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ImmunoPET Imaging of Endogenous and Transfected Prolactin Receptor Tumor Xenografts

Sarah M. Cheal^{†,‡,§}, Shutian Ruan^{†,‡,§}, Darren R. Veach^{†,‡}, Valerie A. Longo^{||}, Blesida J. Punzalan^{†,‡}, Jiong Wu[†], Edward K. Fung^{†,⊥}, Marcus P. Kelly[#], Jessica R. Kirshner[#], Jason T. Giurleo[#], George Ehrlich[#], Amy Q. Han[#], Gavin Thurston[#], William C. Olson[#], Pat B. Zanzonico^{‡,||,⊥}, Steven M. Larson^{†,‡,§,▽}, Jorge A. Carrasquillo^{*,†,‡,§,▽}

[†] Department of Radiology, Memorial Sloan Kettering Cancer Center (MSK), New York, NY 10065, United States

[‡] Molecular Pharmacology Program, MSK, New York, NY 10065, United States

[§] Center for Targeted Radioimmunotherapy and Diagnosis, Ludwig Center for Cancer Immunotherapy, New York, NY 10065, United States

^{||} Small-Animal Imaging Core Facility, MSK, New York, NY 10065, United States

[⊥] Department of Medical Physics, MSK, New York, NY 10065, United States

[#] Regeneron Pharmaceuticals, Inc., Tarrytown, NY 10591, United States

[▽] Department of Radiology, Weill Cornell Medical Center, New York, NY 10065, United States

Abstract

Antibodies labeled with positron-emitting isotopes have been used for tumor detection, predicting which patients may respond to tumor antigen-directed therapy, and assessing pharmacodynamic effects of drug interventions. Prolactin receptor (PRLR) is overexpressed in breast and prostate cancers and is a new target for cancer therapy. We evaluated REGN2878, an anti-PRLR monoclonal antibody, as an immunoPET reagent. REGN2878 was labeled with Zr-89 after conjugation with desferrioxamine B or labeled with I-131/ I-124. In vitro determination of the half-maximal inhibitory concentration (IC₅₀) of parental REGN2878, DFO- REGN2878, and iodinated REGN2878 was performed by examining the effect of the increasing amounts of these on uptake of trace-labeled I-131 REGN2878. REGN1932, a non-PRLR binding antibody, was used as a control. Imaging and biodistribution studies were performed in mice bearing tumor xenografts with various expression levels of PRLR, including MCF-7, transfected MCF-7/PRLR, PC3, and transfected PC3/PRLR and T4D7v11 cell lines. The specificity of uptake in tumors was evaluated by comparing Zr-89 REGN2878 and REGN1932, and in vivo competition compared

*Corresponding Author Telephone: 212-639-2459; Fax: 212-717-3263; carrasj1@mskcc.org.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.molpharmaceut.7b01133](https://doi.org/10.1021/acs.molpharmaceut.7b01133). IC₅₀ data, Western blot xenografts, PET images xenografts, Biodistribution Zr-89 REGN2878, correlation of PRLR sites and uptake (AUC), and kinetic analysis modeling data (PDF)

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Zr-89 REGN2878 uptake in tumor xenografts with and without prior injection of 2 mg of nonradioactive REGN2878. The competition binding assay of DFO-REGN2878 at ratios of 3.53–5.77 DFO per antibody showed IC₅₀ values of 0.4917 and 0.7136 nM, respectively, compared to 0.3455 nM for parental REGN2878 and 0.3343 nM for I-124 REGN2878. Imaging and biodistribution studies showed excellent targeting of Zr-89 REGN2878 in PRLR-positive xenografts at delayed times of 189 h (presented as mean $\pm$ 1 SD, percent injected activity per mL (%IA/mL) 74.6 $\pm$ 33.8%IA/mL). In contrast, MCF-7/PRLR tumor xenografts showed a low uptake (7.0 $\pm$ 2.3%IA/mL) of control Zr-89 REGN1932 and a very low uptake and rapid clearance of I-124 REGN2878 (1.4 $\pm$ 0.6%IA/mL). Zr-89 REGN2878 has excellent antigen-specific targeting in various PRLR tumor xenograft models. We estimated, using image-based kinetic modeling, that PRLR antigen has a very rapid in vivo turnover half-life of ~14 min from the cell membrane. Despite relatively modest estimated tumor PRLR expression numbers, PRLR-expressing cells have shown final retention of the Zr-89 REGN2878 antibody, with an uptake that appeared to be related to PRLR expression. This reagent has the potential to be used in clinical trials targeting PRLR.

Graphical Abstract

Keywords

immunoPET; prolactin receptor; Zr-89; I-124

INTRODUCTION

The prolactin receptor (PRLR) is a transmembrane receptor belonging to the class-1 cytokine receptor superfamily; its main ligand is prolactin, and its normal function is related to reproductive biology.¹ PRLR is expressed in a subset of breast and prostate cancers and is implicated in the pathogenesis.^{2–4} A phase I immunotherapy study demonstrated the safety of naked antibody administration, although no antitumor response was observed.⁵ We have validated the expression of PRLR in human breast cancers and produced fully human anti-PRLR monoclonal antibodies, including REGN2878, the antibody used in this study.⁶ Our related studies demonstrated the rapid internalization of PRLR and anti-PRLR antibodies and showed that the rapid lysosomal degradation of PRLR antibody drug conjugates (ADC) leads to potent in vitro cytotoxicity, despite PRLR having only moderate cell surface

expression (~30 000 PRLR per cell).⁷ Preclinical pharmacology studies of REGN2878 ADC demonstrated significant antitumor activity in a number of breast cancer xenograft models.⁶ Furthermore, PRLR is also expressed in ovarian cancer, and others have recently taken advantage of a gadolinium magnetic resonance imaging ligand that binds to PRLR and internalizes and visualizes tumor xenografts.⁸ Thus, PRLR is a potential therapeutic and imaging target in PRLR-bearing tumors. Several immunoPET antibodies have been used in preclinical and clinical trials for tumor imaging, theranostics, and assessment of tumor response.^{9–12} Although positron-emitting radionuclides, such as Cu-64 and Ga-68, have been used for immunoPET,^{13,14} their short half-lives, 12.7 and 1.1 h, respectively, are suboptimal for the slow tumor-localizing kinetics of intact immunoglobulins, and thus, there has been great interest in using longer-lived positron emitters, such as Zr-89 and I-124, with half-lives of 78.4 and 100.4 h, respectively. For example, Zr-89 trastuzumab has been used as a biomarker to evaluate inpatient heterogeneity of trastuzumab uptake in HER2-positive breast cancer and to correlate the uptake with the treatment response in patients treated with the trastuzumab emtansine (T-DM1) antibody-drug conjugate. In this setting, combining Zr-89 trastuzumab and FDG PET predicted the response to treatment with T-DM1.¹⁵ In addition, early changes in the Zr-89 trastuzumab uptake in patients with breast cancer have correlated with CT changes in the size of individual lesions in patients treated with an HSP90 inhibitor.¹⁶ Further, a recent report has suggested that patients with prior biopsy-proven, HER2-negative disease may be identified as HER2-positive by Zr-89 trastuzumab imaging and selected for a therapeutic intervention that they would not otherwise receive.¹⁷

REGN2878 is a fully human monoclonal antibody to PRLR that has demonstrated therapeutic potential as an ADC.⁶ The rationale for developing a radiolabeled REGN2878 is to potentially use this reagent for patient selection, assessment of PRLR engagement during therapy, heterogeneity of PRLR expression, and prediction of tumor response in patients undergoing PRLR-directed therapy. As a first step in developing a radiolabeled antibody for imaging or theranostic purposes, preclinical studies are required to determine the feasibility of labeling, biodistribution, and pharmacokinetics. In this report, we investigated the ability of Zr-89 REGN2878 to target PRLR-expressing tumor xenografts and characterized its biodistribution in tumor xenograft models. REGN2878 binds to human PRLR but not to the mouse.⁶ We also determined the relationship of antigen density with the tumor uptake, assessed specificity of the uptake, and compared Zr-89 REGN2878 targeting to radioiodinated REGN2878 to obtain data on the internalization rate in vivo.

EXPERIMENTAL SECTION

Antibody Reagents.

REGN2878 is an IgG1 monoclonal antibody that binds to PRLR and was provided at 50.8 mg/mL.⁶ The isotype-control antibody REGN1932 is a human IgG1 antibody that recognizes the cat allergen Fel d 1 and was provided at 50.9 mg/mL. A 27.7-kD human PRLR ectodomain protein with carboxy-terminal myc and histidine tags (hPRLR) was used in antigen binding assays. All antibodies and PRLR ectodomain proteins were provided by Regeneron Pharmaceuticals, Inc. (Tarrytown, NY).

Conjugation.

The antibodies were conjugated with isothiocyanate-desferrioxamine B (SCN-DFO, Macrocyclics, Dallas, TX) as previously described.¹⁸ The DFO chelate-to-antibody conjugation ratios were determined by isotope titration as previously described.¹⁹ The antibody concentration was determined by ultraviolet (UV) spectroscopy (absorbance at 280 nm).

Radioimmunoassays.

To assess the effect of the conjugation with DFO on REGN2878, an in vitro competition assay was performed to determine the half-maximal inhibitory concentration (IC₅₀) with each of the three DFO to REGN2878 conjugation ratios of 3.53, 4.23, and 5.77 to 1, and the original nonconjugated REGN2878 was a control, as well as a REGN1932 non-PRLR binding antibody. The competition assay was performed using I-131 REGN2878 at a specific activity (SA) of 454 MBq/mg as the tracer. To determine the IC₅₀, 3 ng of I-131 REGN2878 was mixed with increasing concentrations of either nonradioactive REGN2878, the various DFO-REGN2878 conjugates, or I-127 REGN2878 (labeled using stable I-127, as described below), or REGN1932 in concentrations ranging from 1×10^{-6} to $1 \mu\text{M}$ per tube using 5×10^5 MCF-7/PRLR cells in a final volume of 0.5 mL. After a 60 min incubation period at room temperature (RT), the cells were centrifuged and washed with PBS, and the cell pellet was counted in a gamma counter to determine the bound fraction of I-131 REGN2878. The data were then fit using one-site-fit log IC₅₀ (Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA).

Immunoreactivity was determined using increasing numbers of MCF-7/PRLR cells (from 0.5 to 5 million). Approximately 2 ng of antibody was mixed with the cell in a final volume of 0.5 mL for 60 min at RT. The cell-bound activity was counted after centrifugation and discarding the supernatant. Data was analyzed as using the Lindmo method.²⁰ In addition, the immunoreactivity/specificity of binding was confirmed using an in vitro binding assay with excess amounts of hPRLR, followed by size-exclusion high-pressure liquid chromatography (SE HPLC; column: TSKgel 3000SW 7.5 mm $\times$ 30 cm, 10 μm (TOSOH Bioscience); running buffer: 100 mM citrate, 100 mM NaCl pH 6.71; flow rate: 1.2 mL/min; UV detection: 280 nm; HPLC system: Shimadzu LC-20AB Prominence Liquid Chromatograph with SPD-20A UV/vis Detector and Bioscan Flow-Count Radiodetector). The SE HPLC traces of non-radioactive REGN2878, radiolabeled REGN2878, and Zr-89 REGN1932 were compared to UV or radioactivity tracings after these antibodies were mixed together with excess hPRLR to generate high molecular weight antibody—PRLR complexes (Figure 1).

The affinities of three nonradioactive conjugated reagents with DFO to REGN2878 ratios of 3.53, 4.23, 5.77 to 1 and the original nonconjugated REGN2878 as a control were measured in surface plasmon resonance biacore experiments performed on a Biacore T200 instrument (GE Healthcare, Piscataway, NJ) using a dextran-coated (CM5) chip at 37 °C. The running buffer was HBS-ET filtered (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.05% polysorbate 20, pH 7.4). The capture surface was prepared by covalently immobilizing the goat antihuman Fc antibody (GE Healthcare, Piscataway, NJ) to the sensor chip using (1-

ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride)/N-hydroxysuccinimide (EDC/ NHS) coupling chemistry. Antibodies were captured through their Fc regions on the antihuman Fc antibody immobilized sensor chip and were tested for binding to the monomeric extracellular domain of human PRLR with carboxy-terminal myc and a histidine tag. PRLR solutions were prepared at a concentration range between 200 and 6.25 nM and individually injected over antibody (parental REGN2878), DFO conjugate (3 DFO-REGN2878s), and isotype control (REGN1932) captured surfaces. All capture surfaces were regenerated with one 15-s pulse of 10 mM glycine—HCl, pH 1.5 (GE Healthcare). Kinetic parameters were obtained by globally fitting the data to a 1:1 binding model using Biacore T200 evaluation software. The equilibrium dissociation constant (K_D) was calculated by dividing the dissociation rate constant (k_d) by the association rate constant (k_a).

Zr-89 was provided in-house by the Radiochemistry and Molecular Imaging Probes Core Facility using methods previously described with minor modifications.²¹ I-124 was either provided in-house or purchased commercially (IBA Molecular, Richmond, VA). I-131 was purchased from Nordion (Ottawa, ON, Canada). DFO-REGN2878 (DFO to REGN2878 ratio of 3.03) and DFO-REGN1932 (DFO to REGN1932 ratio of 2.89) were labeled with Zr-89 at specific activities of 110 to 223 MBq/mg and were used for biodistribution and imaging.¹⁸ Postprocessing radiochemical purity (RCP) averaged 99%, as determined by instantaneous thin-layer chromatography (ITLC) using 5 mM DTPA. In addition, REGN2878 was labeled with either I-124, I-131, or I-127 using the iodogen method.²² I-124 REGN2878 was prepared at a SA of 140 MBq/mg and was used for imaging and biodistribution; RCP was 98% by ITLC with 0.9% NaCl. For biodistribution and radioimmunoassay, REGN2878 was labeled with I-131; the SA was 179–453 MBq/mg and RCP was 99% by ITLC with 0.9% NaCl. Briefly, to prepare I-127 REGN2878, 2 mg of stock REGN2878 (13.4 nmol) was mixed with 80 μ L of 0.2 M sodium phosphate, pH 7.4, in a precoated iodogen tube (Thermo Scientific Pierce). Next, 29 μ L of 0.1 mg/mL NaI (19.1 nmol) in 1 mM NaOH was added. After 5 min at room temperature, the reaction was transferred to a new tube containing 50 μ L of iodogen-stop buffer [10 mg/mL of tyrosine (saturated), 10% glycerol, 0.1% xylene cyanol in PBS] and purified using a PD10 column that was pre-equilibrated and eluted with PBS.

Cell Lines.

A total of five PRLR-expressing cell lines were studied. Cell lines were cultured as described in the Supporting Information (cell culture conditions) and used for subsequent xenograft generation and/or binding studies. PRLR sites per cell ranged from approximately <5000 to ~185 000 as previously determined by quantitative flow cytometry analysis as described by Andreev et al. and Kelly et al.^{6,7} The cell lines used included T47Dv11 breast cancer cells, a variant of parental T47D cells developed by in vivo passaging⁶ that have approximately 25 000–30 000 PRLR sites per cell. In addition, we used low PRLR-expressing MCF-7 breast cancer (5000–10 000 PRLR sites per cell) and PC3 prostate cancer (estimated as <5000 PRLR sites per cell). Furthermore, these latter two cell lines were transduced to overexpress PRLR,⁶ resulting in MCF-7/PRLR (~185 000 PRLR sites per cell) and PC3/PRLR (~25 000 PRLR sites per cell). In summary, the rank of PRLR

expression from lowest to highest is PC3 < MCF-7 < PC3/PRLR $\approx$ T47Dv11 < MCF-7/PRLR.

Tumor Xenografts.

All animal studies were carried out under an IACUC approved protocol at MSK. Tumor xenografts of MCF-7 and MCF-7/PRLR were generated by injecting female athymic nude mice (Harlan Laboratories, Inc., Madison, WI) that had been pre-implanted (3 d prior to inoculation) with extended release 0.72-mg β -estradiol pellets (Innovative Research of America, Sarasota, FL) and inoculated with 1.5×10^7 cells per mouse subcutaneously (s.c.) in 0.2 mL of 50% Matrigel plus phosphate-buffered saline (PBS). Tumor xenografts of T47Dv11 were generated in female CB17 SCID mice (Taconic Biosciences, Inc., Hudson, NY) that were likewise pre-implanted with extended release 0.72-mg β -estradiol pellets and then received 7.5×10^6 T47Dv11 cells/ mouse in 0.2 mL of 50% Matrigel plus PBS. Tumor xenografts of PC3 and PC3/PRLR were generated in CB17 SCID male mice by injecting 5×10^6 cells per mouse s.c. in 0.2 mL of 50% Matrigel plus PBS.

Western Blotting of Tumor Xenograft PRLR Expression.

The expression of PRLR protein in ex vivo tumor xenografts was assessed using Western blotting. A sample of T47Dv11 cells was used as a positive control. Frozen tumors were homogenized in RIPA buffer (Cell Signaling Technologies, Danvers, MA) with protease and phosphatase inhibitors (Thermo Scientific, Waltham, MA) and sonicated briefly (QSonica, Newton, CT). Lysates were resolved on 4–12% Novex tris-glycine gels and blotted to PVDF membranes (Thermo Fisher, Waltham, MA). Membranes were labeled with primary antibodies for PRLR (1A2B1, Thermo Fisher) or betaactin (GeneTex, Irvine, CA). Labeling with primary antibodies was followed by a HRP-conjugated secondary antibody (Promega, Madison, WI) at 1:2000 and chemiluminescent detection.

Zr-89 REGN2878, I-124 REGN2878, and Zr-89 REGN1932 ImmunoPET Imaging in the MCF-7/PRLR Xenograft Model.

The initial imaging study was performed using MCF-7/PRLR tumor xenografts (established using the PRLR high MCF7/PRLR cell line). In order to determine specificity of tumor uptake, Zr-89 REGN2878 and I-124 REGN2878 were administered and compared to the non-specific uptake of the Zr-89 REGN1932 control antibody. Five animals were injected with each antibody and underwent serial PET imaging (Inveon microPET/microCT, Siemens Medical Solutions USA, Inc., Malvern, PA) on the day of injection and approximately 1, 3, 4, and 8 d post-injection (p.i.) for mice receiving Zr-89-labeled antibodies, and on the day of injection and approximately 1, 3, and 7 d p.i. for mice receiving I-124 REGN2878; differences in days of acquisition were due to logistical reasons. The mice receiving Zr-89 REGN2878, Zr-89 REGN1932, and I-124 REGN2878 were euthanized immediately following the last imaging time at 192 ± 0.1 , 189 ± 0.1 , and 167 ± 0.04 h p.i., respectively, and the major organs were harvested, weighed, and counted in a gamma counter (Wizard Automatic Gamma Counter, Perkin Elmers, Waltham, MA) to determine biodistribution in organs. Tumor sizes were in a similar range, measuring at 0.175 ± 0.047 , 0.259 ± 0.128 , and 0.269 ± 0.124 g for Zr-89 REGN2878, Zr-89 REGN1932, and I-124 REGN2878 groups, respectively. The administered activities were 4.51 ± 0.04 , 4.51 ± 0.26 , and 4.81 ± 0.08 MBq

for Zr-89 REGN2878, Zr-89 REGN1932, and I-124 REGN2878, respectively. Whereas the mass administered of Zr-89 REGN2878, Zr-89 REGN1932, and I-124 REGN2878 was statistically different among the three groups (one-way analysis of variance (ANOVA): $p < 0.003$), the differences were small, consisting of 26.8 ± 0.2 , 24.5 ± 1.4 , and $34.2 \pm 0.5 \mu\text{g}$, respectively. Note that the masses of the antibody administered were smaller for biodistribution/nonimaging studies (see below) because less radioactivity was needed for those than for the imaging. No blocking of the thyroid was performed.

Zr-89 REGN2878 Biodistribution in Multiple Tumor Xenograft Models.

A second imaging and biodistribution study was performed in order to determine how tumor uptake was related to PRLR expression. Here, comparative biodistribution and imaging studies were performed in all five PRLR-expressing cell line xenograft models. Uptake in the low-endogenous PRLR-expressing parental cell line MCF-7 xenograft was compared to the high-expressing MCF-7/PRLR xenograft model. In addition, uptake in PC3 xenografts was compared to that in the PRLR moderate PC3/PRLR model. Lastly, the T47Dv11 xenograft model that had moderate endogenous expression of PRLR was also evaluated.

Biodistribution studies with gamma counting of tissues were performed in mice bearing MCF-7, MCF-7/PRLR, PC3, PC3/PRLR, and T47Dv11 tumor xenografts at 18–20, 41–45, 65–66, and 138 h p.i. of $522 \pm 26 \text{ kBq}$ and $4.7 \pm 0.2 \mu\text{g}$ of Zr-89 REGN2878. The tumor weights at the time of euthanasia (138 h) were 0.12 ± 0.08 , 0.15 ± 0.08 , 0.42 ± 0.16 , 0.35 ± 0.15 , and $0.30 \pm 0.13 \text{ g}$ for MCF-7 ($n = 4$), MCF-7/PRLR ($n = 5$), PC3 ($n = 5$), PC3/PRLR ($n = 5$), and T47Dv11 ($n = 5$) xenografts, respectively.

Imaging studies were performed in groups of three mice bearing xenografts of MCF-7, MCF-7/PRLR, PC3, PC3/PRLR, and T47Dv11 (T47Dv11 had only two animals imaged), using Zr-89 REGN2878 $5.51 \pm 0.41 \text{ MBq}$ and $24 \pm 2 \mu\text{g}$. Imaging was performed at approximately 1, 2, 3, and 6 d p.i. using the Inveon microPET/microCT scanner. These data were used for nonlinear kinetic compartmental modeling analysis of the association and internalization rates, as well as to determine the antigen density for each animal model^{23,24} (Supporting Information).

Specificity of Zr-89 REGN2878 was determined, as described above, by comparing the tumor uptake to control Zr-89 REGN1932 in PRLR-expressing xenografts. In addition, the in vivo specificity of the Zr-89 REGN2878 uptake was also confirmed by comparing the tumor uptake at ~48 h gamma counting in mice bearing PC3/PRLR xenografts ($n = 3$, tumor weight: $0.16 \pm 0.8 \text{ g}$) preblocked with 2 mg of cold REGN2878 (13.4 nmol) administered by iv immediately prior to administration of Zr-89 REGN2878 ($503 \pm 26 \text{ kBq}$, $4.6 \pm 0.2 \mu\text{g}$; $31 \pm 1 \text{ pmol}$) versus the uptake in mice bearing PC3/PRLR tumor xenografts ($n = 5$, tumor weight: $0.27 \pm 0.14 \text{ g}$) after administration of Zr-89 REGN2878 (514 kBq , $5 \mu\text{g}$) without preblocking.

Dual Isotope Biodistribution.

A dual-isotope biodistribution dissection study was performed in mice with MCF-7/PRLR tumor xenografts comparing Zr-89 REGN2878 and I-131 REGN2878. Five animals per group (median tumor weight was $0.247 \pm 0.139 \text{ g}$) were injected with a mean of 725 ± 15

and 181 ± 4 kBq of I-131 REGN2878 (179 MBq/mg) and Zr-89 REGN2878 (201 MBq/mg), respectively, and were euthanized at 2, 24, 119, and 167 h p.i. for assay ex vivo.

Dual isotope counting was performed by counting Zr-89 in a 330- to 850-keV window and I-131 in a 210- to 330-keV window. Pure samples of I-131 and Zr-89 were counted to determine the respective calibration factors and the corrections for cross-talk.

Statistics.

Data were expressed with descriptive statistics using mean, median, and standard deviation. A *t* test comparison was performed when comparing two groups, and one-way ANOVA with Tukey post hoc analysis was used for multigroup comparison and Pearson correlation (Sigmastat 3.5, San Jose, CA) to determine the correlation between the PRLR content and tumor uptake.

RESULTS

Antibody Characterization.

Using a competition assay, the IC₅₀ for the parent nonconjugated antibody was 0.344 nM. With increasing DFO to REGN2878 ratios of 3.53, 4.23, and 5.77, there was an increase in IC₅₀ to 0.4917, 0.6318, and 0.7136 nM, respectively. Iodination of REGN2878 with I-127 yielded an IC₅₀ of 0.3343 nM. In contrast, the nonspecific antibody REGN1932 showed <20% inhibition at 1 μ M concentration (Figure S1).

Extrapolated immunoreactivity using MCF7/PRLR ranged from 67 to 95% for Zr-89 REGN2878; the range for I-131 REGN2878 was 108–120%, reflecting some statistical noise in the extrapolation and essentially no binding (0.1%) for the nonspecific Zr-89 REGN1932 antibody. A SE HPLC assay confirmed the above findings, namely, that most of the REGN2878 was immunoreactive, as shown by complex formation when the nonradioactive parent REGN2878 (data not shown) and the Zr-89 and I-131/I-124 REGN2878 were incubated with the hPRLR, and that no complexes formed when control antibody REGN1932 was used, indicating specificity of the radioimmunoconjugate (Figure 1). The differences in the shape of higher molecular complex peaks in the HPLC tracing (Figure 1), that shows double peaks for I-131REGN2878 incubated with hPRLR, may be related to 2 different size complexes possibly related to slight changes in stoichiometry between the concentration of the antibody and the hPRLR antigen. Using surface plasmon resonance, the equilibrium dissociation constants (K_D) for the parental nonconjugated antibody was 4.20 nM. With increasing DFO to REGN2878 ratios of 3.53, 4.23, and 5.77, there was a minimal decrease in K_D to 4.25, 4.75, and 4.96 nM, respectively (Table S1).

Zr-89 REGN2878, I-124 REGN2878, and Zr-89 REGN1932 ImmunoPET in MCF-7/PRLR.

Representative images of MCF-7/PRLR tumor-bearing mice receiving Zr-89 REGN2878, I-124 REGN2878, and Zr-89 REGN1932 are shown in Figure 2. Serial imaging with both Zr-89-labeled antibodies visually demonstrated similar clearing from the blood pool and accumulation in all normal organs, with the exception of statistically significant changes in the liver and bone (*t* test $p = 0.012$) and a trend in the spleen ($p = 0.053$) (Figure 2, graph

insert). In contrast, MCF-7/PRLR tumor uptake of PRLR-targeted Zr-89 REGN2878 (mean: $74.6 \pm 33.8\%$ of the injected activity per gram (%IA/g) at 189 h) was greater than that of control Zr-89 REGN1932 (mean $7.0 \pm 2.3\%$ IA/g at 189 h) and also greater than that of I-124 REGN2878 (mean $1.4 \pm 0.6\%$ IA/g at 167 h) (Figure 2, graph insert). Visually, the uptake of I-124 REGN2878 did not show definite tumor uptake or an increase in uptake over time. There was, however, increased I-124 thyroid uptake. In addition, the whole-body clearance of the I-124 radioactivity was more rapid than that for the two Zr-89-labeled antibodies, with an area under the curve (AUC) for the period of observation of $9191 \pm 721\%$ IA * h for I-124 REGN2878 compared to $11720 \pm 504\%$ IA * h for Zr-89 REGN2878 and $12161 \pm 2244\%$ IA * h for Zr-89 REGN1932, with significant differences between the I-124 REGN2878 and both Zr-89-labeled antibodies (ANOVA: $p = 0.017$ and 0.042).

Serial ImmunoPET in Various Tumor Xenograft Models.

These studies sought to relate PRLR expression of the models to in vivo uptake of Zr-89 REGN2878. Western blot analysis of the tumor xenografts showed that the ranking of PRLR expression in the xenografts was consistent with the ranking of the in vitro cell lines (PRLR expression: PC3 < MCF-7 < PC3/PRLR $\approx$ T47Dv11 $\ll$ MCF-7/PRLR; Figure S2). Serial MIP images of Zr-89 REGN2878 in tumor xenografts (Figures S3, S4, and S5) show a similar pattern of tracer distribution with a blood pool uptake that is maximal at the earliest time and decreases over time. In addition, tumor uptake is not seen or is minimal at the earliest time point (1 h p.i.), with a definite tumor uptake by ~ 24 h p.i. that increases slightly over time. Tumor uptake in mice undergoing imaging with Zr-89 REGN2878 ($\sim 14.1 \pm 0.7$ μ g) and euthanized at 6 d p.i. showed $16.4 \pm 10.4\%$ IA/mL in MCF-7 xenografts ($n = 3$; 140.8 h) and $61.4 \pm 13.4\%$ IA/mL in MCF-7/PRLR xenografts ($n = 3$; 141 h); as an example, Figure S3 shows higher tumor uptake at all times in the higher PRLR-expressing MCF-7/PRLR cell xenografts. An average uptake of $6.3 \pm 1.1\%$ IA/mL was seen in PC3 xenografts ($n = 3$; 142.6 h) compared to $27.4 \pm 3.3\%$ IA/mL in PC3/PRLR xenografts ($n = 3$; 143 h) (Figure S4) and $34.0 \pm 0.03\%$ IA/mL in TD47v11 xenografts ($n = 2$; 143 h); as an example of T47Dv11, see Figure S5.

Tumor uptake at ~ 138 h p.i. based on the biodistribution studies with Zr-89 REGN2878 (4.7 ± 0.2 μ g) were $40.0 \pm 2.4\%$ IA/g in MCF-7 xenografts ($n = 4$) and $29.0 \pm 18.1\%$ IA/g in MCF-7/PRLR xenografts ($n = 5$) (Figure 3A,B, respectively); $7.6 \pm 1.5\%$ IA/g in PC3 xenografts ($n = 5$) and $27.1 \pm 6.5\%$ IA/ g in PC3/PRLR xenografts ($n = 5$) (Figure S6A,B, respectively); and $29.6 \pm 6.4\%$ IA/g in TD47v11 xenografts ($n = 5$) (Figure S6C). All other organs showed a low uptake of Zr-89 REGN2878 accumulation and very little difference between transfected and non transfected lines (Figures 3 and S6).

In vivo competition with pre-administration of 2 mg of cold REGN2878 showed significant blocking of the tumor uptake in PC3/PRLR tumor xenografts of $10.2 \pm 1.6\%$, compared to $25.2 \pm 3.4\%$ IA/g without blocking ($p = 0.001$). The blocked tumor had a similar uptake to that of the nontransfected PC3 tumor xenograft, $8.5 \pm 1.6\%$ IA/g ($p = 0.49$) (Figure 4).

Zr-89 REGN2878 imaging and biodistribution studies typically showed higher tumor uptake in the PRLR-transfected cell lines than the corresponding parental lines (Figure 5). Also, tumor uptake in the intermediate, endogenously PRLR-expressing TD47v11 cell line

(Figures S5 and 5) was generally higher than that in the low-expressing PC3 (Figure S4, upper row) and MCF-7 cell lines (Figure S3, upper row). For all xenografted mice, the blood pool was more prominent at the initial times and decreased over time, and mild liver uptake was noted through the entire series of images but did not show notable increases over time, as confirmed in the biodistribution studies (Figures 3 and S6). The uptake in all other normal organs was relatively low and visually similar among the various tumor xenograft groups.

When tumor uptake, based on 6 d of serial imaging, as shown in Figure 5, was integrated for MCF-7, MCF-7/PRLR, PC3, PC3/PRLR, and TD47v11, the AUCs were 2235, 6635, 1017, 2875, and 3694%IA*h/mL, respectively. When we correlate this AUC with PRLR sites previously measured in the MCF-7, MCF-7/PRLR, PC3/PRLR, and TD47v11 cell lines, there was a significant correlation (Pearson correlation $r = 0.98$, $p = 0.02$) (Figure S7).

Dual-Isotope Biodistribution Studies.

Dual-isotope biodistribution studies comparing I-131 and Zr-89-labeled REGN2878 in the same mice bearing MCF-7/PRLR showed very little concentration of I-131 REGN2878 in the tumor compared to the high concentration seen with Zr-89 REGN2878 at the various time points (Table 1); for example, at 168 h, the uptake was 1.3 ± 0.3 vs $60.3 \pm 22.9\%$ IA/g, respectively. Organ concentrations were higher for Zr-89 REGN2878 compared to I-131 REGN2878.

DISCUSSION

In the current study, we demonstrated the ability of Zr-89 REGN2878 to target PRLR-expressing tumor xenografts and, using a panel of xenografts with different levels of expression of PRLR, also demonstrated the positive relationship between the level of PRLR expression and tumor uptake. In contrast to Zr-89 REGN2878, PRLR targeting of radioiodinated REGN2878 was considerably lower.

To the best of our knowledge, this is the first study with a radiolabeled antibody to evaluate targeting to PRLR. Compared to other radiolabeled antibodies targeting other antigens, the degree of tumor uptake of Zr-89 REGN2878 in PRLR-bearing xenografts is high, and we considered it feasible to use this reagent in a clinical trial. Alternative approaches to imaging PRLR, based on the tight binding of human placental lactogen (hPL) to the PRLR receptor, using MRI or a near-infrared fluorescence imaging agent have been reported.⁸ Although direct comparison of our work to the latter is difficult because of different tumor models and reagents, one advantage of PET imaging is its quantitative abilities. Furthermore, additional studies evaluating safety issues due to the risk of tumor stimulation mediated by activating PRLR signaling by hPL would also need to be addressed with imaging approaches involving hPL.

We demonstrated the specificity of uptake in two ways: first by showing that the tumor uptake could be blocked by preadministration of the same specific REGN2878 antibody, and second by comparing the uptake of Zr-89 REGN2878 to that of Zr-89 REGN1932, a non-PRLR-specific antibody, in PRLR-positive tumor xenografts. Aside from the significant difference in the tumor uptake of Zr-89 REGN2878 and the nonspecific Zr-89 REGN1932,

there were some smaller but significant differences in the uptake in some normal tissues, including the liver, bone, and spleen. The latter differences were not further investigated but could be due to differential catabolism/radiometabolites of the tumor-targeting REGN2878 and the passive clearance of the nontargeted REGN1932.

Prior to in vivo studies, the binding of parental REGN2878 and REGN2878 radioimmunoconjugates was characterized to ensure that radiolabeling did not adversely affect antibody binding. Analysis of DFO-conjugated REGN2878 by competitive binding studies detected some dose-dependent increase of the IC₅₀, but at a 3.53 to 1 ratio, it was only 1.4-fold different. Furthermore, increasing DFO to REGN2878 ratios of 3.53–5.77 showed small changes in K_D compared to the nonconjugated parent REGN2878 (Table S1). It should also be noted that in spite of the slight drop in IC₅₀, the immunoreactivity of the Zr-89 or I-131 REGN2878 was excellent.

Our imaging data is consistent with antigen-dependent tumor uptake, as evidenced by the 3.7–4.3 lower Zr-89-REGN2878 uptake in the low antigen-expressing PC3 and MCF-7 xenografts compared to their higher PRLR-expressing PC3/PRLR and MCF-7/PRLR counterparts. Interestingly, the tumor uptake in the TD47v11 tumor xenografts (~25 000–30 000 sites per TD47v11 cell line) was $34.0 \pm 0.03\%$ IA/g, which was somewhat similar to the $27.4 \pm 3.3\%$ IA/g in the PC3/PRLR xenograft (~25 000 sites per PC3/PRLR cell line). This is consistent with the premise that PC3/PRLR cells have similar but lower PRLR levels than T47Dv11 cells and is supported by the ex vivo analysis of PRLR levels by Western blot. This Western blot data also demonstrated that the tumor xenografts showed generally similar rankings of PRLR expression as the cell lines in vitro, suggesting that the significant alteration of PRLR expression did not occur in vivo. This is of interest, as estrogen has been shown to increase the expression of PRLR in MCF7 cells.²⁵ It was possible that the estrogen pellets implanted to support the tumor xenograft growth may have led to the increased expression of PRLR in MCF7 tumors in vivo, although Western blots did not confirm this. In addition, our modeling data (Figure S8) shows a similar ranking of PRLR sites per cell in the xenografts as in the cell lines, with the exception of slightly higher estimates for the MCF-7 cell line. Interestingly, when we ranked the AUC order of antibody uptake %IA**h*/mL in xenografts versus the estimated number of sites per cell for the various corresponding cell lines, we observed a similar rank order. Overall, this suggests that significant alterations in the relative rank order of PRLR expression did not occur between in vitro cell culture and in vivo growth as tumor xenografts, and that Zr-89 REGN2878 uptake in the different tumor models was dependent on the relative PRLR levels.

Biodistribution of I-131- or I-124-labeled REGN2878 both showed poor tumor uptake, which was expected since they have the same chemical properties. We and others have shown that rapidly internalizing radiohalogenated antibodies may have suboptimal localization in the tumor because of the rapid release of halogens once internalization occurs and the breakdown of the antibody occurs in lysosome.^{26,27} Nonetheless, because some studies with internalizing antibodies have shown adequate targeting in vivo,¹² we considered it important to evaluate the iodinated REGN2878 to assess the degree of targeting and in order to obtain information to assess the speed of internalization. Using kinetic modeling of the imaging data, we documented rapid internalization (14 min) (Figure S9) consistent with

the previous results.²⁸ This internalization half-life^{7,28} is much faster than other tumor-receptor antibody–antigen complexes that we have studied, such as J591/PSMA, which showed an internalization half-time of 8–9 h.²³ The rapid internalization of PRLR in vivo corresponds well to our previous work, demonstrating the rapid and constitutive internalization of PRLR to lysosomes in cellular assays.⁷ Together, these results further validate that PRLR may be a suitable target for REGN2878 antibody—drug conjugates, since rapid and significant internalization is required for the release of active cytotoxics in ADCs.

In conclusion, residualizing Zr-89 REGN2878 antibody is an excellent reagent to specifically target PRLR in tumor xenografts. In contrast, neither I-124 or I-131 REGN2878 were adequate for tumor imaging, consistent with rapid intracellular dehalogenation following internalization. Together, this work suggests that Zr-89 REGN2878 may have utility in assessing the expression of PRLR in normal tissues and tumors in vivo, and that tumor localization of the antibody could be assessed by immuno-PET imaging. This work supports further studies aiming to correlate the uptake of Zr-89 REGN2878 observed in PRLR-positive tumor xenografts with responses to PRLR-directed ADCs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Size-exclusion HPLC tracings of Zr-89 REGN2878, I-124- or I-131-labeled REGN2878, and REGN1932 with retention times (min) shown in the boxes above the peaks. Zr-89 REGN2878, I-124- or I-131-labeled REGN2878, and REGN1932 showed predominantly a single peak that had a retention time as expected for a 150-kD-molecular weight antibody (10.1–10.7 min). After incubation of the radiolabeled antibodies with hPRLR (binding/immunoreactivity assay), the radioactivity retention time shifted to the left, indicating high molecular weight complexes (typically 7.3–7.6 min). In the case of the nonspecific antibody REGN1932, there was no shift in the retention time, indicating no significant binding with hPRLR.

Figure 2.

Serial imaging data of representative MCF-7/PRLR tumor-bearing nude mice with Zr-89 or I-124 REGN2878 and nonspecific Zr-89 REGN1932. Imaging data were parametrized in terms of %IA/mL, and the image gray scale was set to a maximum value of 50%IA/mL for all mice. The highest tumor uptakes are seen with the Zr-89-labeled specific antibody. Much lower uptake is seen with the Zr-89-labeled nonspecific antibody. At the end of imaging (8 d for Zr-89 REGN2878 and 7 d for I-124 REGN1932), animals were euthanized, and harvested organs were weighed and counted in a gamma counter, with data expressed as %IA/g (graph insert). Nonspecific accumulation in the liver and bone is seen with the Zr-89-labeled antibodies. The uptake of Zr-89 in REGN2878 vs REGN1932 was similar in all of the normal organs, except for mild differences in the liver, bone, and spleen. I-124 REGN2878 shows a faster clearance from the whole body than Zr-89-labeled antibodies and a lower uptake in the tumor, liver, and other organs. In contrast, there is increasing accumulation in the thyroid over time (the thyroid was not blocked). Focal pelvic uptake seen at the 2 h time point in mice receiving Zr-89 antibodies represents excretion in the bladder.

Figure 3.

The biodistribution study performed after the injection of Zr-89 REGN2878 in mice xenografted with MCF-7 (A) and MCF-7/PRLR (B). Animals were euthanized at 20, 41, 65, and 138 h after injection. The harvested normal organs and tumor were weighed and counted in a gamma counter. Visually, normal organs and blood pool had similar uptakes in both cell line xenografts. In contrast, the tumor uptake (%IA/g) was significantly higher for those animals with MCF-7/PRLR (with an exception of the 138 h) as was the area under the time activity curve (data not shown).

Figure 4.

The tumor uptake 2 days following the injection of Zr-89 REGN2878 was high in the PC3/PRLR xenografts $25.1 \pm 3.4\%$ IA/g compared to the lower uptake of $10.2 \pm 1.6\%$ IA/g when blocked by 2 mg of the competing excess cold REGN2878, which was administered by iv immediately prior to Zr-89 REGN2878. The uptake upon the blockade was in the same range of the very low PRLR-expressing PC3 parent cell line.

Figure 5.

Serial tumor xenograft uptake of intravenous Zr-89 REGN2878, over 6 days, in mice transfected with various cell lines, is plotted (mean and standard deviation). It should be noted that the transfected cell lines with the highest expression of PRLR have higher tumor concentrations than their respective parental cell line. In contrast, the T47Dv11 xenograft with moderate PRLR expression had uptakes that were higher than those of the parental MCF-7 and PC3 but similar to the transfected PC3/PRLR. Only the 6-day tumor concentration is higher for MCF-7 compared to MCF-7/PRLR, which is probably an outlier, given the large standard deviation.

Table 1.

Dual-Isotope Biodistribution in Tumor Xenografts of MCF-7/PRLR

time (h)	tumor uptake	
	I-131 REGN2878%IA/g $\pm$ SD	Zr-89 REGN2878%IA/g $\pm$ SD
1.6	3.64 $\pm$ 1.30	5.58 $\pm$ 2.42
24	4.37 $\pm$ 0.71	40.02 $\pm$ 13.79
120	1.85 $\pm$ 0.38	73.24 $\pm$ 16.60
168	1.31 $\pm$ 0.32	60.33 $\pm$ 22.86