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Radiation Dosimetry Study of [⁸⁹Zr]rituximab Tracer for Clinical Translation of B cell NHL Imaging using Positron Emission Tomography

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Abstract

Purpose—We evaluated the dosimetry of [⁸⁹Zr]rituximab, an anti-CD20 immunoPET tracer to image B cell non-Hodgkin's lymphoma (NHL) using a humanized transgenic mouse model that expresses human CD20 transgenic mice (huCD20TM).

Procedures—Rituximab was conjugated to desferrioxamine (Df) for radiolabeling of Zirconium-89. [⁸⁹Zr]rituximab (2.8±0.2 MBq) was tail vein-injected into huCD20T mice. Positron emission tomography (PET)/CT imaging was performed on the two groups of mice (blocking=2 mg/kg pre-dose of rituximab and non-blocking; n=5) at eight time points (1, 4, 24, 48, 72, 96, 120, and 168 h) post injection.

Results—The novel [⁸⁹Zr]rituximab PET tracer had good immunoreactivity, was stable in human serum, and was able to specifically target human CD20 in mice. The human equivalents of highest dose (mean±SD) organs with and without pre-dose are liver (345±284 μSv/MBq) and spleen (1165±149 μSv/MBq), respectively.

Conclusions—Dosimetry of the human patient whole-body dose was found to be 145 MBq per annum, and the patient dose-limiting organ will be the liver (with rituximab pre-dose blocking) and spleen for non-blocking. The [⁸⁹Zr]rituximab (t_{1/2}=78.4 h) imaging of B cell NHL patients could permit the observation of targeting lesions in NHL patients over an extended period due to longer half-life as compared to the [⁶⁴Cu] rituximab (t_{1/2}=12.7 h).

Keywords

Dosimetry; ImmunoPET tracer; [⁸⁹Zr]rituximab; NHL

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Introduction

The aim of the current research work was to develop and evaluate the dosimetry of an antibody-based immuno-PET tracer for non-Hodgkin's lymphoma (NHL). NHL accounts for 3 % of all cancers worldwide [1]. NHL is originated from B cell or T cell and a heterogeneous group of diseases in which B cell lymphoma is one of the most prevalent NHL cancers. Anti-CD20 monoclonal antibodies (mAb) have been utilized for treatment, and have substantially improved response rates and overall survival; yttrium-90 labeled anti-CD20 mAb ibritumomab tiuxetan (Zevalin®) is approved for treatment of patients with relapsed and refractory NHL [2]. The CD20 phosphoprotein marker is (32 kDa, non-glycosylated) usually expressed by human lymphomas cells; it is used as a target in radioimmunotherapy, and is typically overexpressed on the surface of the mature B cells [3, 4]. Furthermore, radioimmunotherapy (RIT) has been integrated into upfront treatment in indolent NHL [5] or added to high-dose chemotherapy followed by autologous stem cell transplantation, showing significant benefit [6, 7].

Even though a considerable amount of research was carried out on prognosis of NHL, still, the molecular pathogenesis of various NHLs is classified based upon morphological and histological criteria. In order to bypass the limitations of these traditional techniques and thus enhance pathogenic prediction, we have developed a novel immunoPET tracer that is labeled with the Zr-89 radioisotope. To label Zr-89, we have used p-isothiocyanatobenzyl-desferrioxamine (Df-Bz-NCS) [8, 9] chelator. Prior to labeling, the chelator was conjugated to rituximab via amine covalent chemistry. To evaluate this tracer, we have used a transgenic B cell lymphoma mouse model that expresses human CD20 (huCD20TM). In this mouse model, most of the human CD20-expressing B cells homed to the spleen and thus tracer uptake was highest in this organ. Consequently, the huCD20TM mouse model is considered the best to evaluate tracer targeting ability [10, 11]. The literature does reveal some preclinical and clinical studies in which the CD20 antigen of B cells was targeted by rituximab [11–13]. These studies used various forms of the antibody as well as different kinds of isotopes, such as [⁶⁴Cu]-minibody and [¹²⁴I]-scFv-Fc to image B cell NHL in preclinical mouse model [12]. Similarly, [¹²⁴I]-rituximab tracer was tested in rheumatoid arthritis human patients [13]. Although these are pilot studies, they show encouraging results for clinical translation.

Biodistribution studies with [¹¹¹In]ibritumomab tiuxetan and [¹³¹I]ibritumomab tiuxetan in humans have been studied, using gamma cameras, which complicates quantification [14, 15]. However, Perk et al. utilized [⁸⁹Zr]ibritumomab tiuxetan using positron-emitting isotope and demonstrated better quantitative assessment of biodistribution of ibritumomab tiuxetan with positron emission tomography (PET) in mice [16]. Thus, [⁸⁹Zr]ibritumomab tiuxetan PET tracer could be potentially useful for a pilot scan to assess the biodistribution of ibritumomab tiuxetan prior to therapy. This data can be used as a guide for the selection of patients and the amount of [⁹⁰Y]ibritumomab tiuxetan dose needed to administer in the individual patient, which can provide a huge benefit to patients, preventing them from a potentially toxic therapeutic amount of [⁹⁰Y]ibritumomab tiuxetan. Studies on [⁸⁹Zr]Df-mAbs have reported high *in vivo* stability with respect to metal ligand and chelator stability, and relatively low-level accumulation in background tissue in both animals and human. The

bone uptake of ^{89}Zr isotope is also well known and reported in previous studies using various mAbs [8, 9]. However, the nature of the Zr-89 radioactive species accumulating in the bone remains uncertain, but it is reasonable that slow intratumoral metabolism and subsequent recirculation of Zr-89 labeled metabolites may occur.

In the present report, we describe the biodistribution and radiation dosimetry of [^{89}Zr]rituximab in huCD20 transgenic mice. Rituximab, a chimeric murine/human monoclonal antibody (145 kDa), binds to the CD20 antigen approved by the USFDA for the treatment of refractory or relapsed B cell lymphomas. This antibody is composed of murine 2B8 antibody complementarity regions and with human kappa and IgG1 heavy-chain constant region. Two heavy and light chains are consisting of 451 and 213 amino acids, respectively. The binding affinity of the rituximab is nearly 8.0 nM against the CD20 antigen.

We determined the dose-limiting organs of humans in order to translate into human patients using [^{89}Zr]rituximab as immunoPET. To estimate the organs' absorbed doses, Radiation Dose Assessment Resource (RADAR) models were developed at Vanderbilt University and these dose assessments were calculated from the tracer radioactivity residence times of several organs [17, 18]. In order to estimate the human equivalent dosimetry, we extrapolated the mouse residence times to humans, based on simple assumptions of differences between the species. These projected residence times were used to compute the human organ doses, using OLINDA/EXM 1.1 dosimetry software based on adult male phantoms. In this work, we evaluated the *in vivo* biodistribution of [^{89}Zr]rituximab in the huCD20T mouse organs using whole-body PET and CT imaging.

Materials and Methods

Reagents, Antibodies, Radiochemicals, and Cell Lines

All reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Cell culture media and additives were obtained from Invitrogen Corporation (Carlsbad, CA USA). The p-isothiocyanatobenzyl-desferrioxamine (Df-Bz-NCS) and rituximab antibody (Rituxan; 10 mg/ml) were purchased from Macrocyclics (Dallas, TX, USA) and Stanford Hospital Pharmacy (Stanford University, Stanford), respectively [14]. Rituximab is a high-affinity chimeric monoclonal antibody directed against human B cell CD20, which belongs to the IgG1 subtype (human κ light chain and human γ 1 heavy chain). The University of Wisconsin provided the Zr-89 ($t_{1/2}$ =78.4 h, β^+ =22.6 %; ~2.7 GBq/ml in 1 M oxalic acid). The cell lines of Ramos (CD20+ B cells; ATCC number: CRL-1555) and Jurkat (CD20- cells; ATCC number: TIB-152), were purchased from the American Type Culture Collection (www.atcc.com). Ramos and Jurkat cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (4.5 g/l glucose), and MEM/Ham's F-12 (1:1) with 1 % non-essential amino acids, respectively. Both media were complemented with 10 % fetal calf serum (FCS), 2 mmol/l glutamine, 100 units/ml penicillin, 100 μg streptomycin, and 0.25 μg /ml Fungizone.

Instruments

A high-performance liquid chromatography (HPLC) -Ultimate 3000 system connected in line with detectors (ultraviolet and radioactivity) was used for tracer purification and quality control (QC) tests. Immunoconjugate and radiotracer were eluted on size-exclusion chromatography (SEC) 3000 LC column (300×7.8 mm) with 5 µm, hydrophilic-bonded silica support of 400-Å pore size (Phenomenex, Torrance, CA 90501-1430, USA). Immunoconjugate mass was tested on mass spectrometry (AB SCIEX TOF/TOF 5800) of linear mode operation with sinapinic acid as matrix from the Canary Center at Stanford University.

Preparation of Immunoconjugate and ImmunoPET Tracer

Conjugation of Df-Bz-NCS (Df) with rituximab was carried out as described previously [9]. Briefly, rituximab formulation solution (0.1 mM; 350 µl) was buffer exchanged with 0.1 M Na₂CO₃ (pH 9.0). For conjugation reaction, the buffer exchanged rituximab and Df-Bz-NCS (tenfold molar excess of rituximab; 10 mM; in 35 µl of DMSO) were placed in 1.5-ml Eppendorf vial. This mixture was then made up to 0.5 ml of 0.1 M sodium phosphate buffer (pH=8.5) and kept at room temperature for 1 h. A slide-A-lyzer 30 kDa cutoff membrane dialyzer (PIERCE, USA) 0.1 M sodium acetate buffer (pH 7.0) for buffer exchange was used to remove excess Df from the reaction mixture. Prior to Zr-89 labeling, the unconjugated Df purified immunoconjugate was concentrated by centrifugation dialysis, and aliquoted into multiple vials of 0.1 to 0.2 mg/ml. These vials were properly labeled and stored at -80 °C. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was used to test the number of Df chelators (c) coupled per antibody (a), i.e., c/a molecule by comparison of rituximab and Df- rituximab [11].

Immunoreactivity of the Df-Bz-rituximab (Df-rituximab; three to four Df chelates per antibody), pre-labeling, was tested on CD20+ Ramos cell lines and analyzed by flow cytometry. Staining of cells was performed in a V-bottomed 96-well microtiter plate. Briefly, in each well, 100 µl of the antibody (Df-rituximab) and unmodified rituximab were diluted to 500 µl ranging from 50 to 390 ng/ml in Dulbecco's PBS with 2 % FCS and 0.1 % sodium azide solution (fluorescence-activated cell sorting (FACS) buffer). Each well containing antibody was mixed with 50 µl of 5×10⁵ Ramos cells and incubated for 1 h on ice. Antibody-coated cells were washed thrice with FACS buffer, followed by each well receiving 100 µl of goat anti-human IgG/Fc-FITC (Jackson ImmunoResearch, West Grove, PA) pre-diluted to 1:100 in FACS buffer. Cell staining were performed in the dark on ice for 0.5 h and washed thrice with FACS buffer. Propidium iodide was used to stain dead cells from the live cells. Analysis was carried out on a flow cytometry (FACS Aria III, BD Biosciences, San Jose, CA, USA) at the Stanford FACS Facility. Data were analyzed by FlowJo FACS analysis software (Tree Star, Ashland, OR, USA).

To synthesize high specific activity radiotracer, labeling reagents were added in the following sequence in a 1.5 ml Eppendorf tube. First, Zr-89 (90–100 MBq; 750 µl) and 2.5 M Na₂CO₃ (100 µl) were mixed into the tube and kept at room temperature (RT) to balance pH. After 3 min, in the same reaction vial, 10 mM ammonium acetate buffer (150 µl, pH 7.0), and Df-rituximab (250 µl, 100 µg) in 10 mM ammonium acetate buffer (pH 7.0) was

added and shaken on a test tube *rocker* for 45 min at RT. After incubation, 0.1 M *diethylenetriaminepentaacetic acid* (DTPA), pH 7.0, was added to the reaction mixture to achieve a final concentration of 5 mM and kept for 15 min to scavenge unchelated Zr-89.

The radioimmunoconjugate (80–100 µg in 1500-µl volume) was purified on a SEC 3000 column in PBS buffer [0.1 M NaCl, 0.05 M sodium phosphate buffer, pH 7.4] at a flow rate of 1.0 ml/min. The radioconjugate peak at 8.5 min (retention time) corresponding to rituximab antibody was collected, and concentrated using an Amicon Ultra-15 (Millipore, USA) device and centrifuged at 3000×g for 15 min. The concentrated final radioconjugate was passed through a 0.2-µm filter into a sterile vial.

Quality Control of the ImmunoPET Tracer

The [⁸⁹Zr]Df-rituximab ([⁸⁹Zr]rituximab) radiochemical purity was analyzed by thin layer chromatography as well as SEC 3000, radio-HPLC. Immunoreactivity of the immunoPET tracer was assayed by live cell-binding assays as described by previous publications [19, 20]. Tracers and cells were diluted in 1 % bovine serum albumin (BSA) in PBS (PBSA; pH 7.4). Briefly, Ramos (CD20+) and Jurkat (CD20–) cells were diluted to the following concentrations of 5.0, 4.0, 3.0, 2.5, 2.0, 1.5, and 0.5×10⁶ cells/ml of PBSA. In each microfuge tubes, 0.5 ml of the cells and an aliquot of [⁸⁹Zr]rituximab (50 µl; 0.37 kBq [1 µCi], 0.01 µg of mAb) were added (*n*=3; final volume=550 ml) incubated at 37 °C. After addition of tracer, the solutions were gently vortexed and kept for 2 h. Two hours later, the cells were centrifuged at 300 g for 3 min and the supernatant was removed. This process was repeated twice by suspending the cells in ice-cold PBSA. After two washes, the Zr-89 activity accompanied with the cell pellet was counted (counts per minute=cpm) in a γ-counter (1470 WIZARD Automatic Gamma Counter; Perkin Elmer, Waltham, MA). All samples count were background corrected. The experiment sample activity count was compared with a total number of activity associated with control. Competitive inhibition (blocking) assays, were performed with the unmodified rituximab (50 µl, 0.2 mg/ml in PBSA, [1000-fold excess mAb; 10 µg]) to the [⁸⁹Zr]rituximab solutions.

The immunoPET tracer stability in human serum was tested at 37 °C as per published procedure [9]. To test the stability of the radiotracer purified [⁸⁹Zr]rituximab (25 µg/100 µl) was mixed with human serum (400 µl) and 20-fold excess of DTPA (500 µg/100 µl). This mixture was incubated over 7 days at 37 °C; at each time point, a portion of the aliquot was filtered and analyzed by SEC 3000 radio-HPLC at 4, 24, 72, 120, and 168 h.

Animal Studies

Animal experiments were performed with approval from the *Administrative Panel on Laboratory Animal Care* (APLAC) at Stanford University. The huCD20 transgenic mice used in the experiments were purchased from Genentech (South San Francisco) [11]. Reverse transcription polymerase chain reaction (RT-PCR) was performed to screen the mice for huCD20 expression. Two groups of human CD20 transgenic mice (*n*=4) were selected with an average weight of the mice was 25.0±2.0g. These mice received radiotracer of 2.8±0.2 MBq activity with 5–8 µg of Df-rituximab [200 µL] via tail vein injection. After tracer administration, the animals were scanned at 1, 4, 24, 48, 72, 96, 120, 144, and 168 h

using small animal PET/CT at the Stanford small animal imaging center. Tracer uptake for each organ was computed as % injected dose per gram (%ID/g) of tissue. Statistical analysis was done with Student's *t* test (two-tailed, unequal variance).

Small Animal PET Imaging

For PET/CT imaging, each of the huCD20T mice was anesthetized and administered 2.8 MBq radiotracers through tail vein. From then onwards at each time point (1, 4, 24, 48, 72, 96, 120, 144, and 168 h) mice were anesthetized to perform PET/CT. PET/CT scanning and image data analysis were performed with Inveon small animal multimodality system and Inveon Research Work-place (IRW) software (Preclinical Solutions; Siemens Healthcare Molecular Imaging, Knoxville, TN), respectively.

The Inveon PET/CT system provides excellent radial, tangential, and axial resolutions of higher than 1.5 mm at the center of the field of view. PET data was acquired with energy window settings of 350 to 650 keV energy, and 3.4 ns coincidence timing; attenuation correction was applied from the CT measurements. The CT acquisition was performed with 4064×4064 pixel x-ray detector. The images were obtained with 120 projections per bed position, i.e., two bed position; half scan 220 ° of rotation, and with a cone beam micro-x-ray source (50 µm focal spot size). Shepp-Logan filter and cone-beam filtered back-projection was used to reconstruct the CT raw data.

PET/CT images (post tracer injection) scanning were performed with the following time frame, i.e., 5-min scan for 1-h time point, 10-min scan for 4-, 24-, 48-h, time points, and 20-min scan for 72, 96, 120, and 144-h time points. The acquired images were reconstructed with two-dimensional ordered-subset expectation maximization (OSEM 2D) algorithm [21]. The average radioactivity concentration was estimated for each organ from the small animal PET scan by drawing three-dimensional regions of interest (ROI). These ROI data was computed from mean pixel values within the ROI volume and converted to counts per milliliter per minute by using a predetermined conversion factor. The organs (heart, liver, spleen, and whole body) uptake was estimated by drawing ROI over the non-decay-corrected whole-body PET images. The percentage injected dose per gram of tissue (%ID/g) was computed from the injected dose and ROI of the image data.

In vivo Biodistribution Study of [⁸⁹Zr]rituximab

In vivo biodistribution studies were conducted to evaluate the uptake of [⁸⁹Zr]rituximab in huCD20TM at 4, 24, 72, 96, and 168 h post injection. Mice (*n*=4, per group) with blocking (received 2 mg/kg cold dose of rituximab 2 h prior to tracer injection) and non-blocking (tracer alone) were received [⁸⁹Zr]rituximab [200 µl, corresponding to 2.8±0.2 MBq, 5–8 µg of Df-rituximab], via intravenous (i.v.) tail vein injection (*t*=0). Animals were euthanized by CO₂ gas asphyxiation at 4, 24, 72, 96 and 168 h post injection and organs were removed, rinsed in PBS, dried in air for 5 min, weighed and counted in a gamma counter for Zr-89 activity. The [⁸⁹Zr]rituximab dose uptake by each organ was determined by measuring the total number of counts (counts per minute, [cpm]). The percent injected dose per gram (%ID/g) for each organ was computed by normalization to the total activity injected. These data were decay corrected at the time of injection and background subtracted.

Partial Volume Effect Correction (PVC)

Partial volume effect correction (PVC) was performed using a recovery coefficient method [22]. To compute the recovery coefficient, we used four cylindrical phantoms (50-ml tubes) with one or two fillable spheres of different size placed randomly but well separated from each other inside each tube. We filled a solution of water and Cu64 radioactive tracer with concentration of $\sim 1 \mu\text{Ci/ml}$ to the cylindrical tubes as background and $\sim 10 \mu\text{Ci/ml}$ to each fillable sphere with inner diameters of 3.95, 4.95, 6.25, 7.86, 9.89, 12.43, and 15.43 mm. In order to draw an accurate region of interest over the inner diameter of the spheres, we also added CT contrast agent (Iodine $\sim 400 \text{ mg/ml}$) with the sphere water and radioactivity mixture. We taped the four cylinders together and scanned using Inveon microPET/CT emulating the four mice set acquisition we used for our animal PET/CT study. Qualitative and quantitative assessment was performed under the guide of in-house imaging physicist. Region of interest (ROI) was calculated as the tissue radioactivity concentration corrected for the injected activity and whole body weight of the mouse. Percent-injected dose per gram (%ID/g) quantification with PVE correction was performed for the following tissues such as heart, liver, spleen, and whole body of the mouse. During the measurement of each lesion, we carefully segmented not to include any adjacent high uptake organ in the background measurement. Thus, PVC factor was calculated from the phantom study.

Dosimetry

The human equivalent dose was calculated from the injected dose activity of animal organ using the method of Kirchner et al. [23]. The integral value of time activity per organ ($\mu\text{Ci-h}/\mu\text{Ci}$ injected) was computed from the human equivalent dose. Human dose estimation was performed with the OLINDA/EXM version 1 software [24].

Results

Preparation of Immunoconjugate and ImmunoPET Tracer

Synthesis of Df-rituximab and labeling optimization was reported previously [9]. In this study, we prepared Df-rituximab with slight modification to reach at least three Df molecules per rituximab to increase the specific activity. The conjugation and purification of Df-rituximab yielded 65 % of immunoconjugate with $>98 \%$ purity. Subsequent determination of chelate/antibody (c/a) estimated by MALDI-MS showed 3–4 Df chelates per antibody (data not given). Radiolabeling of ^{89}Zr -oxalate with (Df)₃-rituximab was carried out at room temperature at pH 7.5 ± 0.5 for 1 h and the reaction mixture provided $>60 \%$ radiochemical yield with $10 \pm 5 \text{ mCi/mg}$ [$370 \pm 185 \text{ MBq/mg}$].

Quality Control of the ImmunoPET Tracer

The radiochemical was purified by spin-column centrifugation or by SEC 3000 HPLC and performed the quality assurance of the tracer. The [^{89}Zr]rituximab radiochemical purity was analyzed by thin layer chromatography as well as SEC 3000 radio-HPLC and the purity was always $>97 \%$ (97.6 ± 0.5) (Fig. 1). Immunoreactivity of the Df-rituximab was evaluated by FACS on live Ramos (CD20+) cells and compared with unconjugated rituximab, using serial dilutions. FACS binding assay shows (Supplement Fig. 1) strong binding to the target

cells at 5 µg/ml by unmodified rituximab (mean fluorescence intensity, MFI, 445) while at the same concentration, Df-rituximab (cold) at the left of the histogram (MFI, 325), indicated that conjugate retained >70 % biological activity. Immunoreactive assay was performed on [⁸⁹Zr]rituximab against live Ramos (CD20+) and Jurkat (CD20-) cells by Lindmo et al. [20] using infinite antigen excess. This assay showed tracer had >88 % immunoreactive fraction (Supplement Fig. 2). The control experiment performed on CD20- Jurkat cells and Ramos cells (pre-blocked with cold rituximab) showed (mean %±SD) 1.32±0.09 and 2.21±0.16 %, respectively. The stability of [⁸⁹Zr]rituximab was evaluated in the presence of a competitor with 20-fold excess of DTPA at 37 °C over a period of 7 days. Radio-HPLC analysis at 4, 24, 72, 120, and 168 h showed that the tracer was intact. The activity bound to the antibody was >95 %, which indicates that the radioimmunoconjugate was stable in human serum (Supplement Fig. 3).

Small Animal PET Imaging

The [⁸⁹Zr]rituximab tracer was evaluated in huCD20T mice, a model that resembles B cell lymphoma tumor. Prior to the PET/CT imaging, mice were screened for the expression of huCD20 protein by RT-PCR. The average weight of the mice was 25±2 g. Both blocking (pre-dose with 2 mg/kg rituximab) and non-blocking mice groups received 2.8±0.2 MBq of PET tracer followed by each mouse being imaged by PET/CT at 1, 4, 24, 48, 72, 96, 120, and 168 h post tail vein injection. Figure 2 shows the representative images of PET/CT scanned at 4, 24, 72, and 168 h. These images (Fig. 4 and supplemental movie) clearly indicate that the tracer uptake in spleen was significant, which is the major site for B cells. Further, compared to blocking mice spleen, non-blocking spleen uptake is very high demonstrating tracer specificity.

Figure 3a–d shows the tracer uptake in the heart, spleen, liver, and whole body (% ID per gram estimated from ROI of Fig. 4). Fig. 3a–d shows the comparison of the tracer clearance profile between blocking (Fig. 3a, c) and nonblocking (Fig. 3b, d) mice groups at various time points, for up to 6 days. The tracer uptake by (mean %ID/g±SD; decay corrected) spleen measured by ROI at 1, 4, and 24 h are 7.7 ±0.5, 9.5±0.6, 9.1±0.3, and 26.2±2.4, 36.6±1.9, 36.8±2.3 (*P*=0.02, 0.03, 0.03) of blocking and non-blocking mice, respectively. The liver uptake at 24 h by the blocking and non-blocking mice are 14.6±0.7, and 5.6±1.9 %ID/g (*P*=0.01) respectively. In comparison with our previous tracer [11] using [⁶⁴Cu] DOTA-rituximab, with an identical huCD20T mouse model, this long lived [⁸⁹Zr]rituximab tracer has an uptake value in spleen that is higher. The uptake values (%ID/g calculated from ROI) of [⁶⁴Cu] DOTA-rituximab and [⁸⁹Zr]rituximab tracers at 24-h post injection for non-blocking mouse spleen are 16.5±0.45 and 23.8±2.9 (*P*=0.005), respectively, which is a ~44 % higher signal for the latter. After 168 h, PET/CT imaged organs were resected and measured (cpm) by the gamma counter (Fig. 4) and decay corrected. The *ex vivo* organ uptake values (%ID/g; decay corrected) for spleen and liver are 4.1 and 5.3 (blocking), and 67.4 and 1.7 (non-blocking), respectively. The *ex vivo* spleen to blood ratios (%ID/g) at 168 h p.i. for the tracer is 5.4±2.7 and 145±40 for blocking and non-blocking mice which is significant (*P*=0.01). The *ex vivo* organ uptake values (%ID/g; decay corrected) of tracer at 4, 24, 72 and 96 h are given in supplement Table ST2.

Dosimetry

The absorbed radiation dose estimates (corresponds to each organ in humans) for the administration of [^{89}Zr]rituximab are given in Table 1. The area under the curve of each organ activity was computed by trapezoidal model using Prism software. Estimation of absorbed radiation dose was calculated using the OLINDA/EXM 1.0 software. The organs with the highest radiation burden ($\mu\text{Sv}/\text{MBq}$) for blocking and non-blocking organs are 391 ± 66 , 226 ± 9 (liver and spleen) and 249 ± 22 , 1210 ± 42 (liver and spleen), respectively. The anticipated human doses ($\mu\text{Sv}/\text{MBq}$) were computed by administration of with and without pre-dose as cold rituximab. These estimated dose values ($\mu\text{Sv}/\text{MBq}$) corresponds to with and without pre-dose are following: effective dose (35.1 and 59.7, respectively) and as effective dose equivalent (70.3 and 124.5, respectively). The dose-limiting organ is spleen (non-blocking) for both [^{64}Cu] DOTA-rituximab, and [^{89}Zr]rituximab and the radiation doses ($\mu\text{Sv}/\text{MBq}$) are 98 ± 4 and 1210 ± 42 , respectively. Although we provided the comparison of these tracers, one should note that these two tracers were not identical molecules but with the same carrier antibody, having different chelators and isotope may have different PK profile due to the chelator molecules on the antibody.

Discussion

Recently, we reported the development of [^{89}Zr]rituximab immunoPET tracer for B Cell lymphoma targeting in a humanized transgenic (huCD20T) mouse model, which included optimization of tracer synthesis, serum stability, and immunoreactivity [9]. However, our goal was to translate this tracer for imaging of B cell lymphoma patients, and hence we carried out the dosimetry evaluation of [^{89}Zr]rituximab tracer using huCD20 expressing huCD20T mouse. This study provided the estimate of the radiation dose burden in various organs in huCD20T mouse. From this data we were able to evaluate the safe human dose (maximum activity) that can be injected into a human subject annually without surpassing the allowed dose limits.

The specific activity and immunoreactivity of the [^{89}Zr]rituximab are high enough, as we were able to see a very clear difference in spleen uptake between pre-blocked and unblocked mice (Supporting data, Fig. 1a) even after 5 days. After 24-h post tracer injection, the uptake of tracer in the spleen was 9.1 ± 0.3 and 36.8 ± 2.3 %ID/g for blocked and non-blocked mice, respectively. At the same time point (24 h p.i.), the liver uptake was 14.6 ± 0.7 (blocked) and 5.6 ± 1.9 (non-blocked) %ID/g (decay corrected) in the beginning and declined gradually thereafter (Fig. 3). The high radiotracer uptake demonstrated in the spleen of unblocked mice is due to the overexpression of CD20 antigens by splenic B cells, as the spleen is one of the major source organs for B cell production. Compared to Figs. 3 and 4, Fig. 3 shows clearance profile of the tracer compiled from the ROI drawn over the PET-CT image (Fig. 2) the radioisotope residential activity of the organs and whole body, whereas Fig. 4 shows the tracer uptake (decay corrected %ID/g using *ex vivo* organ measurement) in various organs after 168-h post injection measured from the gamma counter. Further, we have provided the tracer uptake values (decay corrected %ID/g) from *ex vivo* organ measurement at 4–96 h post injection is given in Table ST2 (supplement data).

In clinical practice, it is common that cold rituximab has been used to pre-block the spleen (this is one of the major antigen sinks), to avoid tracer accumulation during therapy with the Zevalin which is a radiolabeled (^{90}Y labeled) version of a CD20 targeting antibody. This allows more of the antibody-based tracer to circulate, thus increasing availability to target lymphoma cells. Thus, we anticipate this tracer could be a suitable imaging agent against huCD20-positive lymphomas and can serve as an effective tool for noninvasive *in vivo* monitoring of lymphoma growth and metastasis.

The annual dose limit of the radiation doses (specified in Title 21 CFR 361.1 by federal regulations) to the whole body, gonads, active blood-forming organs, and lens of the eye should not exceed 50 mSv (5 rem), and 30 mSv (3 rem) for a single study [25]. The dose absorbed by all other organs should not exceed 150 mSv (15 rem) annually and 50 mSv (5 rem) for a single study. For our tracer, liver (with pre-dose) and spleen (no pre-dose) are the dose-limiting (or critical) organs. Based on our projected human doses, the maximum injected activity per annum would be limited by the liver and spleen; the limits for injected [^{89}Zr]rituximab activity would be 128 MBq (3.5 mCi; liver is a dose-limiting organ) for pre-dose and 41 MBq (1.1 mCi; spleen is dose-limiting organ) for non-blocking estimated by an adult male model. However, our clinical study aimed to image rituximab pre-dosed patients, thus 37 ± 2 MBq (~ 1 mCi) per study/patient will allow us to do multiple scanning per annum without exceeding the annual dose limit. Our estimated limit for administered activity in a single human study appears to be similar to the range reported elsewhere [26]. Further, according to the International Commission on Radiological Protection Publication 106, the adult-limiting dose for 2-deoxy-2- ^{18}F -fluoro-D-glucose (^{18}F FDG) in adults is 0.13 mSv/MBq which translates to 384 MBq (10.4 mCi) per study. In comparison with this limit, our dose limit per study would be significantly less, so that our proposed human dose should lower the absorbed dose in critical organs. Dosimetry studies in human patients are our forthcoming research plan that will provide valuable information to verify and validate the accuracy of the animal model results presented in this report.

Thus our immunoPET tracer is well suited for imaging of B cell lymphoma, since the decay half-life of Zr-89 and the biological half-life of the antibody (78.4 h and 2–7 days [27], respectively), as well as the specific binding to CD20 expressing B cells together will enable this tracer to be able to find small NHL lesions (<1 cm) in patients which might otherwise not be observable either due to higher background activity or loss of signal. Further, [^{89}Zr]rituximab PET tracer give more specific and accurate data when compared to FDG-PET, since immunoPET binds at specific target [28]. Additionally, this tracer is likely to be more useful to monitor Zevalin therapy, instead of [^{111}In]rituximab, a SPECT tracer which is in current clinical practice.

In a similar idea, many immuno-PET tracers have been under development [29], e.g., I-124 labeled CD20 targeting antibody fragments. This tracer clearly demonstrated the possibility for the use of PET imaging in preclinical models. However, this may take a while for optimization and necessary FDA approval for the use of clinical study. Based on our preclinical study data, the [^{89}Zr]rituximab immuno-PET tracer is potentially useful for imaging to evaluate the CD20 marker to plan for radioimmunotherapy treatment particularly for B cell NHL patients. Also, [^{89}Zr]rituximab tracer is sensitive enough to detect even

small lesions, for example indolent lymphomas, the present tracer of [^{18}F] FDG PET is able to detect as low as 50 % [30, 31]. Overall, we believe our immunoPET tracer is sensitive, specific, and ready to translate for NHL patient's image.

Conclusions

The preclinical evaluation of [^{89}Zr]rituximab in a humanized CD20T mouse model showed specific targeting of huCD20 antigen which is expressed on B cells, i.e., identical to the human B cell NHL. The radiation dosimetry estimates obtained from the huCD20TM and extrapolated to an adult male human model showed that the radiation doses in most organs in humans would range between 1 and 391 $\mu\text{Sv}/\text{MBq}$ with cold rituximab blocking and 1–1210 $\mu\text{Sv}/\text{MBq}$ nonblocking. The dose-limiting organs for adult male human patients were liver (391 $\mu\text{Sv}/\text{MBq}$) and spleen (1210 $\mu\text{Sv}/\text{MBq}$) for blocking and non-blocking studies, respectively. Total predicted dosimetry for human patients are 128 and 41 MBq annually for blocking and non-blocking, respectively. These results clearly indicate that the [^{89}Zr]rituximab PET tracer has the potential to image B cell NHL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Radio-HPLC analysis of [^{89}Zr]rituximab at 24 h after labeling. The representative chromatogram profile of [^{89}Zr]rituximab on SEC-3000 column, at 1 ml/min elution of PBS; the radio-chromatogram peak corresponds to an 8 ml peak showing >95 % of radioactivity associated with rituximab. The peak at 12-ml elution corresponds to ^{89}Zr -DTPA (<3 %) that was added (10 mM) to sequester any free Zr-89.

Fig. 2.

Representative PET-CT images of CD20T mice ($n=4$) at 4, 24, 72, and 168 h post injection; mice received [^{89}Zr]rituximab tracer (2–3 MBq). The *in vivo* live mouse images (maximum intensity projection view) are shown with blocking of cold rituximab 2 mg/kg (*Blok* blocking) and tracer alone (*Nblok* non-blocking). Note the clear delineation of the spleen (*arrow*) with blocking and non-blocking of spleen uptake. PET-CT images of mice without blocking show high uptake in the spleen. *H* heart, *L* liver.

Fig. 3.

Clearance profile of $[^{89}\text{Zr}]$ rituximab tracer (i.v. dose 2.8 ± 0.2 MBq) in huCD20 transgenic mice computed from ROI drawn over PET-CT image of Fig. 2. y-axis is percent injected dose per gram (%ID/g) of each organ, and x-axis is time (t) in hours. **a, c** Data from mice that received 2 mg/kg pre-dose of cold rituximab 2 h prior to tracer injection. **b, d** Data from mice that received only the PET tracer. **a, b** Clearance by spleen and liver; **c, d** Clearance profile of heart and whole body. The data represent the mean $\pm$ SD of six mice per group. WB whole body.

Fig. 4. $[^{89}\text{Zr}]$ rituximab biodistribution data ($n=4$, per group) at 168-h post injection by *ex vivo* organ decay corrected cpm value counted in gamma counter after PET/CT imaging. Dose $[2.8 \pm 0.2 \text{ MBq/per mouse}]$ administered by i.v., tail vein injection to huCD20 transgenic mice. Bar graph y-axis is %ID/g against various organs. Data represents the mean %ID/g $\pm$ standard deviation (SD).

Table 1 Absorbed radiation dose estimates for the administration of [^{89}Zr]rituximab

Radiation dose estimates for adult male human ($\mu\text{Sv}/\text{MBq}$) using OLINDA		
Mean absorbed dose$\pm$SD		
Organ	Blocked	Non-blocked
Adrenals	64.8 $\pm$ 9.0	65.1 $\pm$ 3
Brain	0.7 $\pm$ 0.1	0.6 $\pm$ 0
Breasts	17.3 $\pm$ 1.0	16.6 $\pm$ 0.4
Gallbladder wall	108.7 $\pm$ 17.5	75.4 $\pm$ 5.9
LLI wall	3.9 $\pm$ 0.5	5.1 $\pm$ 0.2
Small intestine	17 $\pm$ 2.6	16.3 $\pm$ 1
Stomach wall	31.2 $\pm$ 2.1	58 $\pm$ 1.4
ULI wall	26.6 $\pm$ 4.2	22.1 $\pm$ 1.5
Heart wall	200 $\pm$ 89.1	261.5 $\pm$ 40.3
Kidneys	45.5 $\pm$ 6.5	63.4 $\pm$ 3.1
Liver	390.5 $\pm$ 65.8	248.5 $\pm$ 21.9
Lungs	35.5 $\pm$ 4.2	33.9 $\pm$ 0.1
Muscle	13.6 $\pm$ 1.6	14.9 $\pm$ 0.4
Ovaries	6.9 $\pm$ 1.06	6.4 $\pm$ 0.4
Pancreas	62.5 $\pm$ 6.4	105.5 $\pm$ 3.5
Red marrow	16.2 $\pm$ 2.1	16.4 $\pm$ 0.5
Osteogenic cells	10.8 $\pm$ 1.4	10.9 $\pm$ 0.3
Skin	7.9 $\pm$ 1.1	7.9 $\pm$ 0.3
Spleen	225.5 $\pm$ 9.2	1210 $\pm$ 42.4
Testes	0.9 $\pm$ 0.1	0.9 $\pm$ 0
Thymus	25.9 $\pm$ 3.1	23.5 $\pm$ 2.2
Thyroid	3.2 $\pm$ 0.3	3.1 $\pm$ 0.1
Urinary bladder wall	3.3 $\pm$ 0.5	3 $\pm$ 0.2
Uterus	6 $\pm$ 0.9	5.9 $\pm$ 0.3
Total body	24.5 $\pm$ 2.7	24.9 $\pm$ 0.8
Effective dose equivalent ($\mu\text{Sv}/\text{MBq}$)	70.3 $\pm$ 2.0	124.5 $\pm$ 2.1
Effective dose ($\mu\text{Sv}/\text{MBq}$)	35.1 $\pm$ 5.0	59.7 $\pm$ 2.4

LLI lower large intestine, ULI upper large intestine