immunotherapeutic drugs, based on physiological parameters. These models may be used to interpret future results and continuously test our understanding.

The increasing role of molecular imaging in drug development

Molecular imaging is establishing a role in the fast transition from investigational new drugs to approved treatments. Among others, by showing early in the expensive drug development process the whole body biodistribution of the drug and whether it reaches its target. Moreover, molecular imaging may guide clinical decisions by selecting patients by assessing target expression in all tumor lesions.

The role of molecular imaging in drug development is likely to increase due to technical advances, expanding practical knowledge, and the growing need for patient stratification as outlined in the previous section. Novel whole-body PET-imaging systems visualize the location of positron emitters faster while requiring a substantially lower radiation dose as a result of more efficient signal capturing.[15] In addition, requiring less radioactivity

will expand the distribution radius of tracers produced in specialized centers, increasing their availability.

The need for molecular imaging combined with greater applicability of tracers can bolster their future development. This availability should allow for more clinical studies to prove their relevance to the clinic.

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