A Novel Engineered Anti-CD20 Tracer Enables Early Time PET Imaging in a Humanized Transgenic Mouse Model of B-cell Non-Hodgkins Lymphoma

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Introduction

The CD20 antigen is present in greater than 90% of B-cell lymphomas and is neither degraded nor internalized after antibody binding, making it an effective target for immunotherapeutic removal of malignant B cells (1–3). In the plasma membrane, CD20 is predicted to contain 2 extracellular loops, a larger one between the third and fourth transmembrane regions, and a much smaller one between the first and second transmembrane regions (4, 5). Immunotherapies using monoclonal antibodies targeting B-cell surface antigens have been widely accepted for the treatment of B-cell non-Hodgkin’s lymphomas (NHL), with anti-CD20 antibodies being most commonly used (1). Rituximab, a chimeric anti-CD20 antibody, has provided the best clinical results to date with single-agent remission induction rates of >60% in patients with indolent lymphomas and 30% to 35% in relapsed aggressive lymphomas (2–4). However, for imaging, radiolabeled antibodies provide modestly high liver uptake (2–11% ID), and slow clearance yields low tumor-to-blood ratios (<0.5; refs. 6, 7) and therefore necessitates late imaging time points (days).

Compared with antibodies, lower molecular weight protein scaffolds can provide faster clearance and demonstrate specific in vivo targeting ability to yield excellent tumor-to-background contrast (8). Validated scaffolds include, for example, affibodies (8–10), knottins (11, 12), nanobodies (13, 14), peptides (15), antibody fragments (16–19), and the 10th type III domain of human fibronectin (FN3; ref. 20). FN3 is a 10 kDa β-sandwich that has been engineered for picomolar to nanomolar affinity binding to many targets (20, 21) and has been validated for molecular imaging in murine xenograft tumor models (22). The size of FN3 (8% of antibody size) balances rapid clearance from blood and background tissues while having enough surface area for in vivo targeting specificity. Its small size aids vascular extravasation (23) and tissue penetration to improve solid tumor delivery (24, 25). FN3 structure

Purpose: The aim of this article was to evaluate the use of a novel engineered anti-CD20 protein based on the 10 kDa human fibronectin type 3 domain (FN3) and subsequently compare with 64Cu-rituximab for positron emission tomography (PET) imaging of CD20.

Experimental Design: The engineered FN3CD20 and FN3WT were produced in Escherichia coli cells at 2 to 5 mg/L, conjugated to DOTA, labeled with 64Cu, and used for PET imaging of huCD20 expression in B cells. Humanized transgenic mice and subcutaneously xenografted mice each received intravenous 64Cu-FN3CD20 or FN3WT (3.7 MBq/4 μg Do-FN3 in 200 μL PBS). Control group received a blocking dose (50-fold excess) of unconjugated FN3CD20 two hours before radiotracer injection. PET imaging was carried out at 1 to 24 hours postinjections.

Results: In vitro assay demonstrated FN3 binds CD20 with 20 nmol/L affinity on CD20-expressing cells. 64Cu-FN3CD20 showed clear, high-contrast visualization of huCD20-expressing B cells in the spleen of transgenic mice as early as 1 hour postinjection [38 ± 3% injected dose (ID)/g] and exhibited a spleen-to-blood ratio of 13 by 4 hours. This is higher uptake (P = 0.04) and 10-fold greater signal-to-background (P = 0.04) than the 64Cu-rituximab antibody radiotracer. Tumor uptake (16.8 ± 1.6 vs. 5.6 ± 1.4%ID/g) and tumor-background ratios were superior for FN3CD20 relative to rituximab in xenograft studies as well.

Conclusions: The 64Cu-Du-FN3CD20 radiotracer represents a novel small, high-affinity binder for imaging human CD20, which may be well suited for B-cell non-Hodgkin’s lymphoma imaging in patients at early time points. Clin Cancer Res; 19(24): 6820–9. ©2013 AACR.
Translational Relevance

In this article, we assessed the translational aspects of the novel 10 kDa anti-CD20 fibronectin type 3 domain (FN3) binder using positron emission tomography (PET) imaging. $^{64}$Cu-FN3CD20 PET imaging was evaluated in a huCD20 transgenic mouse model aimed to assess translation in human lymphoma patients. The results of this study are strongly encouraging for clinical use; for example, at 1 hour postinjection $^{64}$Cu-FN3CD20 spleen uptake was 38 ± 3% injected dose per gram and exhibited a spleen-to-blood ratio of 13 by 4 hours, which is higher uptake ($P = 0.04$) and 10-fold greater signal-to-background ($P = 0.04$) than the $^{64}$Cu-rituximab antibody radiotracer. Thus, $^{64}$Cu-FN3CD20 tracer could be used for early imaging for effective patient care. Furthermore, this study highlights the preclinical understanding of $^{64}$Cu-FN3CD20 PET imaging and provides insights into the strengths and weaknesses of the tracer for possible B-cell non-Hodgkin lymphomas outpatient PET imaging.

contains 3 solvent-exposed loops that can be mutated to introduce new high-affinity binding activity (20, 26, 27). High stability and a single-lysine distant from the paratope loops, and reintroduced into yeast by electroportation with homologous recombination. As binder enrichment progressed in later evolutionary cycles, fluorescence-activated cell sorting for binding to soluble CD20 peptide was also used. Six cycles of selection and mutation were performed. Plasmid DNA was recovered, transformed into bacteria, and individual clones were sequenced by standard DNA sequencing methods.

Preparation of FN3CD20

Bacterial expression plasmids were constructed to express either the 101-amino acid FN3CD20 or a nonbinding control (FN3WT), which is the human wild-type sequence except the arginine–glycine–aspartic acid sequence was mutated to arginine–aspartic acid–glycine. The plasmids also encode a C-terminal His$_8$ epitope tag for purification. Plasmids were transferred into BL21 (DE3) Escherichia coli. Cells were grown in 1 L of lysogeny broth medium and induced with 0.5 mmol/L isopropyl β-D-1-thiogalactopyranoside for 1 hour. Cells were pelleted, resuspended in 10 mL of lysis buffer (50 mmol/L sodium phosphate, pH 8.0, 500 mmol/L sodium chloride, 5% glycerol, 5 mmol/L CHAPS detergent, 25 mmol/L imidazole, and complete ethylenediaminetetraacetic acid–free protease inhibitor cocktail), frozen and thawed, and sonicated. The sample was centrifuged at 12,000 × g for 10 minutes. Fibronectin was purified from the soluble fraction by immobilized metal affinity chromatography and reversed-phase HPLC with a C18 column. Protein mass was verified by mass spectrometry.

Determination of binding constants by surface plasmon resonance

All measurements were performed at 25°C on a BL100 instrument and streptavidin chip (GE Healthcare Biosciences). The streptavidin chip was first cleaned with 3

Materials and Methods

Reagents and radiochemicals

All reagents were obtained from Sigma-Aldrich unless otherwise stated. N-succinimidyl-DOTA (NHS-DOTA) was purchased from Macronyclics. The CD20 positive B-cell lymphoma cell line Ramos and CD20-negative Jurkat cells were obtained from the American Type Culture Collection (ATCC numbers: CRL-1555 and TIB-152, authenticated by short tandem repeat profiling) and were used shortly thereafter. Ramos cells were maintained in Dulbecco’s Modified Eagle’s Medium (4.5 g/L glucose), Jurkat cells in MEM/Ham’s F-12 (1:1), and 1% nonessential amino acids. All media and additives were obtained from Life Technologies.

High-performance liquid chromatography (HPLC) was performed on HPLC-Ultimate 3000 with an ultraviolet detector and an online radioactivity detector. The system used a SEC 2000 LC column (300 × 7.8 mm) with 5 μm hydrophilic bonded silica support and 400 Å pore size (Phenomenex). Matrix-assisted laser desorption ionization mass spectrometry was performed with an AB SCIEX TOF/TOF 5800 operated in linear mode with sinapinic acid as matrix.

Engineering of FN3CD20

The human CD20 peptide extracellular loop was synthesized (amino acids: 165–185; GGYNCEPANPSSKPSST-QYCCS-biotin) and purified by RP-HPLC on a C-18 column and lyophilized as per the published procedure (8). This peptide was characterized by ESI-MS and immobilized on streptavidin magnetic beads for screening of FN3 binders. The yeast surface displayed FN3 G4 library with diversified loops was sorted and matured as described (26, 29). Briefly, yeast displaying 2.5 × 10^8 FN3 mutants were sorted for binding to magnetic beads with immobilized CD20 peptide, followed by fluorescence-activated cell sorting for full-length proteins using the C-terminal c-myc epitope. Plasmid DNA from selected clones was recovered, mutated by error-prone PCR of either the entire FN3 gene or the paratope loops, and reintroduced into yeast by electroportation with homologous recombination. As binder enrichment progressed in later evolutionary cycles, fluorescence-activated cell sorting for binding to soluble CD20 peptide was also used. Six cycles of selection and mutation were performed. Plasmid DNA was recovered, transformed into bacteria, and individual clones were sequenced by standard DNA sequencing methods.

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consecutive 1 minute injections of 40 μL of a solution of 1 mol/L NaCl in 50 mmol/L NaOH before the immobilization procedure. Five minutes after the cleaning process when the sensorgram reached a stable baseline, biotinylated CD20 peptide, diluted in running buffer to 20 μg/mL was injected for 7 minutes using a flow rate of 5 μL/min. The same procedure was performed for immobilization of nonspecific CD20 peptide, except that the nonspecific CD20 peptide was diluted in running buffer to 200 μg/mL. To attain maximum immobilization level of biotin peptides on the surface of streptavidin, multiple injections were performed.

Target antigen of CD20 peptide was immobilized on a streptavidin chip. FN3CD20 was analyzed in 10 mmol/L HEPES, pH 7.4. 150 mmol/L NaCl, 0.005% Tween 20 at 5 concentrations between 0 and 750 nmol/L and nonspecific FN3 was used as control. The flow rate was 15 μL/min; association and dissociation times were 1 and 2 minutes, respectively. Each concentration sample was assayed in duplicate and the response from an empty flow cell and from buffer injections was subtracted from each dataset. The data were analyzed using BIAeval software, and the affinity value were determined by minimizing the sum of squared errors assuming a 1:1 binding interaction. Experiments were performed in triplicate.

Preparation of Do-FN3

The DOTA-NHS ligand has already shown good biological performance when used in protein conjugation of various radionuclides such as 66Ga, 68Ga, 177Lu, 225Ac, and lead radionuclides (30). DOTA-FN3 (Do-FN3) tracer was prepared by conjugating DOTA-NHS to FN3 according to a published procedure (22). Briefly, lyophilized FN3 protein was resuspended in dimethylformamide with 2% triethylamine, and reacted at room temperature for 1 hour with 20 equivalents of DOTA-NHS. DOTA-FN3 was purified by HPLC and lyophilized for 64Cu labeling. The number of DOTA chelators conjugated to each FN3 molecule was calculated by mass spectrometry by comparing the mass of FN3 and Do-FN3 (31, 32).

Radiolabeling of Do-FN3 and Do-rituximab

The radiolabeling of Do-FN3 with 64CuCl₂ (University of Wisconsin, Madison, WI) was carried out as follows: Do-FN3, 25 to 50 μg in 100 μL of 0.25 mol/L ammonium acetate buffer (pH 5.5) was reacted with 92.5 to 185 MBq of neutralized 64CuCl₂ solution at 37°C of pH 5.5 for 1 hour. After incubation, 0.1 M diethylenetriaminepentaacetic acid, pH 7.0 was added to a final concentration of 5 mmol/L and incubated at room temperature for 15 minutes to scavenge unchelated 64CuCl₂ in the reaction mixture. Purification of the 64Cu-Do-FN3 was achieved using reversed-phase HPLC with a flow rate of 1.0 ml/min followed by rotary evaporation of solvent and dilution in PBS [0.1 mol/L NaCl, 0.05 mol/L sodium phosphate (pH 7.4)]. The final radioconjugate of 64Cu-Do-FN3 was filtered through a 0.2 μm filter and analyzed by flow cytometry. The minimum and maximum fluorescence and the affinity value were determined by minimizing the sum of squared errors assuming a 1:1 binding interaction. Experiments were performed in triplicate.
into a sterile vial. Preparation and purification of $^{64}$Cu-Do-rituximab was carried out according to the published procedure (31).

**Radiotracer cell binding assay**

For a cell culture radiolabeled binding assay, $1 \times 10^5$ cells were aliquoted in each tube washed with PBS and incubated with 25 nmol/L $^{64}$Cu-FN3$_{CD20}$ (10–20 MBq/nmol) for 30 minutes. Cells were washed thrice with PBS. Activity in each tube of cell pellet was quantified with a γ ray counter (1470 WIZARD Automatic Gamma Counter; Perkin-Elmer).

**Small animal PET imaging**

Animal studies were performed in compliance with approval from the Administrative Panel on Laboratory Animal Care at Stanford University. huCD20 transgenic mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, huCD20 transgenic mice were screened to confirm the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33). Before the imaging study, mice (Genentech, South San Francisco) were purchased for the experiments (31, 33).

**Xenograft model**

Six- to eight-week-old female Balb/c nude mice were obtained from Charles River Laboratories and maintained under pathogen-free conditions at Stanford University. Two days after whole body irradiation (400 rads), $2 \times 10^6$ Ramos cells were implanted subcutaneously on the left flank. Mice with similar tumors (400 mm$^3$) were chosen for the studies. Mice (3 per group) were administered the dose of $^{64}$Cu-Do-FN3 or $^{64}$Cu-Do-rituximab or (3.7 MBq/4 μg Do-FN3 or Do-rituximab in 200 μL PBS) via a lateral tail vein. One group also received a blocking dose (50-fold excess) of unconjugated FN$_{CD20}$ 2 hours before radiotracer injection. At each time point (1, 2, 4, 16, and 24 hours postinjection), the animals were anesthetized and imaged on a Siemens Inveon small-animal multimodality PET/CT system (Preclinical Solutions; Siemens Healthcare Molecular Imaging). This PET/CT system combines 2 independently operating PET and CT scanners with radial, tangential, and axial resolutions of 1.5 mm at the center of the field of view of the PET module. The CT raw images were acquired at 80 kVp and a 200 μm focal spot size and a 2.048 × 3.072 pixel X-ray detector. Computed tomography (CT) raw datasets were reconstructed using Shepp-Logan filter and two-dimensional ordered-subset expectation maximization (OSEM 2D) algorithm (34). Image files were analyzed using a Medical Image Data Examiner (AMIDE; ref. 35) open source software or Inveon Research Workspace (IRW). For each small animal PET scan, three-dimensional regions of interest (ROI) were drawn over the heart, liver, spleen, kidneys, and muscle on decay-corrected whole-body images. The average radioactivity concentration in the ROI was determined from the mean pixel values within the ROI volume. These data were converted to counts per milliliter per minute by using a predetermined conversion factor. The results were then divided by the injected dose to obtain an image region of interest-derived % ID/g. Statistical analysis was done with Student t test (2-tailed, unequal variance).

**Results**

**Engineering and characterization of FN3$_{CD20}$**

After 6 iterations of selection and maturation, 5 dominant clones were identified by sequence analysis (Table 1). Affinity titrations with yeast surface display and flow cytometry indicated that one clone showed the best dissociation constant ($K_d$) of 14.3 ± 1.3 nmol/L. In these studies, the clone (FN3$_{CD20}$) with the highest affinity and specificity was tested for in vitro CD20-alien lymphoma cell binding assay using FACS. FN3$_{WT}$ (control) and FN3$_{CD20}$ (CD20 binder) were used for physico-chemical characterization and in vivo animal imaging study. The sequence in the diversified BC, DE, and FG loops are presented as well as mutations within the framework as a result of mutagenic PCR during evolution.

**Table 1. FN3 variable domains sequence alignment**

<table>
<thead>
<tr>
<th>FN3 clones</th>
<th>BC</th>
<th>DE</th>
<th>FG</th>
<th>Framework</th>
</tr>
</thead>
<tbody>
<tr>
<td>FN3$_{WT}$</td>
<td>DAPA</td>
<td>GSKST</td>
<td>GRDGSPASSK</td>
<td>Framework</td>
</tr>
<tr>
<td>FN3$_{CD20}$</td>
<td>HYTCAGS</td>
<td>HYYGWDRYSH</td>
<td>A74V</td>
<td></td>
</tr>
<tr>
<td>FN3$_{CD20}$-02</td>
<td>HYTCADS</td>
<td>HYYGWDRYSH</td>
<td>A74V</td>
<td></td>
</tr>
<tr>
<td>FN3$_{CD20}$-03</td>
<td>HYTCAGS</td>
<td>HYYGWDRYSH</td>
<td>A74V</td>
<td></td>
</tr>
<tr>
<td>HSSDVSY</td>
<td>YWFTN</td>
<td>YRDCSSE</td>
<td>E9G, A57T, V75A</td>
<td></td>
</tr>
</tbody>
</table>

FN3$_{WT}$, wild-type (WT), and FN3$_{CD20}$ to FN3$_{CD20}$-03 clones listed in the table were selected against the CD20 peptide from G4 library. Among the 4 unique clones, the clone (FN3$_{CD20}$) with the highest affinity and specificity was tested for in vitro live lymphoma cell binding assay using FACS. FN3$_{WT}$ (control) and FN3$_{CD20}$ (CD20 binder) were used for physico-chemical characterization and in vivo animal imaging study. The sequence in the diversified BC, DE, and FG loops are presented as well as mutations within the framework as a result of mutagenic PCR during evolution.
not show appreciable binding toward a scrambled CD20 peptide. This clone, named FN3_{CD20}, was produced in bacterial culture with a His6-tag and purified by nickel column chromatography and reversed-phase HPLC with >95% purity. Mass spectrometry showed 11,560 Da molecular weight (expected 11,561 Da). Surface plasmon resonance (SPR) demonstrates the affinity of FN3_{CD20} for CD20 peptide (amino acids 165–185) was 22 nmol/L (Fig. 1A). A flow cytometry assay with live Ramos cells, which express CD20 antigens, indicates a binding affinity for cellular CD20 as 20 ± 2.0 nmol/L (Fig. 1B).

Production and characterization of ^{64}Cu-Do-FN3

The anti-CD20 FN3 binder was conjugated with DOTA-NHS (Fig. 2A) for in vitro live cell-binding assay and in vivo animal-imaging study. DOTA was conjugated to primary amines on FN3_{CD20} with a yield of 1.8 DOTA molecules per FN3 protein as measured by mass spectrometry. Radiolabeling of Do-FN3 with ^{64}Cu in ammonium acetate buffer (pH 5.5) was performed with 80.0 ± 3.1% yield. The highest radiochemical yield achieved was 85% at 37°C, of pH 5.5, at incubation for 60 minutes. Radiochemical purity was 97.0 ± 0.5% as determined by HPLC.

The immunoreactivity and specificity of ^{64}Cu-Do-FN3 radio tracer was tested in live Ramos (CD20^+) and Jurkat (CD20^-) cells. Twenty-five nanomolar ^{64}Cu-Do-FN3 readily bound to Ramos cells, whereas the nonbinding control ^{64}Cu-Do-FN3_{WT} exhibited only background signal (P < 0.001); moreover, binding was inhibited by the addition of 1000 nmol/L unlabeled FN3_{CD20} (P < 0.001; Fig. 2B). CD20 specificity was further demonstrated by the reduced binding to Jurkat cells, which lack appreciable CD20 expression. Thus, ^{64}Cu-Do-FN3 was a specific tracer for CD20 antigen. ^{64}Cu-Do-FN3_{CD20} PET tracer is stable in human serum as it remains >95% intact for up to 24 hours (Fig. 2C).

Small animal PET imaging

To evaluate the in vivo targeting ability of the ^{64}Cu-Do-FN3_{CD20} PET tracer, we used a humanized transgenic mouse model that expresses human CD20 antigens on B cells (huCD20TM) to mimic a human B-cell lymphoma tumor. From the PET/CT data (Figs. 3 and 4 and Supplemental Movie), it is evident that ^{64}Cu-Do-FN3_{CD20} had significant uptake in the spleen, the major site for B cells, which express the CD20 antigen. Spleen uptake was 38 ± 3%ID/g within 1 hour postinjection (46 ± 2, 75 ± 2%ID/g for 4 and 16 hours, respectively).
hours, respectively) and increased to 85 ± 4%ID/g at 24 hours. Notably preadministration of 50-fold excess of unla-
beled FN3CD20 dramatically reduces spleen uptake 24/C6, 12/C6, 11/C6 %ID/g (P = 0.005, 0.0004, and 0.01), for 4, 16, and 24 hours, respectively; Fig. 4A). Specificity is further demonstrated as the nontargeted control 64Cu-Do-FN3WT exhibits low spleen uptake: 4.8 ± 0.6, 8.3 ± 0.3, 10.4 ± 0.9%ID/g (P = 0.02, 0.01, and 0.01) at 4, 16, and 24 hours, respectively.

At 24 hours, the tracer uptake value of liver and kidney of nonblocking mice are 50 ± 2 and 14 ± 3, respectively, while blocking mice exhibit 10.6 ± 0.5 and 5.3 ± 0.9 for liver and kidney, respectively measured by ROI. After 24 hours postinjection, 2 groups of mice (blocking and nonblocking) organs were resected and counted for the tracer uptake (Fig. 5A). Overall study results of both in vivo and ex vivo correlate well. Moreover, ex vivo studies show low tracer uptake by background tissues: 1.2 ± 0.6%ID/g in blood and 0.28 ± 0.11%ID/g in muscle. The ratio of tracer target-to-background tissue (spleen/blood) is 56/C6, which is significantly decreased in preblocked mice (8.8 ± 1.7, P = 0.03) by ex vivo measurement.

To assess the impact of the protein scaffold and evaluate translational potential for PET imaging, 64Cu-Do-FN3CD20 was compared with our previous data (31) for 64Cu-rituximab within the identical huCD20TM model.
The $^{64}$Cu-Do-FN3CD20 tracer uptake value in spleen was significantly higher, compared with the $^{64}$Cu-rituximab mice group. The uptake values of $^{64}$Cu-FN3 and $^{64}$Cu-rituximab are 38.0 ± 1.8 and 31.8 ± 1.6 ($P = 0.041$) for 1 hour, and 46 ± 2 and 35 ± 2 ($P = 0.037$) for 4 hours, respectively (Fig. 5B). The spleen-to-blood ratios for the FN3 and $^{64}$Cu-rituximab tracers are 13 ± 1 and 1.34 ± 0.03 at 4 hours (Fig. 5C), which are also highly statistically significant ($P < 0.005$).

Furthermore, $^{64}$Cu-FN3CD20 and $^{64}$Cu-Do-rituximab tracers were evaluated in mice-bearing Ramos CD20 (+) tumors ($n = 3$, 3 groups) averaging 145 (±30) mg in weight. Each mouse was injected with approximately 3.7 MBq of $^{64}$Cu-FN3CD20, $^{64}$Cu-FN3WT, or $^{64}$Cu-Do-rituximab (specific activity = 0.93 MBq/µg). Whole body images were obtained at 4, 16, and 24 hours. After the final scan, mice were sacrificed and organs were harvested, weighed, and counted in a $\gamma$ counter to calculate %ID/g. The PET images obtained at 4 and 24 hours by $^{64}$Cu-FN3CD20 and $^{64}$Cu-Do-rituximab tracers showed specific uptake in tumors (Supplementary Fig. S1). The activities in the tumor, liver, spleen, and kidneys calculated from biodistribution and ROIs are shown in Supplementary Table S1. The mean tumor uptake (%ID/g ± SD) by $^{64}$Cu-FN3CD20 and $^{64}$Cu-Do-rituximab were 16.8 ± 2.2 and 6.8 ± 1.3 at 4 hours and 6.8 ± 1.3 and 6.8 ± 0.5 at 24 hours ($P < 0.05$, at 24 hours) respectively, determined by ROI analyses, on mouse images presented in Supplementary Table S1. After 24 hours scan, the ex vivo tumor uptake was 6.2 ± 1.2 and 8.7 ± 0.3 for $^{64}$Cu-FN3CD20 and $^{64}$Cu-Do-rituximab, respectively. The
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tumor-to-blood (T/B) ratio at 24 hours by $^{64}$Cu-FN3CD20 was 4.1, which is 2-fold higher than the $^{64}$Cu-Do-rituximab T/B ratio (2.3) at the same time point ($P = 0.04$). ROIs of the $^{64}$Cu-FN3WT resulted in 1.9 ± 0.8 and 1.2 ± 0.4%ID/g at 4 and 24 hours, respectively.

Discussion

CD20 has proven to be a promising target for therapy of B-cell NHL (36). Development of an imaging agent would have significant value for monitoring disease progression and therapeutic efficacy. Several antibodies targeting CD20 have been tested for their potential for therapy and molecular imaging (37). In this report, we describe the development of a novel radiotracer based on the FN3 protein scaffold, which is 8% of the antibody size and thereby provides more rapid clearance and potentially more effective distribution. On the contrary, intact antibodies generally have slow distribution and clearance. The accumulation in tumor tissue and clearance from the circulation can take several (3–10) days (38, 39). Our intention is to develop a tracer to achieve quantitative visualization of tumors by PET at an early time point, preferably within 12 hours after tracer injection; this would be beneficial clinically and could substantially reduce normal tissue radiotoxic burden.

The FN3-based protein scaffold system has been explored by various research groups and demonstrates potential as a useful scaffold for imaging agents (20, 40). FN3 domains can be readily engineered for specific, high-affinity binding, retain good stability, and are derived from a human parental sequence, which may limit immunogenicity (40). Here, we have developed FN3-based binders for CD20, screened and selected against immobilized CD20 peptide, and intact lymphoma cells by established methods (22). The resulting soluble FN3 protein provides high-affinity CD20 binding as tested by SPR (Fig. 1A, $K_D = 22$ nmol/L) and a live cell binding assay (Fig. 1B, $K_D = 20$ nmol/L). Monovalent FN3CD20 exhibits comparable binding affinity to rituximab antibody ($K_D = 8$ nmol/L; ref. 41); and faster clearance of FN3 binder from blood and background tissues enables visualization of the target tissue more clearly and sooner than antibody based PET (Figs. 3 and 5). The stability of the FN3 scaffold and the DOTA chelator yield a radiotracer that remains >95% intact for 24 hours (radioactive peak corresponds to 7 cm) in human serum (Fig. 2C). At 24 hours, we observe increased aggregation from 3% to 5% (peak corresponds to 3 cm).

$^{64}$Cu-FN3CD20 tracer rapidly and durably targeted the spleen, which is the dominant location of CD20-positive B cells, as evidenced by 38%ID/g uptake at 1 hour and increasing signal to 83%ID/g at 24 hours (Figs. 4 and 5A). This targeting is specific as preblocking with cold FN3CD20 substantially reduces the spleen signal (Figs. 3 and 4). Moreover, nontargeted control $^{64}$Cu-FN3WT uptake by spleen was only 10%ID/g (Fig. 3) at 24 hours.

Previously, we have developed the $^{64}$Cu-DOTA-rituximab tracer, tested it in the huCD20TM model, and are now performing a clinical trial (31) under a US FDA IND (#104995). The current FN3-based tracer performs favorably relative to this antibody tracer. Spleen uptake in the huCD20TM model is higher at 1 and 4 hours (Fig. 5B) and target tissue-to-blood ratio is 10-fold higher (Fig. 5C). It should be noted that these results were obtained with a transgenic mouse model, which should prove to be more representative of human tumor development than typical subcutaneous xenograft models.

Although $^{64}$Cu-Do-FN3CD20 rapidly clears from most tissues, liver, and kidney signal are more prevalent. Liver activity may be because of dissociation of $^{64}$Cu from DOTA (42) or charge effects of the engineered protein and the DOTA chelator. Kidney retention is a common problem for small proteins (37) because they pass through the glomerulus and can be reabsorbed in the renal tubules. The renal retention of $^{64}$Cu-Do-FN3CD20 is actually much lower than many other comparably sized $^{64}$Cu-DOTA–labeled molecules (37). Spleen uptake remains substantially higher than liver (1.7 ± 0.1 fold) and kidney (3.7 ± 2.1; Fig. 5A). It should be noted, although, that the specific tumor targeting is far greater with $^{64}$Cu-Do-FN3CD20 than $^{64}$Cu-Do-rituximab (Supplementary Fig. S1) or $^{64}$Cu-Do-minibody (37): 4 hours tumor:liver of 7.6 for FN3CD20 versus 0.4 for rituximab and 0.7 for minibody; 4 hours tumor:kidney of 2.6 for FN3CD20 versus 0.6 for rituximab and 0.5 for minibody; 19 to 24 hours tumor:liver of 5.6 for FN3CD20 versus 1.5 for rituximab and 0.5 for minibody; 19 to 24 hours tumor:spleen of 4.1 for FN3CD20 versus 1.9 for rituximab and 0.9 for minibody; 19 to 24 hours tumor:kidney of 3.3 for FN3CD20 versus 5.5 for rituximab and 0.3 for minibody.

Recently, in another experiment, we tested rituximab with a long half-life isotope ($^{89}$Zr: $t_{1/2} = 78.4$ hours) in the huCD20TM model, which showed increased spleen uptake (mean ± SD) 67 ± 43, 80 ± 23%ID/g at 4 and 24 hours, respectively, which is 50% more uptake compared with the $^{64}$Cu-rituximab (43) however, $^{64}$Cu-FN3CD20 tracer also shows similar uptake values. $^{89}$Zr-rituximab has different radionuclide and linker molecule as compared with the $^{64}$Cu-Do-rituximab or $^{64}$Cu-FN3CD20. Moreover, for small molecules like FN3, long half-life isotope may not be required, because this molecule systemically cleared very fast as compared with the monoclonal antibody. Furthermore, the reason for high uptake of $^{89}$Zr-rituximab may be high stability of $^{89}$Zr chelation with desferrioxamine linker compared with the $^{64}$Cu DOTA chelation; more study is warranted to test this hypothesis.

The results of our study and others (37, 44) demonstrate that smaller binders can provide better imaging results compared with high molecular weight antibodies both in terms of tumor-to-blood ratio and absolute tumor uptake. From the patient perspective, the development of a PET tracer to visualize CD20 at early time-points in human patients could provide valuable clinical insight while reducing the radioactivity burden by healthy tissue. This novel tracer might be used to take advantage of the sensitivity of PET in cases of low tumor burden or in cases of indolent...
lymphomas, in which the sensitivity of \(^{18}F\)-FDG PET can be as low as 50% (45, 46). Furthermore, this tracer could be used to study responses to therapies in human lymphoma or rheumatoid arthritis, and other autoimmune diseases, for example B cell targeting vaccines (47).

Overall, PET imaging with both huCD20TM and Ramos xenograft CD20+ tumor models indicated favorable tumor visualization for the \(^{64}\text{Cu-FN3}_{\text{CD20}}\) at early time points (between 4 and 24 h) compared with the \(^{64}\text{Cu-Do-rituximab}\) that showed high background signal even at 24 hours.

Conclusion

In summary, we have developed a novel CD20-targeted PET radiotracer based upon a small FN3 protein scaffold. The radiotracer specifically binds to CD20 proteins on B cells both in culture and in vivo. This novel tracer may provide superior CD20 molecular imaging signal to background for B-cell NHL at early time points compared with an intact antibody. Further development of this tracer is therefore warranted for clinical translation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Conception and design: A. Natarajan, B. Hackel, S.S. Gambhir

Development of methodology: A. Natarajan, B. Hackel, S.S. Gambhir

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Natarajan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A. Natarajan, B. Hackel, S.S. Gambhir

Writing, review, and/or revision of the manuscript: A. Natarajan, B. Hackel, S.S. Gambhir

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Natarajan, S.S. Gambhir

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