89Zr-PET imaging to predict tumor uptake of 177Lu-NNV003 anti-CD37 radioimmunotherapy in mouse models of B cell lymphoma

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[177Lu]Lu-DOTA-NNV003, a radioimmunoconjugate targeting CD37, is developed as novel radioimmunotherapy (RIT) treatment for patients with B cell non-Hodgkin’s lymphoma (NHL). Since patients are at risk for developing hematological toxicities due to CD37 expression on normal B cells, we aimed to develop 89Zr-labeled NNV003 for positron emission tomography (PET) imaging as a surrogate tool to predict [177Lu]Lu-DOTA-NNV003 RIT whole-body distribution and tumor uptake. NNV003 antibody was first radiolabeled with 89Zr. [89Zr]Zr-N-sucDf-NNV003 tumor uptake was evaluated by PET imaging of mice bearing human CD37-expressing REC1 B cell NHL or RAMOS Burkitt’s lymphoma xenograft tumors followed by ex vivo analysis. Finally, CD37-targeting of [89Zr] Zr-N-sucDf-NNV003 and [177Lu]Lu-DOTA-NNV003 RIT were compared. [89Zr]Zr-N-sucDf-NNV003 accumulated in REC1 tumors over time, which was not observed for non-specific, 111In-labeled IgG control molecule. In RAMOS tumor-bearing mice, [89Zr]Zr-N-sucDf-NNV003 tumor uptake was higher than [111In]In-DTPA-IgG at all tested tracer protein doses (10 µg, 25 µg and 100 µg; P < 0.01), further confirming [89Zr]Zr-N-sucDf-NNV003 tumor uptake is CD37-mediated. [89Zr]Zr-N-sucDf-NNV003 and [177Lu]Lu-DOTA-NNV003 RIT showed similar ex vivo biodistribution and tumor uptake in the RAMOS tumor model. In conclusion, [89Zr]Zr-N-sucDf-NNV003 PET imaging can serve to accurately predict CD37-targeting of [177Lu]Lu-DOTA-NNV003. To enable clinical implementation, we established a good manufacturing practice (GMP)-compliant production process for [89Zr]Zr-N-sucDf-NNV003.

Abbreviations

68Ga  Gallium-68
111In  Indium-111
131I  Iodine-131
90Y  Yttrium-90
89Zr  Zirconium-89
BSA  Bovine serum albumin
CI  Confidence interval
CT  Computed tomography
EDTA  Ethylenediaminetetraacetic acid
FCS  Fetal calf serum
FFPE  Formalin-fixed paraffin-embedded
GMP  Good manufacturing practice
HAMAs  Human-anti-mouse antibodies
ID  Injected dose
IRF  Immunoreactive fraction

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Novel treatment options for patients with B cell non-Hodgkin’s lymphoma (NHL) are warranted, especially for those with poor prognosis, since large subgroups of these patients become refractory after initial CD20-based immunotherapy. Therapies directed against other targets expressed by B cells, for example leukocyte antigen CD37, may provide a therapeutic alternative. CD37-directed radioimmunotherapy (RIT) with the fully murine antibody lutetium-177 (177Lu; T1/2: 6.65 d)-lixotomab satetraxetan, was recently evaluated in a phase 1/2a study in relapsed/refractory indolent NHL patients and showed encouraging results1–5. Instead of a murine antibody, next-generation 177Lu-Lu-DOTA-NNV003 consists of a chimeric mouse-human anti-CD37 antibody (NNV003) conjugated with p-SCN-Bn-DOTA that chelates the β- emitting radionuclide 177Lu.

CD37 is a highly glycosylated transmembrane protein selectively expressed by normal B cells and the majority of B cell lymphomas and is specifically of interest for RIT, since CD37 receptor-antibody complexes are highly internalized in tumor cells6,9. In combination with 177Lu’s favorable physical properties for RIT, [177Lu]Lu-DOTA-NNV003 has a potentially improved toxicity profile. Compared to historically used radionuclides for RIT in B cell malignancies such as yttrium-90 (90Y; T1/2: 2.66 d), 177Lu has a relatively short β− range7. Also, 177Lu becomes intracellularly trapped in lysosomes through residualization, whereas iodine-131 (131I; T1/2: 8.02 d) diffuses passively out of cells after catabolization7. This may result in enhanced tumor irradiation, while sparing surrounding healthy tissues.

RIT in B cell malignancies is generally effective in only a subset of patients, while often resulting in off-target, tumor toxicities due to target expression on normal B cells7. Insight in CD37 expression may help to select patients who are more likely to respond or are at risk for developing CD37-induced hematological toxicities. Therefore, RIT could benefit from molecular imaging as a non-invasive approach to provide whole-body information on target presence and RIT distribution. Dosimetry for organs-at-risk in 177Lu radionuclide therapy is often calculated using single photon emission computed tomography (SPECT)/computed tomography (CT) imaging. Still, this procedure has limited sensitivity and quantitative evaluation of target-mediated uptake can be challenging8,11.

We aimed to develop a surrogate image tool for the assessment of [177Lu]Lu-DOTA-NNV003 RIT whole-body distribution and tumor uptake, which could aid its clinical development and use (Fig. 1). The positron emission tomography (PET) radioisotope zirconium-89 (89Zr) is particularly suited for antibody imaging, as it yields high sensitivity and accurate quantification, while its physical half-life of 3.27 d matches the time that antibodies need for tumor accumulation. Studies in patients and mice showed the utility of 89Zr-PET imaging to predict the distribution of therapeutic radionuclides such as 177Lu and 90Y coupled to antibodies12–14.

To assess whether [89Zr]Zr-N-sucDf-NNV003 PET imaging can serve to predict [177Lu]Lu-DOTA-NNV003 RIT biodistribution, we evaluated their whole-body distribution and tumor-targeting properties in mice bearing human B cell lymphomas. Furthermore, a good manufacturing practice (GMP)-compliant production process was established for [89Zr]Zr-N-sucDf-NNV003 to enable administration to patients.

**Results**

**Development and quality control of 89Zr-labeled NNV003.** For in vivo studies, NNV003 was conjugated to tetrafluorphenol-N-succinyl desferal (TFP-N-sucDf) followed by radiolabeling with 89Zr. NNV003 was incubated with increasing molar ratios of TFP-N-sucDf, which resulted in approximately 60% conjugation efficiency (Supplementary Fig. S1A). NNV003-N-sucDf intermediate product was obtained with a mean yield of 54% (Supplementary Fig. S1B). To enable 89Zr-chelation by TFP-N-sucDf, protective Fe(III) must be removed from the hydroxamate groups. At least 40% Fe(III) was removed from NNV003-N-sucDf by incubation with EDTA (Supplementary Fig. S1C). The NNV003-TFP-N-sucDf ratio did not impair immunoreactivity of NNV003-N-sucDf to CD37-expressing RAMOS cells, as the mean immunoreactive fractions (IRF) were similar for all tested ratios and all higher than 0.8 (Supplementary Fig. S1D). NNV003-N-sucDf intermediate product was obtained with ≥ 95% purity and no aggregation or fragmentation was observed (Supplementary Fig. S1E). NNV003 conjugated to ~ 1.2 TFP-N-sucDf chelators per antibody consistently bound 500 MBq [89Zr]Zr-N-sucDf-NNV003 to enable administration to patients.

**[89Zr]Zr-N-sucDf-NNV003 PET imaging and biodistribution in REC1 tumor-bearing mice.** [89Zr]Zr-N-sucDf-NNV003 whole-body distribution and tumor-targeting were evaluated in BALB/c nude mice bearing CD37-expressing human REC1 B cell NHL xenograft tumors. PET imaging revealed [89Zr]Zr-N-sucDf-
uptake is CD37-mediated (Fig. 3D). Furthermore, tumor uptake was comparable for 10, 25 and 100 µg [89Zr]Zr-DTPA-IgG at day 5 pi (Fig. 2C). Bone uptake is common for 89Zr-labeled molecules, as they are metabolized in vivo and unbound 89Zr has a high affinity for hydroxyapatite. Levels similar to the [89Zr]Zr-DTPA-IgG uptake in bone were found in mouse models for other 89Zr-labeled antibodies. For this reason, potential instability of [89Zr]Zr-DTPA-IgG linker chelation is considered to be limited. Ex vivo tumor uptake and tumor-to-blood ratio were higher for [89Zr]Zr-N-sucDf-NNV003 compared to [111In]In-DTPA-IgG (10.9 vs. 3.9%ID/g; P < 0.05 and 2.4 vs. 0.5; P < 0.01) (Fig. 2D), indicating [89Zr]Zr-N-sucDf-NNV003 tumor uptake is CD37-mediated. Furthermore, [111In]In-DTPA-IgG tumor-to-blood ratio was lower than 1, meaning tumor uptake is not target-mediated. [111In]In-DTPA-IgG can therefore be considered a suitable control molecule.

**Dose- and CD37-dependent uptake in RAMOS tumor-bearing mice.** To study CD37-mediated tumor uptake, we evaluated [89Zr]Zr-N-sucDf-NNV003 biodistribution at three total protein dose levels in human RAMOS Burkitt’s lymphoma tumor-bearing mice. A radiolabeled antibody dose of 10 µg [89Zr]Zr-N-sucDf-NNV003 or [111In]In-DTPA-IgG was supplemented with 0, 15 or 90 µg of unlabeled NNV003 or IgG to obtain total protein doses of 10 µg, 25 µg and 100 µg for each tracer. PET quantification showed [89Zr]Zr-N-sucDf-NNV003 accumulation in CD37-expressing RAMOS tumors for all tested protein doses, with the highest tumor-to-blood ratios found for day 5 pi (Fig. 3A). Comparable tumor uptake was observed for 10, 25 and 100 µg [89Zr]Zr-N-sucDf-NNV003, with SUVmean of 1.8 (±0.3), 1.7 (±0.1) and 1.6 (±0.2) respectively at day 5 pi (Fig. 3B). The highest tumor-to-blood ratio (1.7 ± 0.3) was observed for 10 µg [89Zr]Zr-N-sucDf-NNV003 at day 5 pi.

No relevant differences in ex vivo biodistribution of [89Zr]Zr-N-sucDf-NNV003 and [111In]In-DTPA-IgG were observed, except for uptake in bone and tumor (Fig. 3C). [89Zr]Zr-N-sucDf-NNV003 bone uptake demonstrated some variation between 10 µg, 25 µg and 100 µg dose groups, potentially due to slight differences in radiochemical purity, which varied from 95 to 98%. Also, [89Zr]Zr-N-sucDf-NNV003 distribution to healthy tissues was not affected by the addition of unlabeled antibody dose. Ex vivo [89Zr]Zr-N-sucDf-NNV003 tumor uptake was higher compared to [111In]In-DTPA-IgG for all protein dose groups, confirming that [89Zr]Zr-N-sucDf-NNV003 tumor uptake is CD37-mediated (Fig. 3D). Furthermore, tumor uptake was comparable for 10, 25 and 100 µg [89Zr]Zr-N-sucDf-NNV003 with 10.6 (±2.7), 12.4 (±3.4) and 10.1 (±2.1) %ID/g respectively, indicating CD37 is not saturated in tumor cells by addition of unlabeled antibody at these protein doses. The highest [89Zr]Zr-N-sucDf-NNV003 tumor-to-blood ratio of 1.9 (±0.3) and 1.9 (±0.5) was observed for the 10 and 25 µg total protein doses, compared to 1.3 (±0.2) found for 100 µg [89Zr]Zr-N-sucDf-NNV003, suggesting CD37 in RAMOS tumors may be saturated by [89Zr]Zr-N-sucDf-NNV003 at higher protein doses (Fig. 3E). [89Zr]Zr-N-sucDf-NNV003 tumor uptake was the highest in the 25 µg dose group compared to [111In]In-DTPA-IgG (12.4 vs. 3.3%ID/g; P < 0.01).

[89Zr]Zr-N-sucDf-NNV003 tumor uptake was comparable for REC1 and RAMOS tumors, with both tumor types expressing similar levels of CD37 as measured by flow cytometry and immunohistochemistry (Fig. 4).
Lastly, we compared tumor uptake determined by PET quantification with ex vivo tumor uptake to confirm if $[^{89}Zr]$Zr-N-sucDf-NNV003 PET imaging accurately visualizes tumor uptake. Ex vivo biodistribution of $[^{89}Zr]$Zr-N- succDf-NNV003 uptake expressed as SUVmean correlated with PET-derived tumor SUVmean, but not SUVmax, which can be explained by the fact that SUVmax represents the highest voxel and may not reflect whole-tumor uptake (Fig. 5).

Comparison of $[^{89}Zr]$Zr-N-sucDf-NNV003 and $[^{177}Lu]$Lu-DOTA-NNV003 RIT biodistribution in the RAMOS tumor model. Next, we evaluated $[^{89}Zr]$Zr-N-sucDf-NNV003 as a surrogate for $[^{177}Lu]$Lu-DOTA-NNV003 RIT whole-body distribution in RAMOS tumor-bearing mice. Ex vivo tissue analysis showed decreasing $[^{177}Lu]$Lu-DOTA-NNV003 activity in the blood pool between 1 h to 3 days pi, which coincided with decreasing uptake in well-perfused organs such as heart, lung and kidney (Fig. 6A). $[^{177}Lu]$Lu-DOTA-NNV003 uptake in RAMOS tumors increased over time with the highest uptake of 13.6 ± 8.3%ID/g found at 3 days pi. Blood pool activity of $[^{89}Zr]$Zr-N-sucDf-NNV003 at 5 days pi was comparable to $[^{177}Lu]$Lu-DOTA-NNV003 at 3 days pi (5.7 ± 1.7%ID/g versus 7.6 ± 5.0%ID/g), validating a relevant comparison of both tracers’ ex vivo biodistribution results.

Comparison of ex vivo biodistribution results revealed highest uptake of $[^{89}Zr]$Zr-N-sucDf-NNV003 and $[^{177}Lu]$Lu-DOTA-NNV003 in tumor compared to normal tissues (Fig. 6B). Also, similar uptake was found for $[^{89}Zr]$Zr-N-sucDf-NNV003 and $[^{177}Lu]$Lu-DOTA-NNV003 in RAMOS tumors (10.6 vs. 13.6%ID/g; $P = 0.48$), indicating tumor-targeting is not affected by chelator or radioisotope (TEP-N-sucDf for $^{89}$Zr versus p-SCN-Bn-DOTA for $^{177}$Lu). Furthermore, $[^{177}Lu]$Lu-DOTA-NNV003 and $[^{89}Zr]$Zr-N-sucDf-NNV003 showed similar biodistribution in healthy tissues, except for bone and spleen. Bone uptake was higher for $[^{89}Zr]$Zr-N-sucDf-NNV003, which is common for $^{89}$Zr-labeled antibodies, as discussed previously15. In patients, tracer uptake in red bone marrow can be clearly separated from bone uptake on PET. Therefore, toxicity from myelosuppression can be potentially recognized by high $[^{89}Zr]$Zr-N-sucDf-NNV003 uptake in red bone marrow.

Higher spleen uptake was observed for $[^{177}Lu]$Lu-DOTA-NNV003 compared to $[^{89}Zr]$Zr-N-sucDf-NNV003 (5.6 vs. 2.1%ID/g; $P < 0.05$). Murine CD37 is expected to be expressed on B cells present in the spleen, however NNV003 is not cross-reactive with murine CD37. Furthermore, $[^{89}Zr]$Zr-N-sucDf-NNV003 spleen uptake was similar to $[^{111}In]$In-DTPA-IgG, indicating this uptake is not CD37-mediated. In mice, the spleen plays an important role in antibody pharmacokinetics due to its high blood flow and loose capillaries, but also due to the
expression of Fcγ receptors. Therefore, differences in tracer spleen uptake are easily induced by slight differences in the immune status of mice.

Clinical-grade [89Zr]Zr-N-sucDf-NNV003 for patient studies. To enable PET imaging in patients, we developed and characterized the production of clinical-grade [89Zr]Zr-N-sucDf-NNV003 (Supplementary Fig. S2). Quality control for three individual batches of NNV003-N-sucDf intermediate product and [89Zr]Zr-N-sucDf-NNV003 final product was according to specifications, indicating a robust manufacturing process (Supplementary Table S1). NNV003-N-sucDf intermediate product demonstrated stability up to 6 months (Supplementary Table S2). Therefore, NNV003-N-sucDf shelf-life is currently set at 6 months, and may be extended if future stability time points remain within specifications. Furthermore, [89Zr]Zr-N-sucDf-NNV003 final product was stable up to 96 h when stored at 2–8 °C and up to 4 h when prepared in the syringe (Supplementary Table S3).
Our study demonstrates the potential of using \(^{89}\text{Zr}\)Zr-sucDf-NNV003 PET imaging to predict whole-body distribution and tumor uptake of \(^{177}\text{Lu}\)Lu-DOTA-NNV003 RIT. We showed that \(^{89}\text{Zr}\)Zr-sucDf-NNV003 accumulated in REC1 and RAMOS tumor tissues in a CD37-dependent manner, resulting in high tumor-to-blood ratios. Furthermore, \(^{89}\text{Zr}\)Zr-sucDf-NNV003 biodistribution and tumor-targeting were similar to \(^{177}\text{Lu}\)lilotomab satetraxetan. The murine version of \(^{177}\text{Lu}\)Lu-DOTA-NNV003, is currently in phase II for patients with anti-CD20 refractory follicular lymphoma and has previously been studied in phase I/IIa clinical trials in patients with relapsed, CD37-positive, indolent and aggressive NHL (ClinicalTrials.gov Identifier: NCT01796171, NCT02658968). These studies showed high RIT uptake in tumors, but also in red bone marrow, liver, spleen, and kidneys. Predosing with unlabeled lilolomab significantly reduced the absorbed radiation dose in healthy CD37-expressing tissues due to \(^{177}\text{Lu}\)lilotomab satetraxetan. Also, \(^{177}\text{Lu}\)lilotomab satetraxetan dosimetry, biodistribution, and tumor targeting were improved by lilolomab predosing compared with rituximab predosing or no predosing. Informing clinicians on whether tumors are effectively targeted by \(^{177}\text{Lu}\)Lu-DOTA-NNV003 and gaining insight into the amount of cold antibody dose required to saturate CD37 expression in healthy tissues are essential to optimize future \(^{177}\text{Lu}\)Lu-DOTA-NNV003 RIT dose-regimens. We showed that \(^{89}\text{Zr}\)Zr-sucDf-NNV003 PET imaging can serve as a surrogate for \(^{177}\text{Lu}\)Lu-DOTA-NNV003 RIT whole-body distribution and represents a potentially attractive tool to assess distribution to tumors and healthy tissues.

The combined approach of RIT and diagnostics such as molecular imaging may support precise cancer therapy in both palliative and curative settings. SPECT/CT imaging was routinely used for assessing biodistribution.
and dosimetry of 90Y- and 131I-based RIT antibodies in the early 2000s20,21, but low quantities of γ-photons emitted by 177Lu complicate quantification. As a therapeutic agent, even low dose pre-treatment imaging of 177Lu may result in local toxicity, while this risk is limited for the low energy β+-rays of PET radioisotopes. In this respect, gallium-68 (68Ga)/177Lu is a commonly used theranostic pair for studying receptor expression or drug distribution. However, 68Ga, given its relatively short physical half-life of 67.6 min, provides no insight in internalizing properties of an antibody. As internalization is an essential factor for both efficacy and toxicity of 177Lu-based RIT agents, longer-lived PET radioisotopes such as 89Zr may better reflect 177Lu RIT in vivo behavior.

In a recent study, response at the tumor lesion level after treatment with 177Lu-lilotomab satetraxetan was evaluated by FDG PET/CT and did not correlate with tumor-absorbed dose11. They hypothesized that the combination regimen of radiolabeled and cold antibodies might preclude such a correlation. In our study, we were able to quantitatively visualize tumor uptake in the presence of unlabeled antibody using [89Zr]Zr-N-sucDF-NNV003 PET imaging. In patients with relapsed B cell NHL, a pre-therapy scan with 89Zr-ibritumomab tiuxetan was used to predict radiation dosimetry during 90Y-ibritumomab tiuxetan therapy11. Importantly, 89Zr-ibritumomab tiuxetan whole-body distribution was not affected by simultaneous 90Y-ibritumomab tiuxetan therapy. These findings emphasize the potential of an [89Zr]Zr-N-sucDF-NNV003 pre-therapy scan to predict CD37-targeting by [177Lu]Lu-DOTA-NNV003 RIT, thereby allowing for patient stratification. Furthermore, a post-therapy [89Zr]Zr-N-sucDF-NNV003 scan may inform on therapy response by evaluating CD37-mediated tumor uptake after [177Lu]Lu-DOTA-NNV003 RIT.

Next-generation anti-CD37 RIT antibody [177Lu]Lu-DOTA-NNV003, based on the chimeric mouse-human antibody NNV003, was hypothesized to be less immunogenic than the fully murine 177Lu-lilotomab satetraxetan. After a single dose of 177Lu-lilotomab satetraxetan, development of human-anti-mouse-antibodies (HAMAs) was reported for seven out of 74 subjects1. Preclinically, [177Lu]Lu-DOTA-NNV003 in silico immunogenicity prediction tools revealed a lower immunogenicity potential compared to 177Lu-lilotomab satetraxetan. These results warrant further evaluation of [177Lu]Lu-DOTA-NNV003 RIT in patients with CD37-expressing B cell malignancies, and [89Zr]Zr-N-sucDF-NNV003 PET imaging could assist its clinical development and use.

Conclusion

We showed that [89Zr]Zr-N-sucDF-NNV003 PET imaging can accurately predict whole-body distribution and tumor uptake of [177Lu]Lu-DOTA-NNV003 therapy in B cell lymphoma xenograft models. To enable clinical implementation of this theranostic strategy, we established a GMP-compliant production process for [89Zr]Zr-N-sucDF-NNV003. [89Zr]Zr-N-sucDF-NNV003 pre-therapy PET imaging in patients with B cell NHL may help to identify those more likely to respond. Furthermore, as a surrogate imaging tool, [89Zr]Zr-N-sucDF-NNV003 may aid in optimizing [177Lu]Lu-DOTA-NNV003 RIT dose-regimens and post-therapy response evaluation.
Materials and methods

Cell lines and flow cytometry experiments. Human CD37-expressing cell lines RAMOS (Burkitt’s lymphoma) and REC1 (Mantle cell lymphoma) were obtained from the American Type Culture Collection. RAMOS and REC1 cell lines were tested and authenticated in July and October 2019 respectively using short tandem repeat profiling. Cells were cultured in Roswell Park Memorial Institute (RPMI) medium, supplemented with 10% fetal calf serum (FCS) and incubated at 37 °C in a humidified atmosphere with 5% CO₂.

CD37 expression by RAMOS and REC1 cells was determined by flow cytometry. Cells were harvested in 2% FCS in phosphate-buffered saline (PBS) and kept on ice prior to use. NNV003 and non-specific human IgG control molecule (Nanogam®, Sanquin) was diluted with 2% FCS in PBS to 20 μg/mL and incubated with 2 × 10⁶ cells/mL for 1 h at 4 °C. Bound NNV003 and control antibodies were detected using a phycoerythrin-conjugated goat anti-human IgG secondary antibody (SouthernBiotech; 2040-09) diluted 1:50 with 2% FCS in PBS and analyzed on a BD Accuri C6 flow cytometer (BD Biosciences). Data analysis was performed using FlowJo v10 (Tree Star) and surface receptor expression was expressed as mean fluorescent intensity (MFI).

Radiolabeling of NNV003 and IgG control for animal studies. NNV003 antibody (IgG1, mouse variable regions, κ, and human constant region, κ; Nordic Nanovector) was conjugated to TFP-N-sucDf (ABX GmbH) and subsequently radiolabeled with ⁸⁹Zr as described previously. To date, several ⁸⁹Zr-labeled antibodies are produced according to this methodology and were evaluated in animals and patients without any sign of toxicity. In short, NNV003 was incubated with a twofold molar excess of TFP-N-sucDf at pH 9.0–9.5. After incubation for 1 h at room temperature (RT), pH was set to 4.0–4.5. Ethylenediaminetetraacetic acid (EDTA; Hospital Pharmacy UMCG) 25 mg/ml was added and incubated for 30 min at 35 °C to transchelate Fe(III) from the TFP-N-sucDf hydroxamate groups. NNV003-N-sucDf was subsequently purified using a Vivaspin-2 concentrator (Sartorius GmbH), aliquoted and stored at −80 °C until use. On the day of tracer injection, NNV003-N-sucDf was radiolabeled using GMP-grade ⁸⁹Zr oxalate (Perkin Elmer). RCP of [⁸⁹Zr]Zr-N-sucDf-NNV003 was determined by trichloroacetic acid precipitation test. Furthermore, NNV003 antibody was conjugated to p-SCN-Bn-DOTA (Macrocyclics) and subsequently radiolabeled using ⁷⁷Lu chloride (Perkin Elmer) as described previously.

Non-specific IgG control molecule was conjugated with a 50-fold molar excess of p-SCN-Bn-DTPA (Macrocyclics) as described previously. Radiolabeling of IgG-DTPA was performed using ⁴⁷In chloride (Mallinckrodt) by incubation during 1–2 h in ammonium acetate pH 5.5. Radiochemical purity of [¹¹¹In]In-DTPA-IgG was assessed by instant thin-layer chromatography using 0.1 M citrate buffer pH 6.0 as eluent.

Cell lines and flow cytometry experiments.

Materials and methods

All experiments were performed in accordance with relevant guidelines and regulations. All methods were reported in accordance with recommendations in the ARRIVE guidelines. Animal studies involving [⁸⁹Zr]Zr-N-sucDf-NNV003 were approved by the Institutional Animal Care and Use Committee of the University Medical Center Groningen. Male BALB/c Olahsd-Foxn1nu mice (Envigo) 8–10 weeks of age were inoculated with 10⁶ cells. Murine NK cells were depleted to enhance tumor take-rate. This was achieved by administering the mice anti-asialo GM1 treatment on 1 day before and 4, 11, 18 and 25 days post tumor inoculation. When tumors measured a volume of at least 200 mm³ (~14 days post inoculation for REC1 tumors and ~19 days for RAMOS tumors), mice received intravenous injections of 10 μg (~5 MBq) [⁸⁹Zr]Zr-N-sucDf-NNV003 supplemented with either 0, 15 or 90 μg of unlabeled NNV003 and co-injected with an equal total protein dose (~1 MBq) of [¹¹¹In]In-DTPA-IgG control (n = 5–6 mice per group). By co-injection of [⁸⁹Zr]Zr-N-sucDf-NNV003 and [¹¹¹In]In-DTPA-IgG, tumor uptake and biodistribution results can be compared within the same animal, thereby providing valid results on target-specific uptake. Also, the number of animals required for these studies can be reduced using this strategy. Mice underwent microPET scanning at 1, 3 and 5 days post injection (pi), followed by ex vivo biodistribution.

MicroPET scans were performed using a Focus 220 rodent scanner (CTI Siemens). Scans were reconstructed using a 2-dimensional ordered-subset expectation maximization reconstruction algorithm with Fourier rebinning, 4 iterations, and 16 subsets. Data sets were corrected for decay, random coincidences, scatter, and attenuation. For in vivo quantification, regions of interest were drawn for tumor based upon ex vivo weight, assuming 1 g/cm³ tissue density, and heart using AMIDE medical image data examiner software v1.0.4. Tracer uptake was
Ex vivo tissue preparation and immunohistochemistry. For ex vivo tissue analysis, formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were prepared. FFPE blocks were sliced into 4 μm tumor tissue sections, fixated on microscope slides and dried overnight at 60 °C. For CD37 immunohistochemistry, tumor tissue sections were deparaffinized in xylene and rehydrated. Heat-induced antigen retrieval was performed in 10 mM citrate (pH 6.0) for 15 min at 95–100 °C. Endogenous peroxidase was blocked by 10-min incubation with 10% hydrogen peroxide in PBS. Slides were incubated with rabbit anti-human CD37 antibody (Proteintech; 21044-1) or rabbit IgG antibody control (Abcam; ab172730) diluted to 0.8 µg/mL in 1% BSA in PBS for 1 h at RT. Thereafter, slides were incubated with Dako EnVision horseradish peroxidase system (Agilent Technologies) for 30 min at RT, followed by 10-min incubation with diaminobenzidine chromogen. Hematoxylin counterstaining was applied routinely. For histological analysis of tumors, hematoxylin/eosin staining was performed on subsequent tissue sections. Digital scans of slides were acquired by a Hamamatsu NanoZoomer 2.0-HT multi-slide scanner and analyzed with NanoZoomer Digital Pathology viewer software.

Production and stability testing of clinical grade [⁸⁹Zr]Zr-N-sucDf-NNV003. NNV003 was conjugated to TFP-N-sucDf at a 1:2 molar ratio and subsequently radiolabeled with GMP-grade ⁸⁹Zr. Quality control was performed on both NNV003-N-sucDf intermediate product and [⁸⁹Zr]Zr-N-sucDf-NNV003 final product. This included analysis on appearance, yield, purity, concentration, pH, radiochemical purity, residual solvents, sterility, endotoxin content and IRF. Analytical procedures were validated to demonstrate suitability for use in quality control testing of NNV003-N-sucDf and [⁸⁹Zr]Zr-N-sucDf-NNV003. The production processes for NNV003-N-sucDf and [⁸⁹Zr]Zr-N-sucDf-NNV003 were validated according to GMP guidelines by the production of three consecutive validation batches.

NNV003-N-sucDf intermediate product was stored in sterile vials (BioPure) at −80 °C. Stability of NNV003-N-sucDf was analyzed at 0, 1, 3 and 6 months after production. [⁸⁹Zr]Zr-N-sucDf-NNV003 final product was stored in sterile vials (BioPure) at −2–8 °C and stability was analyzed at 0 and 96 h. Stability of [⁸⁹Zr]Zr-N-sucDf-NNV003 in the syringe at RT was analyzed at 0 and 4 h. Stability tests consisted of quality control according to release specifications.

Statistical analysis. Data were analyzed for statistical significance in GraphPad Prism v7.0 using the Mann–Whitney U test for non-parametric data followed by Bonferroni post-test correction for comparison of more than two groups. Ex vivo biodistribution of [⁸⁹Zr]Zr-N-sucDf-NNV003 and [¹⁷⁷Lu]Lu-DOTA-NNV003 were compared with Welch’s t-test for unequal variances. Correlation was assessed by Spearman’s rank-order correlation test. In vitro experiments were repeated at least 3 times. p values <0.05 were considered significant.

Data availability
Data that support the findings of this study are available from E. G. E. de Vries upon request.

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Author contributions

D.G., M.N.L.-d.H., M.N., H.H., J.D., E.G.E.d.V. and M.P. participated in the design and/or interpretation of the reported experiments or results. D.G. and M.P. participated in the acquisition and/or analysis of data. H.H. and J.D. participated in $^{177}$Lu-Lu-DOTA-NNV003 animal studies and provided data on ex vivo biodistribution. D.G., M.N.L.-d.H., H.H., J.D., E.G.E.d.V and M.P. provided administrative, technical or supervisory support. All authors participated in drafting and/or revising the manuscript. All authors read and approved the final manuscript.

Competing interests

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Additional information

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