Radiopharmaceuticals for translational imaging studies in the field of cancer immunotherapy

E.L. van der Veen, PhD
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University of Groningen

Promotores:
Prof. E.G.E. de Vries, MD, PhD
M.N. Lub-de Hooge, PhD

Immune checkpoint inhibitors allow a relevant new treatment strategy for patients with cancer (1). Several immune checkpoint inhibitors, targeting cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein (PD-1)/programmed death-ligand 1 (PD-L1) have been approved for treatment of patients with various tumors. Many other immunotherapeutics are in development. Moreover, a large number of clinical immunotherapy studies are evaluating immunotherapeutic agents or combinational strategies with other immunotherapeutics, chemotherapeutic drugs, targeted agents or radiotherapy (2).

Although these treatments have shown to clearly affect outcome in various settings, major challenges are that not every patient responds to immune checkpoint inhibitor therapy and that this treatment can lead to serious side effects. Therefore, strategies are required to improve patient selection and predict response to these immunotherapeutics. Currently, clinically approved biomarkers are based on PD-L1 measurement by immunohistochemistry (IHC) or microsatellite instability-high and mismatch repair deficient status measurement by IHC and polymerase-chain-reaction-based assays. However, these biomarkers are based on a single biopsy and do not capture the highly dynamic, complex and heterogeneous immune response.

PET and SPECT with specific radiopharmaceuticals can provide non-invasive whole-body information about the biodistribution of immune checkpoint inhibitors in the body, heterogeneity of target expression, effects of immunotherapy on immune cells, and therapy effects on other cells in the tumor microenvironment. Molecular imaging with radiolabeled molecules targeting players in the tumor immune response, might be a tool for patient selection and response prediction in immunotherapy treatment. Aim of this thesis was to develop new radiopharmaceuticals for molecular imaging in the field of cancer immunotherapy.
In chapter 2 of this thesis we summarized research to identify the potential of non-invasive whole-body molecular imaging for cancer immunotherapy to produce quantitative outputs as such imaging biomarkers might help to improve patient selection for treatment (3). We searched for both PET and SPECT imaging and divided three main groups for imaging strategies. Firstly, imaging with radiolabeled immune checkpoint targeting molecules. Secondly, imaging of immune cells with *ex vivo* or *in vivo* radiolabeled tracers and thirdly, imaging extracellular matrix components, including adhesion molecules, growth factors and cytokines. These molecular imaging strategies – used alone, in combination or serially – could potentially contribute to patient selection upfront or early during immunotherapy.

As first imaging strategy we developed different radiopharmaceuticals targeting immune checkpoints, such as radiolabeled anti-PD-L1 or anti-PD-1 antibodies.

The disadvantage of molecular imaging with full monoclonal antibodies is that, given their half-life, to obtain adequate contrast, PET imaging is performed 4-7 days after tracer injection. Molecular imaging with radiolabeled smaller molecules, with faster pharmacokinetics and shorter half-lives, allows earlier imaging. In chapter 4 we evaluated the usability of the PD-L1 targeting adnectin-based PET tracer ^{18}F-BMS-986192 to determine different PD-L1 expression levels, as well as therapy-induced changes in tumor PD-L1 expression (4). Therefore, *in vitro* binding assays for ^{18}F-BMS-986192 were performed in human tumor cell

lines with different PD-L1 total protein and membrane protein expression levels. Subsequently, PET imaging was executed in immunodeficient mice xenografted with these cell lines. We found that ^{18}F-BMS-986192 binding reflected PD-L1 membrane levels in tumor cell lines, and tumor tracer uptake in mice was associated with PD-L1 expression measured immunohistochemically. *In vitro* interferon gamma (IFNγ) treatment increased PD-L1 expression in the tumor cell lines and caused increased tracer binding. *In vivo* IFNγ did neither affect PD-L1 tumor expression measured immunohistochemically nor ^{18}F-BMS-986192 tumor uptake. *In vitro* treatment with the MEK1/2 inhibitor selumetinib downregulated cellular and membrane levels of PD-L1 of tumor cells by 50% as measured by Western blotting and flow cytometry. In mice, selumetinib lowered cellular, but not membrane PD-L1 levels of tumors and consequently no treatment-induced change in ^{18}F-BMS-986192 tumor uptake was observed. In conclusion, ^{18}F-BMS-986192 PET imaging allows detection of membrane-expressed PD-L1, as soon as 60 minutes after tracer injection. Moreover, the tracer can discriminate a range of tumor cell PD-L1 membrane levels.

Other molecular imaging strategies can be used to target immune cells specifically. Targeting interleukin-2 (IL2) receptors on T-cells using PET with N-(4-^{18}F-fluorobenzoyl)-interleukin-2 (^{18}F-FB-IL2) could be such a strategy. In chapter 5 we describe the challenging translation of the partly manual labeling of ^{18}F-FB-IL2 for preclinical studies into an automated procedure following GMP, resulting in a radiopharmaceutical suitable for clinical use (5). To overcome several challenges, major adaptations in the production process were executed. The final analytical and production methods were validated and documented. All data with regard to the quality and safety of the final drug product were documented in the IMPD. Restrictions in the ^{18}F-FB-IL2 production were imposed by hardware configuration of the automated synthesis equipment and by use of disposable cassettes. Critical steps in the ^{18}F-FB-IL2 production comprised the purification method, stability of recombinant human IL2 and the final formulation. With the GMP compliant production method, ^{18}F-FB-IL2 could reliably be produced with consistent quality, complying to all specifications. This enabled the first use of ^{18}F-FB-IL2 in clinical studies.

Although successfully applied in both preclinical and clinical studies, the production of this ^{18}F-FB-IL2 is complex and time-consuming. Therefore, new strategies for IL2 radiolabeling have been investigated. In chapter 6 two radiolabeled IL2 variants, namely aluminum mono-^{18}F-fluoride-(restrained complexing agent)-IL-2 (^{18}F-AlF-RESCA-IL2) and ^{68}Ga-gallium-(1,4,7-triazacyclononane-4,7-diacetic acid-1-glutaric acid)-IL-2 (^{68}Ga-Ga-NODAGA-IL2) were studied (6). Their in vitro and in vivo characteristics were evaluated and compared to ^{18}F-FB-IL2. ^{68}Ga-Ga-NODAGA-IL2 and ^{18}F-AlF-RESCA-IL2 were produced with a radiochemical purity >95% and a radiochemical yield of 13.1±4.7% and 2.4±1.6% within 60 and 90 minutes, respectively. Both tracers were stable in serum with >90% remaining intact after 1 h. *In vitro*, both tracers bound to activated hPBMCs. *Ex vivo* biodistribution studies 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. Dynamic PET imaging revealed uptake in activated hPBMCs in severe-combined immune deficient (SCID) mice. Low uptake was seen after a blocking dose of IL2 or in the control group. In addition, ^{18}F-AlF-RESCA-IL2 yielded highest contrast PET images of target lymph nodes. In conclusion, we found that the production of ^{18}F-AlF-RESCA-IL2 and ^{68}Ga-Ga-NODAGA-IL2 was simpler and faster than ^{18}F-FB-IL2. ^{18}F-AlF-RESCA-IL2 shows good *in vitro* and *in vivo* characteristics, indicating its potential as a PET tracer for imaging of T-cells.

In chapter 7 we describe the results of a first-in-human study to assess the feasibility of imaging with anti-PD-L1 ^{89}Zr-atezolizumab, including biodistribution, and test its potential to predict response to PD-L1 blockade (7). For this purpose, we developed and produced ^{89}Zr-atezolizumab according to GMP guidelines. We imaged 22 patients across three tumor types before start of atezolizumab therapy. The PET tracer uptake was high in lymphoid tissues and at sites of inflammation. In tumors, uptake was generally high but heterogeneous, varying within and among lesions, patients, and tumor types. Intriguingly, clinical responses in our patients correlated better with pretreatment PET tumor uptake than with IHC- or RNA-sequencing-based predictive biomarkers. Autoradiography of two tumor samples showed heterogeneous tracer distribution, and PD-L1 as well as CD8 IHC showed heterogeneous staining, partly corresponding with regions of high tracer uptake. This study is encouraging further development of molecular PET imaging for assessment of PD-L1 status and clinical response prediction.

In conclusion, in this thesis we describe the development of new radiopharmaceuticals for molecular imaging in the field of cancer immunotherapy. We show the challenging development and preclinical evaluation of radiolabeled immune checkpoint protein

antibodies and small molecules. Furthermore, we describe the potential for immune cell imaging in preclinical studies. Additionally, we showed the feasibility of molecular imaging with a PD-L1 antibody in a small size clinical imaging trial. In the future, this approach might lead to better patient selection to improve therapy outcomes for cancer immunotherapy.

e.l.van.der.veen@umcg.nl ♦

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