Abstract

Purpose: Macrophage mannose receptor (MMR, CD206) expressing tumor-associated macrophages (TAM) are protumorigenic and were reported to negatively impact therapy responsiveness and are associated with higher chances of tumor relapse following multiple treatment regimens in preclinical tumor models. Since the distribution of immune cells within the tumor is often heterogeneous, sampling errors using tissue biopsies will occur. In order to overcome this limitation, we propose positron emission tomography (PET)/X-ray computed tomography (CT) imaging using $[^{68}\text{Ga}]$-labeled anti-MMR single-domain antibody fragment (sdAb) to assess the presence of these protumorigenic TAM.

Procedures: Cross-reactive anti-MMR-sdAb was produced according to good manufacturing practice (GMP) and conjugated to $\rho$-SCN-Bn-NOTA bifunctional chelator for $^{68}\text{Ga}$-labeling. Biodistribution and PET/CT studies were performed in wild-type and MMR-deficient 3LL-R tumor-bearing mice. Biodistribution data obtained in mice were extrapolated to calculate radiation dose estimates for the human adult using OLINDA software. A 7-day repeated dose toxicity study for NOTA-anti-MMR-sdAb was performed in healthy mice up to a dose of 1.68 mg/kg.

Results: $[^{68}\text{Ga}]$Ga-NOTA-anti-MMR-sdAb was obtained with 76 ± 2 % radiochemical yield, 99 ± 1 % radiochemical purity, and apparent molar activity of 57 ± 11 GBq/μmol. In vivo biodistribution analysis showed fast clearance via the kidneys and retention in MMR-expressing organs and tumor, with tumor-to-blood and tumor-to-muscle ratios of 6.80 ± 0.62 and 5.47 ± 1.82, respectively. The calculated effective dose was 0.027 mSv/MBq and 0.034 mSv/MBq for male and female, respectively, which means that a proposed dose of 185 MBq in humans would yield a radiation dose of 5.0 and 6.3 mSv to male and female patients, respectively. In the toxicity study, no adverse effects were observed.

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Conclusions: Preclinical validation of \([^{68}\text{Ga}]\text{Ga-NOTA-anti-MMR-sdAb}\) showed high specific uptake of this tracer in MMR-expressing TAM and organs, with no observed toxicity. \([^{68}\text{Ga}]\text{Ga-NOTA-anti-MMR-sdAb}\) is ready for a phase I clinical trial.

Key words: Single-domain antibody (sdAb), Macrophage mannose receptor (MMR), Tumor-associated macrophages (TAM), PET

Introduction

Increasing data is becoming available reporting the intense crosstalk between cancer cells and the tumor microenvironment. Tumor-associated macrophages (TAM) in the microenvironment play a key role in tumor growth and progression. Besides local immunosuppression, they influence angiogenesis and cancer cell mobility. Importantly, the TAM population consists of pro- and anti-tumoral populations residing in different tumor regions. In this respect, TAM with a high expression of MMR (MMR\textsuperscript{hi} TAM) were shown to reside in hypoxic tumor areas and were highly angiogenic and strongly immunosuppressive, characteristics that suggest a strong protumoral activity [1–3].

MMR\textsuperscript{hi} TAM were reported to negatively impact therapy responsiveness and allow tumor relapse following irradiation, anti-angiogenic, or vascular disrupting therapies and chemotherapy in preclinical tumor models [4]. A clear association between the presence of MMR\textsuperscript{hi} TAM and the (lack of) responsiveness to treatment has not been demonstrated so far in patients, likely because it is difficult to fully capture the heterogeneity of TAM presence using core needle biopsy and immunohistochemistry (IHC).

To determine MMR\textsuperscript{hi} TAM density, we propose the use of \(^{68}\text{Ga}\)-labeled anti-MMR single-domain antibodies fragments (sdAbs) for the non-invasive imaging of MMR\textsuperscript{hi} TAM in all cancer lesions within a patient. sdAbs, such as anti-MMR-sdAbs, are antigen-binding fragments derived from heavy-chain only antibodies of the Camelidae. Compared to conventional antibodies or antibody fragments, sdAbs are small (12–15 kDa) and they have the ability to bind antigens on hidden or unusual epitopes [5]. sdAbs have proven their role in molecular imaging and targeted radionuclide therapy in a preclinical setting [6–9], and a \(^{68}\text{Ga}\)-labeled compound for positron emission tomography (PET)/X-ray computed tomography (CT) imaging of HER2 was found safe and it is used straightforward in a phase I clinical trial, with low radiation burden for the patient [10].

Previously, we have reported the generation of cross-reactive human-mouse-anti-MMR-sdAb, described in [11], was cloned in the pAOXZalpha plasmid in frame with the \(\alpha\)-factor secretory signal peptide. pAOXZalpha vector is a derivative of the pPICZalpha plasmid (Thermo Fisher), with minor changes in the multiple cloning site. \(\textit{Pichia pastoris}\) strain GS115 + His4 was transduced and a clone with stable genomic integration and high sdAb secretion in the medium was selected. The untagged sdAb was purified from the fermenter supernatant by 0.2-\(\mu\)m filtration, mixed-mode chromatography (MMC) capturing and polishing using anion-exchange chromatography (AEX) on NatriFlo a HD-Q Recon column (Natrix). The sdAb was buffer-exchanged to phosphate buffer saline (PBS) by tangential flow filtration and concentrated to 1.9 mg/ml. The GMP-grade sdAb was generated by Q-Biologicals (Ghent).

Materials and Methods

All commercially obtained chemicals were of analytic grade. \(p\)-SCN-Bn-NOTA was purchased from Macrocyclics. \(^{68}\text{Ga}\) was obtained from a \(^{68}\text{Ge}/^{68}\text{Ga}\) Galli Eo™ generator (IRE, Belgium). Buffers used for coupling reactions or for radiolabeling were purified from metal contamination using Chelex 100 resin (Aldrich). High purity water (Fluka) was used for radiolabeling.

Production and Purification of GMP-Grade anti-MMR-sdAb

The DNA sequence coding for the cross-reactive human-mouse-anti-MMR-sdAb, described in [11], was cloned in the pAOXZalpha plasmid in frame with the \(\alpha\)-factor secretory signal peptide. pAOXZalpha vector is a derivative of the pPICZalpha plasmid (Thermo Fisher), with minor changes in the multiple cloning site. \(\textit{Pichia pastoris}\) strain GS115 + His4 was transduced and a clone with stable genomic integration and high sdAb secretion in the medium was selected. The untagged sdAb was purified from the fermenter supernatant by 0.2-\(\mu\)m filtration, mixed-mode chromatography (MMC) capturing and polishing using anion-exchange chromatography (AEX) on NatriFlo a HD-Q Recon column (Natrix). The sdAb was buffer-exchanged to phosphate buffer saline (PBS) by tangential flow filtration and concentrated to 1.9 mg/ml. The GMP-grade sdAb was generated by Q-Biologicals (Ghent).

Chromatographic Analysis

Superdex Peptide 10/300GL (GE), using 0.1 M ammonium acetate pH 7 as eluate and flow 0.5 ml/min, was used for NOTA-anti-MMR-sdAb purification and quality control. Superdex Peptide 3.2/300 column (GE), using 0.02 M PBS/0.28 M NaCl pH 7.4 as mobile phase was used for the quality control of \(^{69,71}\text{GaNOTA-anti-MMR-sdAb}\) and \(^{68}\text{Ga}\)Ga-NOTA-anti-MMR-sdAb. The same column was also used when performing stability studies of \(^{68}\text{Ga}\)Ga-NOTA-anti-MMR-sdAb. Instant thin-layer chromatography (iTLC) was performed on silica gel (SG) (Pall Corp. Life Sciences) using 0.1 M sodium citrate pH 5.0 as eluent.
**Conjugation of p-SCN-Bn-NOTA to anti-MMR-sdAb**

Anti-MMR-sdAb (3 mg, 0.24 μmol) was buffer-exchanged to 0.05 M sodium carbonate buffer, pH 8.7, using PD-10 size exclusion disposable columns (GE). Protein solution (2 ml) was added to a 20-fold molar excess p-SCN-Bn-NOTA (2.6 mg, 47 μmol), pH adjusted to 8.5–8.7 with 0.2 M Na₂CO₃. After 2-h incubation at room temperature (RT), the pH of the reaction mixture is lowered to pH 7.4 by adding HCl 1 N. The NOTA-anti-MMR-sdAb protein solution is loaded on a size exclusion column. The collected fractions containing monomeric NOTA-anti-MMR-sdAb protein are pooled and the solution is passed through a 0.22-μm filter. The protein concentration is determined by UV absorption at 280 nm (ε = 40,660 M⁻¹ cm⁻¹). Three validation tests were performed in which the product was fully characterized as required for clinical application. Product was stored at −20 °C and stability tests were performed at selected time points (T0M, T3M, T6M at −20 °C and T3d, T7d at 4 °C).

**Synthesis of ⁶⁹,⁷¹GaNOTA-anti-MMR-sdAb**

To a solution of NOTA-anti-MMR-sdAb (1.2 mg, 91.4 nmol) in 1 ml of 0.1 M ammonium acetate (pH 7), 250 μl of 1 M sodium acetate buffer pH 5 and 35 μl of a 60 mM solution of Ga(NO₃)₃·10H₂O (1.83 μmol) were added, and incubated for 2 h at RT. Size exclusion purification using PD-10 column pre-equilibrated with 0.1 M ammonium acetate (pH 7) was used to remove free gallium acetate. ⁶⁹,⁷¹GaNOTA-anti-MMR-sdAb was characterized by ESI-Q-ToF-MS, SDS-PAGE/Western Blot, surface plasmon resonance (SPR), and SEC. The compound was used as the reference for the identification of the radio-SEC chromatogram signals of [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb (retention time [tᵣ] = 8.4 min).

**SPR**

SPR measurements were performed on a Biacore T200 instrument (GE) as described previously [11].

**Preparation of [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb**

⁶⁸Ga eluate (500–877 MBq) was added to NOTA-anti-MMR-sdAb (100–130 μg, 7.7–11 nmol) in 1 M sodium acetate buffer pH 5 (1 ml). The homogenized solution is incubated for 10 ± 1 min at RT to form [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb. The solution is loaded on a PD-10 column (GE), which is pre-equilibrated with 0.9 % NaCl 5 mg/ml ascorbic acid at pH 6. The column is eluted with 0.9 % NaCl 5 mg/ml ascorbic acid at pH 6 by gravity flow and the eluate is passed through a 0.22-μm filter (Millex GV, Millipore). Radiochemical purity was evaluated using iTLC and by radio-SEC (SEC: [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb tᵣ = 10.2 min; [⁶⁸Ga]Gacitrate tᵣ = 15.9 min; iTLC-SG: [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb Rᵣ = 0, [⁶⁸Ga]Gacitrate Rᵣ = 1).

**In Vitro Stability of [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb**

Stability of [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb was tested in 0.9 % NaCl 5 mg/ml ascorbic acid at pH 6, with a 1000-fold molar excess diethylenetriaminepentaacetic acid (DTPA) and in human plasma. [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb (349 ± 55 MBq, n = 4) in 0.9 % NaCl 5 mg/ml ascorbic acid pH 6 at RT was followed by iTLC up to 4 h. [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb (22.7 MBq) was added to 500 μl of human plasma incubated at 37 °C up to 1 h and analyzed by SEC. To a solution of [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb (22 ± 4 MBq, n = 2) was added a 1000-fold molar excess of DTPA and incubated at RT for 4 h and followed by iTLC.

**Animal Models**

Wild-type (WT) female C57BL/6 mice (Janvier) were used for blood curves (9 weeks old) and dosimetry studies (7 weeks old). To evaluate biodistribution and targeting specificity, female C57BL/6 WT and MMR-deficient (MMR-KO) (Janvier) (7 weeks old) mice were subcutaneously inoculated in the right flank with 3 × 10⁶ of the 3LL-R clone of Lewis lung carcinoma cells suspended in HBSS medium while anesthetized with 2.5 % isoflurane (AB-BOTT). Tumors were allowed to grow for 12 days (tumor weight of 0.662 ± 0.267 g for WT and 0.730 ± 0.268 g for MMR-deficient).

The animals were housed at 22 °C in 50–60 % humidity with a light/dark cycle of 12 h. They were kept under pathogen-free conditions and were given autoclaved food pellets and water ad libitum. For animal handling and processing of data, technicians and researchers were not blinded.

All procedures followed the guidelines of the Belgian Council for Laboratory Animal Science and were approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (license 13-272-5).

**Biodistribution Studies in Tumor-Bearing Mice**

3LL-R tumor-bearing mice (C57BL/6 WT (n = 5) and MMR-KO (n = 5)), anesthetized with 2.5 % isoflurane, were injected intravenously with [⁶⁸Ga]Ga-NOTA-anti-MMR-sdAb (11.6 ± 0.6 MBq, 5 μg of NOTA-anti-MMR-sdAb) via the tail. Mice were euthanized at 3 h after injection, and major organs were collected, weighed, and counted against a standard of known activity in a gamma counter. Tissue/
organ uptake was calculated and expressed as a percentage injected activity (%IA) per gram, corrected for decay.

**Blood Curves**

WT C57BL/6 mice (n = 6) anesthetized with 2.5% isoflurane were injected intravenously with [68Ga]Ga-NOTA-anti-MMR-sdAb (4.7 ± 0.2 MBq, 5 µg of NOTA-anti-MMR-sdAb) via the tail. Blood samples were collected 2, 5, 10, 20, 30, 40, 60, 120, and 180 min after injection, via a microcapillary, and analyzed in the gamma counter to obtain a blood time–activity curve. The half-life was calculated by biexponential nonlinear regression fit (GraphPad Prism; GraphPad Software).

**Dosimetry Studies**

WT C57BL/6 mice (n = 6 per time point) anesthetized with 2.5% isoflurane were injected intravenously with [68Ga]Ga-NOTA-anti-MMR-sdAb (5.1 ± 0.5 MBq, 5 µg of NOTA-anti-MMR-sdAb) via the tail. Animals were euthanized at 10, 60, 120, and 180 min post injection and major organs were collected, weighed, and counted against a standard of known activity in a gamma counter. Tissue/organ uptake was calculated and expressed as %IA and %IA/g, corrected for decay. Radiation dose estimates for the adult female and male were calculated from the biodistribution data of mice using OLINDA 1.0 software. Organ doses, effective dose, and effective dose equivalent were calculated using the appropriate weighing factors for the various organs. Full methods are described in supplemental data.

**Micro-PET/CT Imaging**

Mice were injected with [68Ga]Ga-NOTA-anti-MMR-sdAb (11.62 ± 0.58 MBq, 5 µg NOTA-anti-MMR-sdAb) of the tracer via the tail vein, and scanned after 60 and 150 min. Micro-PET/CT imaging was performed on a MILabs VECTor/CT. The CT scan was set to 55 keV and 615 μA, resolution of 80 μm, with a total body scan duration of 108 s. PET images were obtained using the high-energy PET collimator in spiral mode, 94 positions for whole-body imaging, with 9 s per position. Images were reconstructed with 0.6-mm voxels with 4 subsets and 2 iterations, without post reconstruction filter. Image viewing was performed with AMIDE imaging software.

**Toxicity Study with NOTA-anti-MMR-sdAb**

Full materials and methods of the toxicity study are described in supplemental data. A 7-day intravenous repeated dose toxicity study of the NOTA-anti-MMR-sdAb was performed (Eurofins BioPharma Product testing). The ICH M3 (R2) guideline was applied (EMA/CPMP/ICH/286/95).

NOTA-anti-MMR-sdAb or vehicle solution (PBS) was administered at 1.68-mg/kg body weight daily via intravenous route to 20 male and 20 female Balb/c mice for 7 days. Toxicity was assessed using observation, blood sampling (hematology and clinical biochemistry), necropsy, and urine analysis. Histopathological examination was performed for 40 selected organs. In addition, 10 recovery animals per group and gender were observed for 14 days following the last administration and were assessed thereafter.

Also, blood cytokine levels were determined in 5 satellite animals per group and per gender at 2 h and 6 h after single administration. Cytokine determination was performed using V-PLEX Plus Proinflammatory Panel 1 (mouse) kit.

**Statistical Analysis**

Quantitative data are expressed as mean ± SD and compared using the independent t test using Prism 5 (GraphPad Software, Inc.)

**Results**

**Synthesis and Characterization of NOTA-anti-MMR-sdAb and 69,71GaNOTA-anti-MMR-sdAb**

The GMP-produced anti-MMR-sdAb was conjugated to p-SCN-Bn-NOTA on the ε-amino groups of the lysines forming a thiourea bond. The conjugation reaction resulted in a mixture of molecules with 0 and 1 NOTA chelators, as determined by ESI-Q-ToF-MS analysis. NOTA-anti-MMR-sdAb was characterized and stability studies performed (Suppl. Table 1). The product is stable at 4 °C for at least 7 days, and at −20 °C for at least 6 months. For clinical translation, 3 validation tests of NOTA-anti-MMR-sdAb were performed to confirm the reproducibility of the results (Suppl. Table 2).

69,71GaNOTA-anti-MMR-sdAb was prepared and fully characterized as detailed in Suppl. Table 3. It was subsequently used as a reference compound for [68Ga]Ga-NOTA-anti-MMR-sdAb in SEC and assesses its MMR-binding kinetic parameters.

**MMR Protein Binding Characteristics of NOTA-anti-MMR-sdAb and 69,71GaNOTA-anti-MMR-sdAb**

SPR experiments were performed on immobilized recombinant human MMR protein to confirm the affinity of chemically modified anti-MMR-sdAb and to determine binding kinetic parameters (Table 1). All compounds bound to the target protein with affinities in the low-nanomolar range, with no pronounced effect from conjugation of the NOTA chelator or complexation with 69,71Ga.
Preparation of \([{^{68}}\text{Ga}]\text{Ga-NOTA-anti-MMR-sdAb}\)

NOTA-anti-MMR-sdAb was radiolabeled with \([^{68}\text{Ga}]\) at RT and pH 5 for 10 min. \([{^{68}}\text{Ga}]\text{Ga-NOTA-anti-MMR-sdAb}\) was obtained with a radiochemical yield of 76 ± 2 % (\(n = 6\), isolated product after purification, decay corrected), a radiochemical purity of 99 ± 1 % (after purification, iTLC), and an apparent molar activity of 57 ± 11 GBq/μmol (end of purification). The chemical identity of the \([^{68}\text{Ga}]\) complex (\(t_R = 10.2\) min) was confirmed by comparison of the SEC profile with that of the \([^{69,71}\text{Ga}]\) NOTA-anti-MMR-sdAb (\(t_R = 8.2\) min). The radiolabeled compound was stable in 0.9 % NaCl 5 mg/ml ascorbic acid at pH 6 and in the presence of 1000-fold molar excess DTPA at RT over 4 h (Table 2).

Incubation of the radiolabeled compound in human plasma showed high metabolic stability, as well as high stability with regard to trans-chelation. After 1 h at 37 °C, gel filtration analysis showed that more than 95 % of the activity corresponded to intact compound (Table 2).

Dosimetry Analysis

An estimation of organ-absorbed doses was performed by extrapolation of the biodistribution data of \([^{68}\text{Ga}]\) NOTA-anti-MMR-sdAb at different time points in mice (Suppl. Table 5 in ESM) to the human male and female adult phantom using OLINDA software (Table 3). The organ-absorbed doses were calculated using a voiding bladder model with 100 % renal clearance, a voiding bladder interval of 1 h and the biological half-life of 2.49 h. Table 3 summarizes the organ-absorbed doses. The effective dose was estimated at 0.0270 mSv/MBq for male and 0.0342 mSv/MBq for female, which means that a proposed patient dose of 185 MBq would yield an estimated radiation dose of 5.0 mSv and 6.3 mSv to male and female patients, respectively.

Toxicity Study of NOTA-anti-MMR-sdAb

Overall, no treatment-related toxicologically relevant changes were observed in clinical signs, growth, hematology, clinical chemistry, organ weights, gross macroscopy,

### Table 1. Surface plasmon resonance results for the different anti-MMR-sdAb compounds binding to immobilized human recombinant MMR protein

| sdAb                        | \(k_a (M^{-1} s^{-1})\) | \(k_d (s^{-1})\) | \(K_D (\text{nM})\) |
|-----------------------------|-------------------------|-------------------|---------------------|
| anti-MMR-sdAb-His6          | 3.80 × 10^5             | 2.74 × 10^{-4}    | 0.72                |
| anti-MMR-sdAb (GMP-produced)| 3.33 × 10^5             | 2.87 × 10^{-4}    | 0.86                |
| NOTA-anti-MMR-sdAb          | 2.89 × 10^5             | 2.90 × 10^{-4}    | 1.00                |
| \(69,71\text{Ga-NOTA-anti-MMR-sdAb}\) | 3.88 × 10^5             | 5.18 × 10^{-4}    | 1.33                |

\(k_a\), association rate constant; \(k_d\), dissociation rate constant; \(K_D\), equilibrium dissociation constant

### Table 2. In vitro stability of \([^{68}\text{Ga}]\) NOTA-anti-MMR-sdAb

| Incubation condition       | Radioactivity in \([^{68}\text{Ga}]\) NOTA-anti-MMR-sdAb (%)* | Method of analysis |
|----------------------------|---------------------------------------------------------------|-------------------|
| Medium                     | Time (h) and temperature                                     |                   |
| 0.9 % NaCl 5 mg/ml ascorbic acid pH 6 | 1 h, RT                                                      | iTLC              |
|                            | 2 h, RT                                                      | iTLC              |
|                            | 3 h, RT                                                      | iTLC              |
|                            | 4 h, RT                                                      | iTLC              |
| Human plasma               | 1 h, 37 °C                                                   | >95               |
|                            | 2 h, RT                                                      | SEC               |
| 1000-fold molar excess DTPA | 1 h, RT                                                      | 97 ± 1            |
|                            | 4 h, RT                                                      | iTLC              |

*% of the total applied radioactivity associated with the \([^{68}\text{Ga}]\) NOTA-anti-MMR-sdAb peak or spot after incubation in the applied condition RT, room temperature; iTLC, instant thin-layer chromatography; SEC, size exclusion chromatography; DTPA, diethylenetriaminepentaacetic acid
and microscopic observations. Based on the absence of treatment-related toxicologically relevant changes in the endpoints examined, the no observed effect level (NOEL) could be established to be >1.68 mg/kg body weight. Moreover, at the dose tested, NOTA-anti-MMR-sdAb was well tolerated at the injected site and not associated with any acute immunological reaction.

**Discussion**

In oncology research, increasing attention is going to the tumor microenvironment, with a high interest in the local immune interactions, to better understand primary and acquired resistance to different types of immunotherapy. It is hypothesized that the *in vivo* imaging of key molecular markers such as programmed death-ligand 1 (PD-L1) and
tumor-infiltrating lymphocytes can help to understand such resistance mechanisms [12]. Also TAM are believed to play a role in primary resistance to immunotherapy. Macrophages found in the tumor microenvironment display a broad spectrum of molecular markers, and some of these markers are associated with a protumoral and immune-suppressive behavior. As such, macrophage mannose receptor (MMR) has been identified as a distinct marker, present on M2-polarized TAM that promote tumor growth and metastasis [1–3]. The presence of such MMR-expressing TAM could therefore be indicative for the success rate of immune-activating therapies. To further investigate this role in cancer patients, we have developed a PET/CT imaging method using an MMR-targeting sdAb and 68Ga-labeling enabling PET/CT quantification.

The MMR-specific sdAb that recognizes both the mouse and the human MMR target was GMP-produced without any c-terminal tag. The bifunctional chelator p-SCN-Bn-NOTA was conjugated to the GMP-grade compound and radiolabeled with 68Ga. The compound proved to be stable both in the final buffer and in human plasma, and the compound was resistant to trans-chelation, confirming high stability of the [68Ga]Ga-NOTA complex. The affinity of the different compounds was around 1 nM and not affected either by conjugation of NOTA or complexation with 68Ga. These in vitro characterization tests confirm its suitability for further translation to patient use.

In mice, [68Ga]Ga-NOTA-anti-MMR-sdAb accumulate specifically in MMR-expressing organs such as the liver and spleen, as well as in the 3LL-R tumors that are known to be infiltrated with high amounts of MMR” TAM [1]. Specificity was confirmed by the absence of signal in MMR-deficient mice (MMR-KO), both in healthy organs and in 3LL-R tumors. The tracer was rapidly cleared from the blood via the kidneys and urine, as observed by both PET/CT imaging and ex vivo biodistribution analysis. The confirmation of specific targeting and rapid clearance of unbound compound provides the necessary evidence of its potential added value for subsequent use in patients.

When comparing uptake values of the [68Ga]Ga-NOTA-anti-MMR-sdAb with the previously reported [99mTc]Tc(CO)3-anti-MMR-sdAb and [18F]FB-anti-MMR-sdAbs, the tumor uptake for all three compounds is very similar in WT 3LL-R tumor-bearing mice [11]. Uptake in extra-tumoral MMR-expressing organs, such as the liver, spleen, lymph nodes, and bone, is slightly higher for the 68Ga compound when compared to the 18F version, but substantially lower than what was observed for the 99mTc compound. These differences may be due to different radiochemistry methods or differences in apparent molar activity (70.1, 31.5, 10.1 GBq/μmol for 99mTc, 68Ga, 18F, respectively (injection time)) [11]. Kidney uptake was also different for the three variants, with the lowest value obtained for the [18F]FB-anti-MMR-sdAb (7.98 ± 0.86 %IA/g at 3 h p.i.), which is about 20-fold lower than that seen with [99mTc]Tc(CO)3-anti-MMR-sdAb and 10-fold lower than with the here presented [68Ga]Ga-NOTA-anti-MMR-sdAb [11]. Similar lower kidney retention for 18F-labeled sdAb has also been reported for anti-HER2, although
only for the $[^{18}F]$FSB, and not using $[^{18}F]RL$-I prosthetic group, confirming that the radiochemistry is a defining factor [13–15]. Depending on the chemical structure created, different catabolites will be produced in the kidney cells, with some $^{18}$F-catabolites clearing faster from kidneys.

Although the $[^{18}F]FB$-anti-MMR-sdAb compound holds promise for clinical translation and might even perform better given its lower kidney retention, our research group has chosen to initiate clinical trials with the $[^{68}Ga]Ga$-NOTA-anti-MMR-sdAb because of the ease of production and radiolabeling that could be implemented in many radiopharmacies worldwide, even in the absence of cyclotrons or synthesis modules.

For subsequent human use of $[^{68}Ga]$Ga-NOTA-anti-MM-sdAb, the average radiation dose for a human adult was estimated based on extrapolation of normal distribution and retention in mice. The average effective dose was 0.034 and 0.027 mSv/MBq for female and male respectively, resulting in a total body radiation dose per injection of 185 MBq of $^{68}$Ga compound of 6.3 and 5.0 mSv for female and male, respectively. The organs that would receive the highest dose are the kidneys (up to 90 mGy) and the urinary bladder wall (up to 42 mGy), staying well below the kidney threshold of 7–8 Gy for potential deterministic effects [16].

The total body irradiation dose is in the same range as a standard 2-deoxy-2-$[^{18}F]$Fluoro-D-glucose PET scan (7 mSv for 370 MBq of administered activity), and could be used in daily clinical practice for diagnosis and follow-up of patients. In the toxicity study, NOTA-anti-MM-sdAb showed no adverse effects after the 7-day repeated injected up to a dose of 1.68 mg/kg. This dose is a 1000-fold excess of what a 60-kg patient would receive when injected with 0.1 mg of the compound. Based on all preclinical data reported here, $[^{68}Ga]$Ga-NOTA-anti-MM-sdAb can be regarded as safe for clinical translation. A phase I trial to assess safety, biodistribution and dosimetry in cancer patients will be initiated in the near future.

Other research groups have also investigated macrophage imaging using mannoside receptor targeting. Many have focused on mannosylation of the targeting moiety, thereby utilizing the natural ligand of MMR [17–19]. However, mannoside does not only bind MMR, but also multiple other mannoside-binding proteins, such as mannoside-binding lectins. Such lectins are serum proteins that will bind to mannoside and other types of sugars that occur on the cell surface of bacteria and yeasts, thereby facilitating opsonization by phagocytes. It can be foreseen that using mannosylated compounds for imaging will therefore not only identify MMR-expressing macrophages, but will also target other phagocytes that will engulf lectin-bound compounds.

Fluorescence imaging using more specific MMR-targeting agents has been reported using a full monoclonal anti-MMR antibody and using an MMR-binding peptide [20–22]. Recently, a $^{125}$I-labeled monoclonal antibody was used for SPECT imaging [22]. These studies confirm the promise of specifically targeting MMR for the imaging of protumorigenic macrophages, but the use of sdAb offers multiple advantages compared to approaches using mAbs given their faster blood clearance and good tissue penetration characteristics.

Conclusions

In conclusion, the $[^{68}Ga]$Ga-NOTA-anti-MM-sdAb is a very interesting PET tracer with a high potential to specifically image the protumorigenic macrophages in the tumor microenvironment in patients. Its safety and efficacy have been confirmed in mouse studies and the extrapolated radiation doses are within acceptable ranges for repeated imaging. The envisioned trials in cancer patients will help to define the role of MMR in TAM in cancer progression and its susceptibility to immuno-oncological treatments.

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Compliance with Ethical Standards. All procedures followed the guidelines of the Belgian Council for Laboratory Animal Science and were approved by the Ethical Committee for Animal Experiments of the Vrije Universiteit Brussel (license 13-272-5).

Conflict of Interest

KM received travel and accommodation expenses from Bayer; KM and CV have received research funding from Camel-IDS. DN, LT, and RG are co-founders of and/or employed by Camel-IDS. DN has received funding from Boehringer-Ingelheim, Complix, Agenus, and Telix Pharma. KM, LT, DN, RG, VGJ, XC, and BJ have patents on nanobody imaging and therapy. LT received honoraria from Camel-IDS, IBA, and Institut des Radioéléments (IRE).

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