Theranostic Radiolabeled Anti-CD20 sdAb for Targeted Radionuclide Therapy of Non-Hodgkin Lymphoma

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Abstract

Anti-CD20 radioimmunotherapy is an effective approach for therapy of relapsed or refractory CD20pos lymphomas, but faces limitations due to poor tumor penetration and undesirable pharmacokinetics of full antibodies. Camelid single-domain Ab fragments (sdAb) might circumvent some of the limitations of radiolabeled full antibodies. In this study, a set of hCD20-targeting sdAbs was generated, and their capacity to bind hCD20 was evaluated in vitro and in vivo. A lead sdAb, sdAb 9079, was selected on the basis of its specific tumor targeting and significant lower kidney accumulation compared with other sdAbs. SdAb 9079 was then radiolabeled with 68Ga and 177Lu for PET imaging and targeted therapy. The therapeutic potential of 177Lu-DTPA-sdAb 9079 was compared with that of 177Lu-DTPA-rituximab and unlabeled rituximab in mice bearing hCD20pos tumors. Radiolabeled with 68Ga, sdAb 9079 showed specific tumor uptake, with very low accumulation in nontarget organs, except kidneys. The tumor uptake of 177Lu-DTPA-sdAb 9079 after 1.5 h was 3.4 ± 1.3% ID/g with T/B and T/M ratios of 13.3 ± 4.6 and 32.9 ± 15.6. Peak tumor accumulation of 177Lu-DTPA-rituximab was about 9 times higher, but concomitantly with high accumulation in nontarget organs and very low T/B and T/M ratios (0.8 ± 0.1 and 7.1 ± 2.4). Treatment of mice with 177Lu-DTPA-sdAb 9079 significantly prolonged median survival compared with control groups and was as effective as treatment with rituximab or its 177Lu-labeled variant. Taken together, sdAb 9079 displays promising features as a theranostic drug to treat CD20pos lymphomas. Mol Cancer Ther; 16(12): 2828–39. ©2017 AACR.

Introduction

CD20 antigen is an integral transmembrane, nonglycosylated protein that is specifically expressed on the pre-B-cell stage while its expression is absent on plasma cells and hematopoietic stem cells (1, 2). CD20 is expressed by over 90% of B-cell non-Hodgkin lymphoma (NHL; ref. 3). The humanized mAb rituximab is the first approved anti-CD20 mAb and is being used as a first-line treatment against different forms of B-cell NHL (3). Rituximab given as a single agent shows response rates ranging from 50% to 80% (4–8), while in combination with chemotherapy, it shows response rates of more than 90%. Resistance to rituximab eventually occurs in about half of the B-cell NHL patients (9–11).

The combination of immunotherapy with cytotoxic radiation, that is, anti-CD20 radioimmunotherapy (RIT), has been shown to be an effective approach for the treatment of relapsed or refractory B-cell NHL (12). Currently, there is only one FDA-approved radiolabeled anti-CD20 mAb in the market, 90Y-Ibritumomab tiuxetan (Zevalin). Zevalin is administrated as a single dose in combination with rituximab. The therapeutic dose of Zevalin depends on the platelet counts of the patients, but the maximum allowed injected activity is 32 mCi (13). Zevalin has proven to be successful, with more than 80% of patients responding to therapy and 30% showing a complete response (12–14).

Even though the administration of Zevalin is well tolerated, myelosuppression remains the main dose-limiting toxicity (15). In addition, to reduce the slow blood clearance of mAbs, Zevalin consists of mouse mAb ibritumomab as a targeting vehicle, which is the murine counterpart of rituximab. Murine mAbs interact weaker with human Fc-receptors and are therefore cleared faster. However, murine mAbs can induce a human anti-murine antibody response (HAMA), which can result in altered pharmacokinetics (16, 17).

To overcome the limitations of radiolabeled mAbs, smaller mAb fragments have been generated. In many cases, the improved kinetics were counterbalanced by increased instability and decreased target affinity (18). Camelid single-domain Abs fragments (sdAb) are the smallest functional antigen-binding fragments, derived from Camelid...
heavy chain-only Abs (19). They usually show high stability and solubility and low immunogenicity. They bind their antigens with very high specificities, and with affinities comparable with mAbs (20, 21). Because of their small size (<15 kDa), they clear fast from blood and penetrate deep into tissues (20). For imaging of cancer, sdAbs have been directed to a variety of type-I transmembrane cancer cell biomarkers (20–22). A first-in-human PET study with 68Ga-labeled anti-HER2 sdAb for breast cancer imaging was recently finalized in our university hospital. The results of the clinical trial showed the specific targeting of HER2-positive lesions by the 68Ga-labeled sdAb, with very good contrast and no adverse side-effects (23). We also endeavored the use of sdAbs as vehicles for targeted radionuclide therapy (TRNT) in preclinical cancer models, through the therapeutic β -particle-emitting radionuclide 177Lu. These proof-of-concept studies showed strong signs of tumor growth inhibition with absence of toxicity in nontarget tissues (21, 24).

The integration of molecular imaging with a related diagnostic radiotracer sharing similar pharmacokinetic characteristics as the therapeutic could increase the success of TRNT (25). This theranostic approach allows a preliminary examination of the expression pattern of the molecular target for which the therapeutic radiotracer is intended. In addition, it can generate important information about the pharmacokinetics and biodistribution profiles of the therapeutic, predicting possible adverse effects and monitor treatment response (25).

In this study, we describe the generation, production, and purification of a set of sdAb-fragments targeting the human CD20 (hCD20) receptor. In addition, their capacity to bind hCD20 was evaluated in vitro and in vivo, after which two sdAbs, 9077 and 9079 were selected and radiolabeled with 68Ga and 177Lu for PET imaging and targeted therapy. Finally, the therapeutic potential of 177Lu-labeled sdAbs was evaluated in mice bearing hCD20-pos tumors.

Materials and Methods

Cell lines and culture conditions

Daudi (hCD20pos), Reh (hCD20neg), and the murine B16-F10 cell line were obtained from ATCC, and cultured using RPMI1640 (Daudi, Reh) or DMEM (B16-F10) supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine and 0.1 mg/mL streptomycin. The hCD20pos B16 cell line was generated by transfecting the B16-F10 cell line with a plasmid containing the expression cassettes for hCD20 and the neomycin resistance gene. Stable transfected cells were selected and maintained with G418 (2 mg/mL) containing medium, followed by FACS sorting of hCD20pos cells. CD20 expression of Daudi or Reh cells were incubated with 1 ng/ml of rituximab for 1 hour at 4°C. Potential competition with rituximab for hCD20 receptor binding was analyzed by incubating with a 100-fold molar excess of rituximab for 1 hour at 4°C, prior to the addition of sdAbs. After washing, sdAb binding was detected by adding 2 μg FITC-labeled anti-HisTag mAb for 30 minutes at 4°C. After washing, mean fluorescence intensity (MFI) was measured using a FACSCanto Flow Cytometer (BD Biosciences) and analyzed with FlowJo 7 software (Tree Star Inc.). The affinity of the anti-hCD20 sdAbs was measured by incubating a serial dilution (0.5–1,000 nmol/L) with 5 x 105 Daudi cells for 1 hour at 4°C and further processed as described above. Data were plotted using GraphPad Prism.

Preparation of 99mTc-sdAbs

Anti-hCD20 and ctrl sdAbs were radiolabeled with 99mTc for biodistribution studies. 99mTc was incorporated at their His6-tag via tricarbonyl chemistry (28). 99mTc-ctrl sdAbs were separated from unbound (99mTc(H2O)3(CO)3) with 1 mmol/L isopropanol-

Preparation of 68Ga-NOTA- and 177Lu-DTPA conjugates

Rituximab (MabThera, Roche), untagged anti-hCD20 sdAbs 9077 and 9079, and ctrl sdAb were reconstituted in sodium carbonate buffer (0.05 mol/L, pH 8.5) and conjugated with p-SCN-Bn-NOTA for 68Ga-labeling and with CHX-A-DTPA for 177Lu-labeling. Briefly, a 10-fold molar excess of chelator was incubated with different sdAbs for 3 hours at room temperature. For rituximab, a 100-fold molar excess of CHX-A-DTPA was used. SdAb conjugates were purified via SEC on Superdex Peptide 10/300 (GE Healthcare) and DTPA-rituximab on Superdex 75 10/30 column (GE Healthcare).
both in ammonium acetate (0.1 mol/L, pH 7.0). The mean degree of DTPA/NOTA conjugation per sdAb was determined by electrospay ionization quadrupole time-of-flight mass spectrometry (ESI-Q-ToF-MS) (Waters).

$^{68}$Ga was eluted from a $^{68}$Ge/$^{68}$Ga generator (Galli Eo, IRE ELIT) in 0.1 mol/L HCl (400–700 MBq) and incubated with NOTA-sdAbs (50–100 μg) for 10 minutes at room temperature. Carrier-free $^{177}$LuCl$_3$ was obtained from ITG (Garching) with a specific activity of 3,000 GBq/mg. The desired activity of $^{177}$Lu (37–350 MBq) was added to a test vial containing ammonium acetate (0.2 mol/L, pH 5.0) and incubated with the DTPA conjugates (50–100 μg) for 30 minutes at room temperature.

Next, the mixtures were purified via NAP-5 SEC for $^{177}$Lu and PD-10 SEC for $^{68}$Ga (GE Healthcare), and filtered through a 0.22-μm filter (Millex, Millipore). Radiochemical purity was evaluated using ITLC and radio-SEC using Superdex 75 5/15 column (GE Healthcare), with 0.01 mol/L PBS/0.3 mol/L NaCl solution used as mobile phase.

**In vitro characterization of $^{177}$Lu-DTPA-anti-hCD20 sdAbs**

Binding specificity, affinity, and degree of internalization of $^{177}$Lu-sdAbs was evaluated on hCD20pos B16 cells. A total of 1.25 $\times$ 10$^4$ cells were seeded overnight in 24-well plates to assess specificity to hCD20. Plates were placed at 4°C and incubated with 20 nmol/L $^{177}$Lu-DTPA-sdAb for 2 hours at 4°C, with and without a 100-fold molar excess of cold rituximab. In parallel, cells were incubated with $^{177}$Lu-DTPA-ctrl sdAb. After washing, cells were lysed with 1 mol/L NaOH, and radioactivity was measured by incubating cells with a serial dilution of $^{177}$Lu-DTPA-sdAbs (0.1–250 nmol/L), with and without a 100-fold molar excess of cold rituximab, and further processed as described above.

A total of 1.25 $\times$ 10$^4$ cells were adhered overnight in 6-well plates to measure the degree of internalization of $^{177}$Lu-DTPA-sdAbs in hCD20pos B16 cells. Cells were incubated with 20 nmol/L of $^{177}$Lu-DTPA-sdAb during 2 hours at 4°C, with or without a 100-fold molar excess of cold rituximab, after which the supernatant was removed and cells were washed twice before incubation during 1, 2, 4, 6, and 24 hours at 37°C. Next, supernatant was collected and cells were washed twice to obtain the dissociated fraction. To obtain the membrane-bound fraction, cells were incubated with 0.05 mol/L Glycine (pH 2.8) for 5 minutes at 4°C. Again, the cells were washed twice after which the cells were lysed and collected as the internalized fraction. All fractions were processed as mentioned above.

Long-term stability in serum was evaluated by incubating $^{177}$Lu-sdAbs with human serum at 37°C up to 144 hours, and analyzed via radio-SEC.

**Animal models**

For biodistribution experiments, female C57BL6 and CB17 SCID mice were inoculated subcutaneously in the right hind limb with 5 $\times$ 10$^5$ hCD20pos B16 or 10 $\times$ 10$^5$ Daudi cells, respectively, under 2.5% isoflurane anesthesia (Abbott). Tumors reached a maximal size of 250 ± 100 mm$^3$. Prior to imaging, mice were anesthetized with a mixture of 18.75 mg/kg ketamine hydrochloride (Ketamine 1000 Ceva, Ceva) and 0.5 mg/kg medetomidine hydrochloride (Domitor, Pfizer).

Female C57BL6 mice were subcutaneously inoculated with 3 $\times$ 10$^5$ hCD20pos B16 cells and grown until 13.2 ± 1.3 mm$^3$ for targeted therapy. All animal study protocols were approved by the ethical committee of the Vrije Universiteit Brussel (Brussels, Belgium).

**In vivo biodistribution of radiolabeled anti-hCD20 sdAbs**

Mice bearing subcutaneous hCD20pos B16 and Daudi tumors were injected intravenously with $^{99m}$Tc-sdAbs (17.0–70.0 MBq; $n = 3$). One hour postinjection (p.i.), SPECT/micro-CT imaging was performed, image quantification was performed using AMIDE software, and data were expressed as % of injected activity (% I/A) per cm$^3$ (29). Maximum intensity projections (MIP) were generated using OsirIX Lite software.

In addition, mice bearing hCD20pos B16 tumors were injected intravenously with either $^{177}$Lu-DTPA-sdAbs (2.1–10.3 MBq, $n = 3$) or $^{68}$Ga-NOTA-sdAbs (8.0–13.0 MBq, $n = 3$). One hour p.i., micro-PET/and -SPECT/CT (Millabs VECTOR/CT) was performed in mice injected with $^{68}$Ga-NOTA-sdAbs and $^{177}$Lu-DTPA-sdAbs, respectively. The CT-scan was set to 55 kV and 615 μA, resolution of 80 μm. The total body scan was 108 seconds. PET images were obtained using the high-energy PET_COLLIMMator in spiral mode, 85 positions for whole-body imaging, with 10 seconds per position. Images were reconstructed with 0.6-mm voxels with 4 subsets and 2 iterations, without postreconstruction filter. SPECT-images were obtained using a rat SPECT_COLLIMMator (1.5-mm pinholes) in spiral mode, 20 positions for whole-body imaging, with 90 seconds per position. Images were reconstructed with 0.4 mm voxels with 2 subsets and 7 iterations, without postreconstruction filter.

After imaging, mice were sacrificed, followed by the isolation of different organs, tissues, and tumors. The radioactivity present in the different samples was measured against a standard of known radioactivity using a $\gamma$-counter and expressed as % IA per gram (%IA/g), corrected for decay.

In parallel, the biodistribution of $^{177}$Lu-DTPA-sdAb 9079 was compared with that of $^{177}$Lu-DTPA-rituximab in mice bearing hCD20pos B16 tumors. Here, animals were injected intravenously with either $^{177}$Lu-DTPA-sdAb 9079 + 150 mg/kg gelofusin, or $^{177}$Lu-DTPA-rituximab (2.9–3.5 MBq; $n = 3$). Mice were sacrificed at different time points after injection and further processed as above.

**Targeted radionuclide therapy**

Data obtained from the comparative ex vivo biodistribution study were time integrated for the dosimetry calculation of $^{177}$Lu-DTPA-sdAb 9079 and $^{177}$Lu-DTPA-rituximab (24, 30). Briefly, the integrations between time 0 and 72 hours for $^{177}$Lu-DTPA-sdAb 9079 and between 0 and 120 hours for $^{177}$Lu-DTPA-rituximab were made using the trapezoid method. In the absorbed dose calculations, S values were obtained from RADAR phantoms (Unit Density Spheres), with S value for a 1 g sphere (0.0233 mCy/MBq) used to calculate all organ doses.

For targeted radionuclide therapy, mice with palpable hCD20pos B16 tumors were randomly categorized into 5 groups ($n = 8$). Mice in four groups received 4 intravenous injections, once every two days, of (i) $^{177}$Lu-DTPA-sdAb 9079 + 150 mg/kg gelofusin (a total cumulative radioactive dose of 144.0 ± 1.8 MBq), or (ii) $^{177}$Lu-DTPA-ctrl sdAb + 150 mg/kg gelofusin.
(a total cumulative radioactive dose of 135 ± 2.74 MBq), of (iii) 200 µg/injection of unlabeled rituximab or (iv) PBS. Another group (v) received a single injection of 7.0 ± 1.48 MBq 177Lu-DTPA-rituximab. Animal weight and tumor volume (caliper measurements) were recorded daily. Endpoint criteria were defined as >20% loss of the initial body weight, a tumor volume exceeding 1,000 mm³, the presence of necrotic tumors, or limb lameness.

To assess potential renal toxicity of 177Lu-DTPA-sdAb 9079, two groups of three healthy mice were injected intravenously with 144 ± 2.1 MBq 177Lu-DTPA-sdAb 9079 + 150 mg/kg gelofusin or PBS. Renal function was assessed 3 and 7 months after treatment by quantification of renal uptake of 99mTc-dimercaptosuccinic acid ([99mTc-DMSA; UIZ, Brussel]). Mice were injected with 7.6 ± 2.9 MBq 99mTc-DMSA, 1 hour post-injection, SPECT/micro-CT imaging and quantification was performed as described above.

Statistical analyses
Statistical analyses were conducted using the two-tailed t test or one-way ANOVA with Bonferroni multiple comparison tests for analyzing two groups or more than three groups with each other, respectively. The event-free survival between groups was analyzed using the log-rank test. The statistical difference in the figures is indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results
Generation and in vitro characterization of anti-hCD20 sdAbs
Following llama intradermal immunization with a hCD20-encoding plasmid and hCD20⁺ cells, sdAbs were selected via creation of an immune library and subsequent biopannings after sdAb phage-display on hCD20⁺ cells.

The ability of the anti-hCD20 sdAbs to recognize the hCD20-receptor was investigated using flow cytometry. Fourteen of 15 generated sdAbs bound to hCD20⁺ Daudi cells, while no binding was observed on hCD20⁻ Reh cells. MFI signal was reduced to background when Daudi cells were preincubated with a 100-fold molar excess of rituximab, confirming specific binding of all 14 anti-hCD20 sdAbs and their competing character with rituximab (Fig. 1A and B).

Figure 1.
Recognition of hCD20 by purified anti-hCD20 sdAbs and affinity determination via flow cytometry. A, SdAb 9077 and 9079 bind to hCD20⁺ Daudi cells (red) but not after blocking with 100-fold molar excess of rituximab (blue), and not to hCD20⁻ Reh cells (black). B, MFI signal of anti-hCD20 sdAbs on Daudi and Reh cells, and on Daudi cells blocked with 100-fold molar excess of rituximab. Daudi cells incubated with FITC-labeled anti-His mAb were used to measure the background signal. C, The concentration–response curve for sdAbs 9077 and 9079 for six different dilutions. D, The calculated Kᵦ of all anti-hCD20 sdAbs, expressed in mol/L.
The binding affinities were estimated by incubating different concentrations of sdAbs with Daudi cells, and analyzed using flow cytometry. The resulting concentration–response curves for sdAb 9077 and 9079 are shown in Fig. 1C. Calculated $K_d$ values of all 14 anti-hCD20 sdAbs are summarized in Fig. 1D.

Biodistribution of $^{99m}$Tc-sdAbs
All purified anti-hCD20 sdAbs and ctrl sdAb were successfully labeled with $^{99m}$Tc, resulting in a radiochemical purity of $>96\%$. Biodistribution and tumor targeting of $^{99m}$Tc-sdAbs was assessed via SPECT/micro-CT (1 hour p.i.) and necropsy studies (1.5 hours p.i.) in mice bearing Daudi or hCD20 B16 tumors. Quantiﬁed uptake values of $^{99m}$Tc-sdAbs are summarized in Supplementary Table S1. Results from necropsy studies are summarized in Supplementary Tables S2 and S3.

As illustrated in Fig. 2A, all 14 $^{99m}$Tc-anti-hCD20 sdAbs showed high levels of radioactivity in kidneys and bladder. Notably, all anti-hCD20 sdAbs showed higher tumor targeting in both mouse models (tumor uptake of $1.2\% \pm 0.6\%$ to $3.5\% \pm 0.1\%$ IA/g, and $1.2\% \pm 0.4\%$ to $3.7\% \pm 1.3\%$ IA/g in Daudi and hCD20 B16 tumor mouse model, respectively) compared with $^{99m}$Tc-ctrl sdAb (lower than $0.5\%$ IA/g in both tumor models) while uptake in nontarget tissues was low (Supplementary Tables S2 and S3). However, in both tumor models, $^{99m}$Tc-sdAb 9077 and 9079 showed a more pronounced tumor uptake with a low background signal compared with the others (Fig. 2B–D; Supplementary Tables S2 and S3). Interestingly, $^{99m}$Tc-sdAb 9079 showed a lower kidney accumulation (kidney uptake of $113.4\% \pm 18.7\%$ to $132.8\% \pm 10.2\%$ IA/g in Daudi and hCD20 B16 tumor mouse model, respectively) compared with other $^{99m}$Tc-sdAbs (kidney accumulation of $167.4\% \pm 4.1\%$ to $329.3\% \pm 48.5\%$ and $216.9\% \pm 14.6\%$ to $368.9\% \pm 23.1\%$ IA/g in Daudi and hCD20 B16 tumor mouse model, respectively; Supplementary Tables S2 and S3). The ex vivo kidney uptake value of $^{99m}$Tc-sdAb 9079 was significantly lower than that for $^{99m}$Tc-sdAb 9077 in both tumor models (Fig. 2E).

Preparation of $^{68}$Ga-NOTA- and $^{177}$Lu-DTPA conjugates
Anti-hCD20 sdAb 9077 and 9079 were selected for further characterization. $^{68}$Ga-NOTA-sdAbs were developed to assess their potential as an imaging tool. In parallel, their $^{177}$Lu-labeled counterparts were developed to evaluate their potential to treat NHL.
Untagged anti-hCD20 sdAb 9077 and 9079 and ctrl sdAb were conjugated with p-SCN-Bn-NOTA (for 68Ga) and with CHX-A"-DTPA (for 177Lu) on ε-amino groups of their lysines. ESI-Q-ToF-MS analysis revealed a mean degree of NOTA/DTPA conjugation per sdAb of 1. After 68Ga/177Lu labeling, iTLC measured a radiochemical purity of >96%, confirmed by radio-SEC. 177Lu-DTPA-rituximab showed a radiochemical purity of >95%, measured by iTLC and radio-SEC.

In vitro characterization of 177Lu-DTPA-anti-hCD20 sdAbs

The specificity of binding was measured on hCD20pos B16 cells. 177Lu-sdAbs 9077 and 9079 were incubated at 20 nmol/L with cells for 2 hours at 4°C. Binding of 177Lu-DTPA-anti-hCD20 sdAbs was measured with(out) a 100-fold molar excess of rituximab, and compared with binding of nontargeting 177Lu-DTPA-ctrl sdAb (Fig. 3A). The affinities of 177Lu-DTPA-sdAbs toward the hCD20-receptor were calculated by incubating serial dilutions with hCD20pos B16 cells 2 hours at 4°C, as presented in Supplementary Fig. S2A. Kd values were 22.7 ± 2.7 nmol/L and 28.5 ± 2.2 nmol/L for 177Lu-DTPA-sdAb 9077 and 9079, respectively.

The degree of internalization was assessed by incubating cells with 20 nmol/L 177Lu-DTPA-sdAb 9077 and 9079. After 1 hour, 40.4% ± 3.0% and 21.0% ± 1.8% of the initial bound activity was internalized for 177Lu-DTPA-sdAb 9077 and 9079, respectively. After 24 hours, the internalized fractions decreased to 17.7% ± 1.2% and 16.6% ± 0.9% for 177Lu-DTPA-sdAb 9077 and 9079, respectively.

Biodistribution of 177Lu-DTPA- and 68Ga-NOTA-anti-hCD20 sdAbs

Micro-SPECT/CT images of mice bearing hCD20pos B16 tumors showed specific tumor targeting for both 177Lu-anti-hCD20 sdAbs 1 hour p.i., with a low background signal, except kidneys and bladder (Fig. 3C). The ex vivo biodistribution data obtained after 15 hours p.i. (Fig. 3D) revealed uptake values in tumor of 3.7% ± 0.9% IA/g and 3.4% ± 1.3% IA/g for 177Lu-DTPA-sdAb 9077 and 9079, respectively. Nontarget 177Lu-DTPA-ctrl sdAb noted a tumor uptake of 0.64% ± 0.09% IA/g, which is significantly lower than both anti-hCD20 sdAbs. The uptake values in the additional organs and tissues were below 0.5% IA/g, except in kidneys. An important difference in kidney uptake was observed between 177Lu-DTPA-sdAb 9079 (8.6% ± 1.1% IA/g) and 9077 (20.7% ± 1.8% IA/g; Fig. 3D).

Biodistribution and tumor targeting of 68Ga-NOTA-anti-hCD20 sdAbs was assessed in parallel. Here, micro-PET/CT images...
revealed tumor targeting of both $^{68}$Ga-NOTA-anti-hCD20 sdAbs with low background signal except kidneys and bladder (Fig. 4A). $^{68}$Ga-NOTA-ctrl sdAb showed no specific tumor accumulation, again confirming their specificity (Fig. 4A). The ex vivo results (Supplementary Table S5) revealed tumor uptake values of 1.7% ± 0.06% IA/g and 2.2% ± 0.2% IA/g for $^{68}$Ga-NOTA-sdAb 9077 and 9079, respectively. $^{68}$Ga-NOTA-ctrl sdAb noted a tumor uptake of 0.2% ± 0.01% IA/g, significantly lower than $^{68}$Ga-NOTA-anti-hCD20 sdAbs (Fig. 4B). Again, a significantly lower kidney accumulation was observed for $^{68}$Ga-NOTA-sdAb 9079 (15.7 ± 0.3% IA/g) compared with $^{68}$Ga-NOTA-sdAb 9077 (75.4% ± 3.8% IA/g).

Comparative biodistribution of $^{177}$Lu-DTPA-sdAb 9079 and $^{177}$Lu-DTPA-Rituximab

The biodistribution data are presented in Table 1. $^{177}$Lu-DTPA-sdAb 9079 showed the highest tumor uptake early after injection, decreasing from 3.4% ± 1.3% IA/g at 1.5 hours to 0.9% ± 0.1%
IA/g at 24 hours and to 0.3% ± 0.04% IA/g after 72 hours p.i. Kidney accumulation was also the highest at early time points and decreased from 8.6% ± 1.0% IA/g at 1.5 hours p.i. to 1.5% ± 0.5% IA/g at 24 hours p.i. and to 0.2% ± 0.05% IA/g after 72 hours p.i. Tracer accumulation in the other nontarget organs and tissues was below 0.5% IA/g at 1.5 hours p.i. and further decreased over time. In contrast, 177Lu-DTPA-rituximab showed lowest tumor uptake at early time points, and increased from 10.6% ± 1.9% IA/g at 1.5 hours p.i. to 27.4% ± 5.5% IA/g at 120 hours p.i. Radioactivity in blood was higher than in tumor at all time-points, with 98.1% ± 13.3% IA/g at 1.5 hours p.i. and 35.1% ± 5.2% IA/g at 120 hours p.i. Moreover, the radioactivity present in the other nontarget organs and tissues was much higher for 177Lu-DTPA-rituximab compared with 177Lu-DTPA-sdAb 9079.

Therapeutic efficacy of 177Lu-DTPA-sdAb 9079

Organ-absorbed doses from the therapeutic doses of 144.0 MBq of 177Lu-DTPA-sdAb 9079 and 7.0 MBq 177Lu-DTPA-rituximab are depicted in Fig. 5A. The absorbed dose to tumor was 7.4 Gy for 177Lu-DTPA-sdAb 9079, while kidneys received a dose of 16.1 Gy. Absorbed doses to other healthy organs and tissues were low. 7.0 MBq of 177Lu-DTPA-Rituximab led to an absorbed dose of 15.0 Gy to tumor, but in parallel to doses of 26.6, 29.0, and 11.1 Gy to blood, bone, and spleen, respectively. Absorbed doses to additional organs and tissues were also much higher for 177Lu-DTPA-rituximab compared with 177Lu-DTPA-sdAb 9079. To assess therapeutic efficacy of 177Lu-DTPA-sdAb 9079, mice bearing hCD20<sup>pos</sup> B16 tumors were treated with 177Lu-DTPA-sdAb 9079, 177Lu-DTPA-ctrl sdAb, 177Lu-DTPA-rituximab, cold rituximab, or PBS. Tumor volumes were quantified using caliper measurements, as a function of time (Fig. 5B). The resulting Kaplan–Meier survival curves are presented in Fig. 5C. A significant difference in median survival was observed between mice treated with 177Lu-DTPA-sdAb 9079 and PBS (P = 0.0016) or 177Lu-DTPA-ctrl sdAb (P = 0.0204). No significant difference in median survival was observed between mice treated with 177Lu-DTPA-sdAb 9079, 177Lu-DTPA-rituximab, or cold rituximab.

Three and seven months after therapy, renal function was evaluated by measuring renal uptake of 99mTc-DMSA. No significant difference in renal uptake of 99mTc-DMSA was observed between mice treated with PBS (18.0% ± 3.7% and 14.9% ± 1.3% IA, 3 and 7 months, respectively) and 177Lu-DTPA-sdAb 9079 (19.5% ± 2.2% and 14.4% ± 2.9% IA, 3 and 7 months, respectively; Fig. 5D).

Figure 5.
Therapeutic efficacy of 177Lu-DTPA-sdAb 9079 in hCD20<sup>pos</sup> B16 tumor-bearing mice. A, Dosimetry calculations after a therapeutic injection of 177Lu-DTPA-rituximab or 177Lu-DTPA-sdAb 9079 coinfused with 150 mg/kg gelofusin. B, To assess therapeutic efficacy of 177Lu-DTPA-sdAb 9079, mice were inoculated with 3 × 10<sup>5</sup> hCD20<sup>pos</sup> B16 cells (day 0). Mice bearing palpable hCD20<sup>pos</sup> tumors (day 8) were divided into five groups. Four groups of mice received four i.v. injections of 177Lu-DTPA-sdAb 9079 (a total cumulative radioactive dose of 144 ± 1.8 MBq), 177Lu-DTPA-ctrl sdAb (135 ± 2.74 MBq), rituximab (200 μg/injection), or PBS (200 μL/injection) on day 8, 10, 12, and 14. One group received one i.v. injection of 177Lu-DTPA-rituximab (7.0 ± 1.48 MBq) on day 8. Tumor volumes were measured daily via caliper measurements. C, Resulting Kaplan–Meier survival curves. A significant difference in median survival was observed between mice treated with 177Lu-DTPA-sdAb 9079 and PBS (P = 0.0016) or 177Lu-DTPA-ctrl sdAb (P = 0.0204). No significant difference in renal uptake of 99mTc-DMSA was observed between mice treated with PBS and 177Lu-DTPA-sdAb 9079. Mice treated with PBS and 177Lu-DTPA-ctrl sdAb showed no significant difference in median survival. D, No significant difference in renal uptake of 99mTc-DMSA was observed between mice treated with PBS and 177Lu-DTPA-sdAb 9079.
Discussion

In this study, we describe the generation, production, and characterization of a panel of anti-hCD20 sdAbs. Fourteen sdAbs were found to bind hCD20pos cells in vitro with a nanomolar range affinity (Kₐ range of 4.3–34.0 nmol/L). On the basis of flow cytometry, a preincubation of Daudi cells with a 100-fold molar excess rituximab resulted in a strong decrease of MFI signal for all 14 sdAbs, suggesting the competition with rituximab for hCD20 receptor binding. The selection of a rituximab-competing anti-hCD20 sdAb has the advantage that rituximab could be used in blocking strategies. Patients treated with Zevalin first receive relatively high doses of rituximab prior to Zevalin administration. In this way, rituximab blocks the CD20 receptor on the normal mid-stage B cells, resulting in decreased radiation of secondary lymphoid organs and improved tumor targeting of Zevalin.

To select a lead sdAb after in vitro characterization, the biodistribution of 99mTc-radiolabeled sdAbs was evaluated in two hCD20pos tumor mouse models. When we analyzed the tumor targeting and the nonspecific accumulation of all 99mTc-sdAbs, sdAbs 9077 and 9079 showed a more favorable biodistribution. Notably, a remarkable and significantly lower kidney uptake was measured for 99mTc-sdAb 9079 compared with other 99mTc-sdAbs. The high kidney retention of sdAbs and other small hydrophilic proteins is a well-described phenomenon. Indeed, due to their small size, sdAbs are rapidly cleared from blood through kidneys, followed by nonspecific reabsorption in negatively charged lumen of proximal tubuli [31–33]. In case of TRNT, the retained therapeutic radioactivity can lead to nephrotoxicity. One of the most applied strategies to reduce kidney uptake is the coinjection of positively charged amino acids (34, 35). In addition, the introduction of carboxyterminal (C-)tags to sdAbs dramatically increase kidney retention upon renal clearance (24). To this end, sdAbs 9077 and 9079 were produced without a C-tag for further characterization.

In vitro characterization of 177Lu-DTPA-sdAb 9077 and 9079 showed specific targeting of hCD20pos B16 cells with affinities of 22.7 ± 2.7 and 28.5 ± 2.2 nmol/L for 177Lu-DTPA-sdAb 9077 and 9079, respectively. In addition, we also analyzed the internalization rate of 177Lu-DTPA-sdAbs. Internalization of residualizing radiometals traps these radioisotopes intracellularly, potentially leading to increased retention of therapeutic radiation in tumor (30). CD20 internalization is highly variable and depends on the targeted cell type, the presence of coreceptors, the nature of the ligand and the targeted epitope (36). It is remarkable that although 177Lu-DTPA-sdAb 9077 internalized better than 9079 in vitro, this was not translated into a better tumor uptake in vivo. Although speculative, in vitro static internalization experiments may not reflect the in vivo situation that is characterized by a fast cell dissociation and washout from the tumor, and sdAb internalization may be less prominent in this tumor type.

In vivo, both sdAb 9077 and 9079, radiolabeled with 177Lu and 68Ga, showed specific tumor targeting with comparable low background signal, except in kidneys. Despite the highly similar amino-acid sequence of all generated anti-hCD20 sdAbs, sdAb 9079 consistently showed lower kidney accumulation compared with other sdAbs. The accessibility of cationic sites may play an important role in the reduced renal uptake of sdAb 9079 compared with other sdAbs (34, 35). Indeed, after analysis the sequences of all anti-hCD20 sdAbs, sdAb 9079 showed a lower number of positively charged residues compared with other anti-hCD20 sdAbs. The theoretical pl of his-tagged sdAb 9079 was lower (7.18) than the pl of sdAb 9077 (8.66), which could explain the lower degree of interaction of sdAb 9079 with the negatively charged lumen of the proximal tubuli in the kidneys compared with the other sdAbs. In addition, after removal of the C-tag and with a coincjection of 150 mg/kg gelofusin, its accumulation in kidneys was further decreased, in agreement with our previous work (24).

On the basis of this, sdAb 9079 was selected as a lead and its biodistribution was compared with that of 177Lu-DTPA-rituximab. 177Lu-DTPA-sdAb 9079 showed the highest tumor uptake already after 1.5 hours, with very low uptake in all nontarget organs and tissues. Tumor uptake of 177Lu-DTPA-rituximab was higher than the 177Lu-DTPA-sdAb 9079 at all time points, with the highest uptake after 120 hours. However, extremely high amounts of radioactivity were measured in blood and well-perfused organs. These values obtained with 177Lu-DTPA-rituximab confirm the results previously described by Kameswaren and colleagues (37).

Different anti-CD20 mAb–derived fragments have already been investigated as PET-agents for imaging of CD20pos lymphomas. Olafsen and coworkers generated two recombinant rituximab-fragments, a scFv-C₃ minibody (80 kDa) and a scFv-Fc fragment (105 kDa). Radiolabeled with 124I, biodistribution of the fragments was analyzed in mice bearing hCD20pos lymphomas. Both fragments revealed rather high accumulation of radioactivity in nontarget tissues after 4 hours. After 21 hours, T/B ratios of 3.3 and 4.8 were observed for the minibody and scFv-Fc fragment, respectively (38). More recently, the same group evaluated two additional rituximab-derived fragments, a scFv–8-dimer (anti-CD20 Db) and a cysteine-diabody (Cys-Db), again labeled with 124I. Necropsy studies revealed an average tumor uptake of 3.9 ± 1.1% and 2.2% ± 0.6% IA/g for Db and Cys-Db, respectively, after 8 hours p.i. The radioactivity present in blood was again high, with T/B ratios less than 1.3 for both fragments (39). Mendler and colleagues characterized a Fab fragment derived from ofatumumab (2F2, Arzerra), a humanized mAb approved for the treatment of patients with chronic lymphocytic leukemia. Labeled with 124I, pool blood activity was higher (1.4 ± 0.4% IA/g) than the tumor uptake (1.3 ± 0.1% IA/g) after 6 hours. After 24 hours, the calculated T/B ratio increased to 4, with a tumor uptake of 0.2% IA/g (40).

Despite the fact that other radioisotopes and other tumor models were used in these studies, we can conclude that our lead sdAb 9079 shows an improved biodistribution. Indeed, a relatively high tumor uptake with very low background uptake, except kidneys, was observed with 99mTc-9079 as early as 1.5 hours p.i. in two different tumor models (T/B and T/M ratios of 4.5 ± 2.3 and 21.1 ± 14.0, respectively, in Daudi tumor model and 6.1 ± 2.3 and 16.1 ± 10.9 in hCD20pos B16 tumor model). In addition, in vivo biodistribution of 68Ga-NOTA-sdAb 9079 showed T/B and T/M ratios of 3.7 ± 0.7 and 7.2 ± 0.9, respectively, after 1.5 hours i.v. Furthermore, 177Lu-DTPA-sdAb 9079 showed T/B and T/M ratios of 13.3 ± 4.6 and 32.9 ± 15.6, respectively, at 1.5 hours p.i.

In addition to anti-CD20 mAb–derived fragments, Natarajan and colleagues (41) described the generation of a small anti-CD20 protein, based on the human fibronectin type 3 domain (FN3-CΔ30). After labeling with 64Cu via DOTA, they evaluated it as a PET radiotracer for B-cell NHL. In a similar animal model as used in our study, tumor uptake was high, 16.8% ± 1.6% ID/g.
after 4 hours, decreasing to about 6% after 24 hours, with a T/B ratio of 4.1 (41). Even though a higher background signal was observed early after administration, $^{64}$Cu-FN3$_{C2D20}$ measured higher tumor uptake compared with our lead sdAb, resulting in a high tumor-to-background tissue ratio. High liver uptake was explained by potential dissociation of $^{64}$Cu-DOTA complex or charge effects of the engineered protein. The authors also tested their tracer in nontumor-bearing hCD20-transgenic mice. These mice more closely mirror the human situation, that is, with normal hCD20pos B cells residing in the spleen. PET/CT images showed a high speciﬁcity of 18F-FDG PET-tracer can be as low as 50% (47, 48) Furthermore, $^{68}$Ga-NOTA-sdAb 9079 might be used to monitor the response to sdAb-based TRNT or other human lymphoma therapies, or in autoimmune diseases in which autoimmune hCD20pos B cells contribute to pathogenesis.

In conclusion, in this study, we show the successful generation and characterization of different anti-hCD20 sdAbs, derived from llama heavy-chain antibodies. After an extensive in vitro and in vivo evaluation, a lead anti-hCD20 sdAb was selected and was successfully applied as a novel CD20-targeted theranostic radiotracer in preclinical mouse tumor models.

Disclosure of Potential Conﬂicts of Interest
No potential conﬂicts of interest were disclosed.

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Theranostic Radiolabeled Anti-CD20 sdAb for Targeted Radionuclide Therapy of Non-Hodgkin Lymphoma

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