Preclinical Activity of the Novel Anti-Prolactin Receptor (PRLR) Antibody–Drug Conjugate REGN2878-DM1 in PRLR-Positive Breast Cancers

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Abstract

The Prolactin Receptor (PRLR) is a type 1 cytokine receptor that is expressed in a subset of breast cancers and may contribute to its pathogenesis. It is relatively overexpressed in approximately 25% of human breast tumors while expressed at low levels in some normal human tissues including the mammary gland. We developed an anti-PRLR antibody-drug conjugate (ADC), to target PRLR-positive breast cancer. REGN2878-DM1 is comprised of a fully human high-affinity function-blocking anti-PRLR IgG1 antibody (REGN2878) conjugated via a noncleavable SMCC linker to the cytotoxic maytansine derivative DM1. Both unconjugated REGN2878 and conjugated REGN2878-DM1 block PRL-mediated activation in vitro and are rapidly internalized into lysosomes. REGN2878-DM1 induces potent cell-cycle arrest and cytotoxicity in PRLR-expressing tumor cell lines. In vivo, REGN2878-DM1 demonstrated significant antigen-specific antitumor activity against breast cancer xenograft models. In addition, REGN2878-DM1 showed additive activity when combined with the antiestrogen agent fulvestrant. These results illustrate promising antitumor activity against PRLR-positive breast cancer xenografts and support the evaluation of anti-PRLR ADCs as potential therapeutic agents in breast cancer.

Introduction

Increased understanding of breast cancer has revealed that it is a heterogeneous disease comprised of various subtypes based on pathology and different molecular profiles (1). A clear example of this is the classification of tumors based on expression of receptor tyrosine kinase HER2 and hormone receptors for estrogen (ER) and progesterone (PR). The relative expression of these biomarkers is an important determinant of therapy, due to the established role of these proteins as drivers of disease. ER/PR-positive tumors are treated with hormonal therapy comprised of agents such as tamoxifen, aromatase inhibitors, and selective estrogen receptor downregulators (SERD) such as fulvestrant (2). Tumors overexpressing HER2 are treated predominantly with anti-HER2 antibodies including trastuzumab and pertuzumab generally combined with chemotherapy regimens (3). However, 15% to 20% of breast cancers do not express significant levels of these receptors (4). These tumors, classified as triple-negative, as well as patients that progress following hormonal or HER2 therapies, highlight the need for the development of additional targeted therapeutics in advanced breast cancers (5).

Prolactin (PRL) is a polypeptide hormone secreted from pituitary, adrenal, and mammary glands that is known to control lactation (6). PRL binds to prolactin receptor (PRLR), a single-pass transmembrane receptor of the type 1 cytokine receptor superfamily that comprises non-kinase receptors including the growth hormone receptor (7). PRLR signals through multiple intracellular kinases, such as Jak2/Stat5, MAPK, and PI3K (8). Cell surface expression of activated PRLR is tightly regulated via a negative feedback loop composed of ligand-induced receptor internalization and degradation, although recent evidence from our group suggests that PRLR also undergoes constitutive internalization and degradation (9–11).

PRLR has been found to be overexpressed in malignant breast epithelium relative to normal breast tissue and to be expressed across molecular subtypes of human breast cancers (12, 13). In addition, several reports suggest that PRL can increase proliferation of human breast and prostate cancer cells in in vitro and in vivo models, suggesting a role for the PRL/PRLR signaling axis in tumor development (14–17). However, the contribution of PRLR signaling in advanced breast cancer remains contentious, and it may be that PRLR expression here is not a driver of advanced disease (18). Expression of PRLR in normal tissues is limited, with low levels of expression seen in mammary glands, placenta, adrenal gland, and kidney relative to that seen in breast cancers (19, 20). Several strategies have been employed in targeting PRLR in cancer and other indications such as endometriosis. These strategies include PRL-based antagonists such as G129R-hPRL and the Pseudomonas exotoxin A conjugated form G129RPE40-KDEL.

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were transduced with PRLR to enhance expression. MCF7 cells from the American Type Culture Collection (ATCC) in 2011 and were authenticated at the ATCC by over 30 ADCs are in clinical development against an array of metastatic breast cancer due to the selective delivery of highly potent cytotoxic agents to tumor cells (25, 26). Currently, many ADCs utilize maytansinoid- or auristatin-based drugs that target microtubules, inhibiting cellular replication and leading to the induction of apoptosis (27). The anti-HER2 ADC Kadcyla (ado-trastuzumab emtansine) that utilizes the maytansinoid DM1 is approved for the treatment of HER2-positive breast cancer, and over 30 ADCs are in clinical development against an array of tumor types (25, 28).

Here, we report the generation and characterization of an anti-PRLR ADC, REGN2878-DM1, an antibody-drug conjugate comprised of a human anti-PRLR monoclonal antibody, REGN2878, conjugated to DM1. Following antigen-specific binding of REGN2878 or REGN2878-DM1 to PRLR-expressing breast cancer cells, the mAb is internalized, trafficked to lysosomes, and degraded. REGN2878-DM1 demonstrates potent in vivo cytotoxicity and antitumor activity in several breast cancer xenograft models.

Materials and Methods

Cell lines and other reagents

MCF7 (HTB-22) and T47D (HTB-133) cells were purchased from the American Type Culture Collection (ATCC) in 2011 and 1998, respectively. Cell lines were authenticated at the ATCC by short-tandem repeat profiling. MCF7/PRLR are MCF7 cells that were transduced with PRLR to enhance expression. MCF7 cells were transduced with full-length human PRLR using the pLEX lentiviral vector (Thermo Scientific) using puromycin selection at 1 µg/mL. MCF7 and MCF7/PRLR cells were grown in RPMI 1640 medium supplemented with 10% v/v FCS, penicillin/streptomycin/glutamine with 1 µg/mL puromycin for MCF7/PRLR. T47D cells and in vivo growth variant T47Dvl11 were grown in RPMI 1640 medium supplemented with 10% v/v FCS, penicillin/streptomycin/glutamine 10 µmol/L HEPEs, and 10 µg/mL insulin.

Generation of REGN2878 and REGN2878-DM1

VeloImmune mice (with genes encoding human immuno-globulin heavy and kappa light chain variable regions; ref. 29) were immunized with recombinant human PRLR extracellular domain. Spleens were harvested for generation of hybridomas or for direct isolation of antigen-binding splenocytes. The cloned human immunoglobulin variable region genes from antibodies exhibiting the desired characteristics were joined to human IgG1 constant region genes for production in Chinese hamster ovary (CHO) cells. The anti-PRLR mAb, herein referred to as REGN2878, was selected as a lead antibody from more than 300 antigen-binding clones following screening by in vitro biochemical and cell-based assays for specificity, affinity, internalization, and blockade of ligand-mediated signaling.

Conjugation of antibodies

REGN2878-DM1 and control ADC are comprised of anti-PRLR REGN2878 or an irrelevant IgG isotype control mAb, respectively, conjugated to DM1 via a noncleavable SMCC linker. For both REGN2878-DM1 and control ADC, antibody (10 mg/mL) in 50 mmol/L HEPEs, 150 mmol/L NaCl, pH 8.0, and 10% (v/v) DNA was conjugated with a 6-fold excess of SMCC-DM1 for 1 hour at ambient temperature. The conjugate was purified by size exclusion chromatography and sterile filtered. Protein and linker payload concentrations were determined by UV spectral analysis and MALDI-TOF mass spectrometry. Size-exclusion HPLC established that all conjugates used were >95% monomeric, and RP-HPLC established that there was <0.3% unconjugated linker payload. All conjugated antibodies were analyzed by UV for linker payload loading values and by mass difference, native versus conjugated (30). Average molar drug-to-antibody ratios were 3.4 for both REGN2878-DM1 and control ADC.

Molecular characterization of antibodies

The affinities of REGN2878 and REGN2878-DM1 for human PRLR were measured in Surface Plasmon Resonance Biacore experiments performed on a Biacore T200 instrument using a dextran-coated (CM5) chip at 37°C. Antibodies were captured by mouse anti-human Fcγ antibody immobilized on the sensor chips and were tested for binding to the extracellular domains of human PRLR in monomeric format. PRLR proteins at a range of concentrations (from 3.125 to 200 nmol/L) were injected over the captured REGN2878 or REGN2878-DM1 surfaces. The kinetic parameters were obtained by globally fitting the data to a 1:1 binding model using Biacore T200 Evaluation software (BiaEvaluation) version 2.0. The equilibrium dissociation constant (Kd) was calculated from the ratio of the dissociation rate constant to the association rate constant (Kd = koff/kon).

ELISA-based blocking assays were developed to determine the ability of REGN2878 and REGN2878-DM1 to block binding of human PRLR to immobilized human PRL. A dimeric human PRL mouse Fc fusion protein (hPRL.mFc) was used along with monomeric MycMychis-tagged human PRL construct (hPRL.mmH). For coating plates with the PRL construct, anti-Myc polyclonal antibody at 1 µg/µL PBS was first passively absorbed overnight at 4°C to microtiter plates followed by a protein block with 0.5% BSA in PBS. Subsequently, 1 µg/mL of hPRL.mmH was captured via the C-terminal MycMychis-tag by anti-Myc antibodies. To generate initial dose-response curves, human PRLR at concentrations ranging from 3.38 pmol/L to 200 nmol/L was added to the plates previously coated in 1 µg/µL PRL. After 1-hour incubation, captured hPRL.mFc was detected with horseradish peroxidase (HRP)–conjugated goat anti-Fcγ antibody and visualized with tetramethylbenzidine (TMB) colorimetric substrate by measuring absorbance at 450 nm. To determine the inhibition of PRLR binding by REGN2878 or REGN2878-DM1, 10 nmol/L of human PRLR was mixed with mAbs or ADC ranging from 520 pmol/L to 30 nmol/L and incubated at room temperature for 1 hour. Mixtures were then added to plates coated with 1 µg/mL PRL, and captured PRLR was detected as described above. Data were analyzed using a 4-parameter logistic equation over an 11-point response curve in GraphPad Prism software, and IC50 values were calculated.

The capacity of REGN2878 and REGN2878-DM1 to block human PRL-induced PRLR signaling was determined in a luciferase reporter assay using a HEK293 cell line engineered to
constitutively express PRLR and stably transduced with a luciferase reporter construct comprising five tandem STAT5-binding sites derived from the promoter of the human IL2 receptor α gene [STAT5 response element (x5)–luciferase; ref. 31]. STAT5 transcription factors are known mediators of intracellular signal transduction downstream of PRLR (32). To generate a dose–response curve, human PRL was added to the reporter cells at concentrations ranging from 0.8 pmol/L to 100 nmol/L. Following addition of PRL, cells were incubated for 6 hours at 37°C and 5% CO₂ and then equilibrated to room temperature for 15 minutes. Luciferase activity was measured following addition of ONE-Glo substrate. To test for inhibition of PRL-mediated PRLR signaling in the cells, mAbs or ADCs were added at concentrations ranging from 3.3 pmol/L to 200 nmol/L (molarity indicates antibody concentration for both ADC and mAb), followed by the addition of a constant concentration of 2 nmol/L PRL. Plates were incubated at 37°C and 5% CO₂ for 5 hours and equilibrated to room temperature for 10 minutes. Luciferase activity was again measured following addition of ONE-Glo substrate.

**PRLR RNAscope**

To assess the expression of PRLR mRNA in human breast cancer, sections of human breast tumors were assessed using RNAscope ISH technology (Advanced Cell Diagnostics). Formalin-fixed Paraffin Embedded (FFPE)-embedded samples were from breast tumor microarrays (TMA) obtained from Indivumed. FFPE pellets of T47D and MCF7 cells were used as controls for PRLR expression levels. Cell pellet and tumor sections were assessed for PRLR RNA expression using specific RNAscope probes and developed using diaminobenzidine chromogenic stains according to standard protocols. Images were scanned on an Aperio XT Scan scope at 40x (Leica Systems), and PRLR expression was assessed in the samples qualitatively.

**Western immunoblotting of human tumor samples for PRLR**

The expression of PRLR protein in human breast cancer samples was assessed using Western immunoblotting. Patient tumor samples were purchased from Bioserve. Pellets of T47D and MCF7 cells were used as controls for PRLR expression levels. The cell pellets and snap-frozen tumors were homogenized in RIPA buffer (Boston BioProducts) with protease and phosphatase inhibitors (Thermo Scientific) and sonicated briefly (QSonica). lysates were resolved on 4% to 20% Novex Tris-Glycine gels and blotted to PVDF membranes (Novex). Membranes were labeled with primary antibodies for PRLR (Cell Signaling Technology; 13552) or beta-actin (Santa Cruz Biotechnology; sc-47778). Labeling with primary antibodies was followed by HRP-conjugated secondary antibody (Promega) at 1:2,000 and chemiluminescent detection.

**Flow cytometry**

The relative expression of PRLR on the surface of cell lines was assessed via flow cytometric analysis. Cells were incubated with REGN2878 as a primary antibody followed by detection with an allophycocyanin (APC)-labeled goat F(ab’2) anti-human IgG secondary antibody. See Supplementary Materials and Methods for details.

**Internalization assays**

To assess internalization of REGN2878 into PRLR-positive cells, antibody directly labeled with Alexa488 fluorophore was incubated with T47D or MCF7/PRLR cells on ice. Cells were then further incubated for 1 hour in PBS + 2% FBS either on ice or at 37°C. Following incubation, cells were washed and nuclei labeled using DRAQ5. Images were then acquired on an ImageXpress Micro (Molecular Devices).

**Cytotoxicity assays**

Cells were seeded into triplicate rows of collagen-coated clear bottom 96-well plates in a volume of 100 μL/well at 5 to 6 × 10⁴ and incubated overnight at 37°C and 5% CO₂. Serial dilutions of REGN2878-DM1 or control ADC were generated and added to the cells. Cells were incubated for 72 hours at 37°C and 5% CO₂. Relative cell viability was determined by measuring levels of Hoechst-labeled nuclei using the ImageXpress Micro XL System and the Columbus Image Data Storage and Analysis System. See Supplementary Materials and Methods for details.

**In vivo efficacy studies**

As parental T47D cells are poorly tumorigenic in mice, T47D cells sourced from the ATCC were serially passaged in vivo to develop a variant with consistent tumorigenicity and growth kinetics thereafter named T47Dv11. See Supplementary Materials and Methods for details. For efficacy studies, mice were implanted with 7.5 × 10⁵ T47Dv11 cells into the left flank of female C.B.-17 SCID mice supplemented with a 90-day release 0.72 mg/pellet estrogen pellet. For MCF7 and MCF7/PRLR tumor xenografts, 2 × 10⁶ cells were implanted s.c. into the left mammary fat pad of female NCr nude mice (7–8 weeks old) supplemented with 90-day release 0.72 mg/pellet estrogen pellets. Tumors were measured with calipers twice a week for the duration of each study. Tumor volume was calculated using the formula TV = (length × width²)/2.

After T47Dv11, MCF7, or MCF7/PRLR tumors grew to an average volume of approximately 150 to 200 mm³ (14–16 days post implantation), mice were randomized into treatment groups (n = 8–9 mice/group) and received either REGN2878-DM1, control ADC, or vehicle control, administered systemically by intravenous tail-vein injection. Single-dose studies were used to establish the relative efficacy in the models, whereas repeat dose studies, where three doses were given every 7 days, were designed to maximize the efficacy observed. Weekly doses of ADCs utilizing the mcat-DM1 linker drug have proven efficacious in anti-CD22 and anti-EGFRvIII ADCs and were subsequently employed in this study (33, 34).

The SERD fulvestrant (Faslodex; Pharmaceutical Buyers Inc.) was administered subcutaneously in its recommended vehicle (10% w/v alcohol, 10% w/v benzyl alcohol, and 15% w/v benzyl benzoate, made up to 100% w/v with castor oil) once weekly for the duration of the studies. Tumor size and body weight were measured twice per week throughout the duration of the study. Efficacy of REGN2878-DM1 was also assessed in a patient-derived xenograft (PDX) model of breast cancer that may better represent the response in patients. NOD SCID gamma (NSG) mice bearing tumor fragments of the TM00107 PDX breast cancer tumor were sourced from The Jackson Laboratories. The TM00107 PDX model was derived from an ER⁻/PR⁻/HER2⁻ metastatic breast adenocarcinoma. To test the efficacy of REGN2878-DM1 in a more challenging model, tumors were allowed to grow to an average volume of approximately 400 to 600 mm³ (21 days after implantation). Mice were then randomized into treatment groups (n = 7 mice/group) and were administered REGN2878-DM1, control ADC, or vehicle control by intravenous injection once weekly for a total of 4 weeks. A separate treatment group was
administered paclitaxel by intravenous injection every 4 days for a total of 4 doses. All procedures were conducted according to the guidelines of the Regeneron Institutional Animal Care and Use Committee.

Pharmacodynamic analysis of REGN2878-DM1

Mice were implanted with T47Dv11 cells, and tumors were grown for 15 days. Mice were then randomized based on tumor volume and dosed intravenously with either vehicle, REGN2878-DM1 at 5 or 15 mg/kg or control ADC at 15 mg/kg (N = 4–5). At 24, 48, or 72 hours after treatment, mice were euthanized and tumors processed as FFPE samples for IHC analysis. Tumor sections were then stained with anti–phospho-histone H3 (pH3) antibody (clone: Ser10; Cell Signaling Technology; # 9701) as a measure of mitotic cells. Images were scanned on an Aperio XT Scan scope at 40x (Leica Systems). pHH3-positive nuclear signals were imaged and quantified with the Halo Cyto-Nuclear Count algorithm (Halo, Indica Labs). The percentage of positive cells in each tumor was quantified for each group and an average calculated for each treatment condition.

Statistical analysis

Statistical analyses were performed utilizing GraphPad software Prism 5.0. Statistical significance was determined by two-way ANOVA with Tukey multiple comparisons if all animals survived until the end of the study or by unpaired nonparametric Mann–Whitney test if mice had to be sacrificed per Institutional Animal Care and Use Committee guidelines before the end of the study (typically due to excessive tumor growth). A threshold of \( P < 0.05 \) was considered statistically significant.

Results

PRLR is expressed in human breast cancers

To examine the extent of PRLR expression in breast cancers, RNAscope, a form of ISH, was performed on a panel of commercially obtained FFPE-embedded breast tumor samples. Representative examples of PRLR expression in breast cancer are shown in Fig. 1A. A range of PRLR expression levels was observed, and some tumors demonstrated stronger expression than observed in MCF7 cells and approached the levels observed in T47D cells. Notably, PRLR expression was observed in tumors across breast cancer subtypes including hormone receptor–positive tumors as well as ER/PR/HER2 “triple negative” samples.

To assess PRLR protein levels in breast cancer, Western blotting of 20 commercially obtained breast cancer samples was performed. Note these were from different patients than the samples assessed by RNA scope. As seen in Fig. 1B, PRLR expression levels varied across the tumor samples. Five of 20 samples (25%) demonstrated PRLR expression greater than MCF7 lysates. Overall, the expression patterns of PRLR assessed by RNAscope and Western blotting are consistent with analysis of PRLR mRNA expression levels in human breast cancer from The Cancer Genome Atlas gene expression database. Notably, many breast cancers demonstrate levels of PRLR expression significantly higher than those observed across normal tissues, where expression is generally low (Supplementary Fig. S1).

REGN2878 and REGN2878-DM1 bind human PRLR with high affinity and inhibit PRLR-STAT5 signaling

Human antibodies against PRLR were generated using VelocImmune mice as described in Materials and Methods. REGN2878 was selected from hundreds of antigen-binding clones and conjugated to DM1 to produce REGN2878-DM1. Unconjugated REGN2878 exhibited high-affinity binding for human PRLR (\( K_D = 1.05 \text{ nmol/L} \)) and monkey (Macaca fascicularis) monomeric PRLR (\( K_D = 1.64 \text{ nmol/L} \)). Similarly, REGN2878-DM1 demonstrated high-affinity binding for human PRLR (\( K_D = 1.24 \text{ nmol/L} \)) and monkey (Macaca fascicularis) monomeric PRLR (\( K_D = 1.92 \text{ nmol/L} \)). In contrast, neither REGN2878 nor REGN2878-DM1 showed detectable binding to rat or mouse PRLR in these assays.

Both REGN2878 and REGN2878-DM1 blocked the binding of human PRLR to monomeric human PRL ligand, as measured by ELISA (Fig. 2). Here, human PRLR bound to human PRL in a dose-dependent manner, with an EC_{50} value of 4.9 nmol/L (solid black squares in insets in Fig. 2A). REGN2878-DM1 and REGN2878 blocked the binding of 10 nmol/L human PRLR to immobilized human PRL with IC_{50} values of 4.4 nmol/L and 5.0 nmol/L respectively (Fig. 2A). Control antibody and control ADC did not demonstrate any blocking activity under identical assay conditions.

To ensure that REGN2878-DM1 also blocked PRL induced STAT5 activity, a HEK293/PRLR/STAT5-Luc reporter cell line was generated. In this reporter line, 2.1 nmol/L of human PRL was determined to be the concentration required for stimulation of human PRLR signaling to 50% of the maximum activity level (EC_{50}; Fig. 2B). Both REGN2878 and REGN2878-DM1 effectively blocked PRL-induced luciferase activity in a dose-dependent manner, with 0.4 nmol/L of REGN2878 and REGN2878-DM1 required for a 50% reduction of PRLR signaling (IC_{50}) in the presence of a constant concentration of 2 nmol/L PRL. As with the blocking ELISA assays, neither control mAb nor control ADC blocked PRL-induced PRLR-STAT5 signaling.

Analysis of PRLR expression, internalization of REGN2878 on cell lines, and REGN2878-DM1 cytotoxicity

The expression of PRLR was assessed on human tumor cell lines used for in vitro and in vivo studies. MCF7 breast cancer cells expressed modest levels of endogenous PRLR, with a 25-fold increase in MFI relative to secondary antibody alone control (Fig. 3A). Higher levels of endogenous PRLR were observed in T47D cells with an MFI fold/secondary alone control ratio of 87 (Fig. 3A). The highest expression of PRLR was seen in engineered MCF7/PRLR cells, with an MFI fold/secondary alone control ratio of 498 (Fig. 3B). Overall, the relative levels of PRLR expression seen here correlate well with quantitative flow cytometry, showing 5,000 to 8,000 PRLR molecules expressed on MCF7 cells and approximately 27,000 PRLR molecules expressed on T47D cells (9). Engineered MCF7/PRLR cells were found to express approximately 185,000 PRLR molecules per cell (Supplementary Fig. S2).

REGN2878-DM1 potently induced cell killing of MCF7/PRLR and T47D cells in culture, with IC_{50} of 0.06 nmol/L and 0.97 nmol/L, respectively (Fig. 3E and F). REGN2878-DM1 did not induce cytotoxicity (IC_{50} > 100 nmol/L) of parental MCF7 cells in this assay (Fig. 3D). The activity of REGN2878 conjugated to the alternate cytotoxins MMAE and DM4 through their standard linkage chemistry was also assessed in T47D cells. The IC_{50} of REGN2878-MMAE and REGN2878-DM4 were 0.5 nmol/L and 0.8 nmol/L, respectively (Supplementary Fig. S3). These values were similar to the IC_{50} observed using REGN2878-DM1 in these cells, and REGN2878-DM1 was subsequently used for initial in vivo
studies. Robust internalization of REGN2878 was observed when assayed in MCF7/PRLR and T47D cells with rapid intracellular localization of fluorescently labeled antibody apparent following 1-hour incubation of the cells at 37°C (Fig. 3G).

REGN2878-DM1 has significant antitumor activity against MCF7 and MCF7/PRLR breast cancer xenografts

The antitumor activity of REGN2878-DM1 was assessed in human breast tumor xenograft models expressing both endogenous and transduced PRLR. To assess the activity of REGN2878-DM1 in a tumor expressing low levels of the PRLR, estrogen-supplemented NCr nude mice bearing established MCF7 tumor xenografts were treated with either a single dose or 3 once-weekly doses of REGN2878-DM1. Although this tumor cell line was not inhibited by REGN2878-DM1 in vitro (see above), a single injection of REGN2878-DM1 at 15 mg/kg significantly inhibited the growth of the MCF7 tumors relative to control ADC (Fig. 4A). More moderate in vivo antitumor activity was observed following a single dose of either 5 or 10 mg/kg of REGN2878-DM1 (Fig. 4A). Repeat treatment (three injections i.v. over 14 days) of MCF7

Figure 1.

A, PRLR mRNA is expressed in breast cancer. Representative PRLR RNAscope ISH in human breast cancers TMAs showing qualitative expression of PRLR. FFPE cell pellets of T47D and MCF7 cells were included as positive controls. All images are 40X magnification. B, PRLR protein expression in human breast cancers as assessed by Western blotting in two separate sets (1-10 and 11-20) of snap-frozen samples. Each set was run with T47D and MCF7 cell lysates as positive controls on the same gels with equal ECL exposure time for accurate comparison. BCa, breast cancer.
tumors study resulted in larger antitumor effects. Here, both 10 and 15 mg/kg of REGN2878-DM1 significantly inhibited MCF7 tumor growth relative to control ADC, although some antitumor activity was induced by control ADC relative to vehicle control (Fig. 4B). The therapeutic efficacy of REGN2878-DM1 was also assessed in MCF7/PRLR tumors transduced to express high levels of PRLR. Here, treatment with a single injection of REGN2878-DM1 at doses as low as 2.5 mg/kg was active. Dose-dependent antitumor activity was observed, with 10 and 15 mg/kg REGN2878-DM1 leading to tumor eradication (Fig. 4C). Repeat dosing increased antitumor efficacy; three injections of either 5 or 15 mg/kg REGN2878-DM1 caused tumor regression. Complete regressions were apparent for some tumors that received the highest dose (Fig. 5B). No activity of control ADC was apparent in the T47Dv11 model, and unconjugated REGN2878 again had no effect on tumor growth.

Because estrogen inhibition is a mainstay of treatment for ER-positive cancer, and estrogen has been reported to interact with PRLR expression and signaling, we tested the combination of REGN2878-DM1 with the SERD fulvestrant in the T47Dv11 xenograft model (35, 36). Here, the activity of either agent at partially effective doses was compared against the activity of the agents together. First, a dose titration study of weekly administration of fulvestrant was performed in order to establish its single-agent activity (Supplementary Fig. S4). Separate in vitro experiments demonstrated that continuous 7-day culture of T47Dv11 cells with cytostatic concentrations of fulvestrant (0.01 or 0.1 μmol/L) did not significantly reduce PRLR expression as assessed by flow cytometry or Western blotting. A similar
Figure 3.
PRLR expression, cytotoxicity of REGN2878-DM1, and REGN2878 internalization in PRLR positive cell lines. PRLR expression in breast cancer cell lines as assessed by flow cytometry in MCF-7 (A), MCF-7/PRLR (B), and T47D (C). F/B indicates the fold change in MFI relative to secondary alone controls. Cytotoxicity of REGN2878-DM1 against PRLR-positive cell lines following 72 hours by determining total cell numbers via staining for Hoechst-positive nuclei in MCF7 (D), MCF7/PRLR (E), and T47D (F). Internalization of PRLR antibody into PRLR-positive MCF7/PRLR and T47D cells at 1 hour (G).
maintenance of PRLR expression was observed with culture of T47Dv1 cells in 0.1 or 1 μmol/L tamoxifen over the same time period (Supplementary Fig. S5). Significant antitumor activity was observed following the individual treatments of 2.5 mg/kg REGN2878-DM1 and 150 or 250 mg/kg fulvestrant. Further, combination of REGN2878-DM1 with fulvestrant at either 150 mg/kg (Fig. 5C) or 250 mg/kg (Fig. 5D) was observed to cause a significant increase in antitumor activity, suggesting that combination of REGN2878-DM1 with estrogen inhibition may have additive efficacy in ER-positive tumors.

REGN2878-DM1 has antitumor activity against TM00107 PDX tumor
REGN2878-DM1 was assessed in the TM00107 PDX tumor model obtained from The Jackson Laboratories. PRLR expression obtained from PRLR Western immunoblotting on Figure 4.

REGN2878-DM1 is active against MCF7 tumors and MCF7/PRLR tumors in vivo. NCr Nude mice bearing established MCF7 or MCF7/PRLR tumors were treated with various dose levels of REGN2878-DM1 and controls. The line graphs depict the average tumor volume ± SEM per group over time. The red arrow indicates the day of dosing. Results of a MCF7 single-dose study (A) and results of a MCF7 multi-dose (q7dx3) study (B). Results of a MCF7/PRLR single-dose study (C) and results of a MCF7/PRLR multi-dose (q7dx3) study (D). Asterisks indicate levels of statistical significance relative to a matched dose of control ADC: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; ns, not significant.
prestudy tumor samples provided by The Jackson Laboratory (Supplementary Fig. S6). Treatment with REGN2878-DM1 or controls began at 21 days after implantation, when tumors were approximately 500 mm³ in size. Control tumors grew very rapidly, and mice that received vehicle control were euthanized at day 30 after implantation due to large tumor volume. At this time, a moderate but significant ($P < 0.01$) decrease in average tumor volume of mice treated with 20 mg/kg REGN2878-DM1 was observed compared with animals administered 20 mg/kg control ADC (Fig. 5E). A trend toward inhibition of tumor growth was observed in mice treated with a lower dose of 10 mg/kg REGN2878-DM1 compared with vehicle control–treated mice, which did not reach statistical significance. Paclitaxel treatment did not have an effect on tumor growth in this study. Retrospective Western immunoblotting analysis of PRLR expression in this model revealed relatively low expression of PRLR, with levels in
these tumors lower than that observed in MCF7 cell lysate (Supplementary Fig. S6). In a second PDX model, (TM00386/BR0555, also from The Jackson Laboratories), which appeared to have PRLR protein levels similar to those seen in TM00107 tumors (Supplementary Fig. S6), treatment with REGN2878-DM1 at 15 mg/kg did not result in significant antigen-specific antitumor activity (Supplementary Fig. S7). Thus, results in the two PDX tumor models, both with relatively modest levels of PRLR expression, are inconclusive and require further investigation.

Pharmacodynamic assessment of REGN2878-DM1 activity in vivo

In order to validate the activity of DM1 on microtubule activity, the induction of mitotic arrest following treatment of T47Dv11 tumors with REGN2878-DM1 in vivo was assessed by pHH3 IHC. In this pharmacodynamic study, tumors treated at a dose of 5 mg/kg REGN2878-DM1 showed clear induction of pHH3 immunoreactivity, with a significant increase relative to vehicle-treated tumors (Fig. 6A). At a higher REGN2878-DM1 dose of 15 mg/kg, a further increase in pHH3 levels in tumors was apparent (Fig. 6B). Importantly, levels of pHH3 peaked at 48 hours after treatment and were somewhat lower at 72 hours, suggesting the time of maximal activity of DM1 on microtubules and cell-cycle arrest. Importantly, the induction of pHH3 in tumors was specific to treatment with REGN2878-DM1, as tumors treated with 15 mg/kg control ADC did not have a significant increase in pHH3 over vehicle-treated tumors. Accordingly, levels of pHH3 expression resulting from treatment with either 5 or 15 mg/kg REGN2878-DM1 were significantly higher than those observed in control ADC–treated tumors. Separate pharmacokinetic analysis of REGN2878-DM1 in T47Dv11 tumor–bearing mice revealed that following a 5 mg/kg dose, ADC serum levels remained above 16 μg/mL (~100 nmol/L) for at least 10 days, indicating that tumors were exposed to ADCs at levels that were sufficient to mediate cytotoxicity in vitro (Supplementary Fig. S8).

Discussion

The targeted delivery of cytotoxic drugs to tumors via ADCs is a clinically proven treatment in breast cancer and certain hematologic malignancies (37, 38). Here, we show that PRLR is a promising target for ADCs due to its elevated expression in some breast tumors and its rapid intracellular internalization into lysosomes. We therefore generated REGN2878-DM1, comprised of a fully human anti-PRLR antibody, REGN2878, and the cytotoxic drug DM1. We found high expression of PRLR in approximately 25% of breast cancers across previously established molecular subtypes defined by HER2, ER, and PR expression, in agreement with other published reports (12, 24). Of note, we found that PRLR may be expressed in some triple-negative breast cancers (TNBC), as these tumors remain in need of novel targeted therapeutics. Unfortunately, although we did identify some TNBC patient samples to be PRLR positive, we were not able to identify TNBC cell lines with significant levels of PRLR to allow modeling of efficacy in this tumor type. However, it may be possible to identify PRLR-positive PDX models of TNBC for such studies.

Although the cell surface levels of PRLR are relatively modest, PRLR undergoes rapid and constitutive internalization. Furthermore, PRLR–REGN2878 complexes are efficiently routed to lysosomes for degradation (9). In comparison, although HER2 is abundantly expressed in certain tumors, it undergoes internalization with relatively slow kinetics (9, 39). Thus, despite the limited number of PRLR molecules on the surface of tumor cells, the rapid lysosomal degradation of REGN2878-DM1 results in efficient release of active drug and potent ADC-mediated cell killing. A similar relationship was recently reported by de Goeij and colleagues, who demonstrated that efficient target turnover and lysosomal degradation of tissue factor results in potent activity of anti-tissue factor ADCs (40). Although we have not
established a lower level expression threshold for efficacy, it is apparent that 10,000 to 20,000 PRLR molecules per cell may be sufficient for potent in vitro activity with anti-PRLR ADCs. In vivo experiments demonstrated significant antitumor activity of REGN2878-DM1 in both MCF7 and T47Dv11 xenografts that express endogenous PRLR. Overall activity of REGN2878-DM1 roughly paralleled PRLR expression levels, with MCF7/PRLR tumors expressing the highest PRLR levels demonstrating the greatest sensitivity to REGN2878-DM1. Interestingly, MCF7 xenografts showed a clear response to REGN2878-DM1 in vivo despite expressing moderate levels of the PRLR and showing limited in vitro sensitivity. It is possible that the longer exposure time to REGN2878-DM1 in vivo is responsible for the differential response observed. Supporting this idea, pharmacokinetic analysis of REGN2878-DM1 in T47Dv11 tumor-bearing mice demonstrated that ADC serum levels remained above 16 μg/mL (~100 nmol/L) for at least 10 days following a 5 mg/kg dose. In addition, estrogen has been reported to increase PRLR levels to levels of the MCF7 xenografts increased PRLR expression to levels required for increased sensitivity to REGN2878-DM1.

As observed from the increased antitumor activity in MCF7/PRLR tumors relative to parental MCF7 tumors, the efficacy of REGN2878-DM1 is likely to correlate with expression. Such a correlation has been observed for other ADCs including anti-REGN2878-DM1 is likely to correlate with expression. Such a correlation has been observed for other ADCs including anti-REGN2878-DM1 is likely to correlate with expression. Such a correlation has been observed for other ADCs including anti-REGN2878-DM1 is likely to correlate with expression. Such a correlation has been observed for other ADCs including anti-HER2 ado-trastuzumab emtansine and highlights the requirement for development of validated companion diagnostics using techniques such as flow cytometry to identify patients most likely to benefit [42]. Although direct comparison is difficult, the preclinical in vivo activity of REGN2878-DM1 utilizing MCC-DM1 linker-drug chemistry appears to generally approximate that of trastuzumab-MCC-DM1 (ado-trastuzumab emtansine), with both ADCs demonstrating tumor regression following treatment with 15 mg/kg of ADC [43].

In the two PDX tumor models examined here, REGN2878-DM1 showed modest activity in one model and little or no activity in the second. Both models expressed PRLR, as assessed by Western blotting of whole tumor lysates, although the levels of PRLR were relatively low (comparable with MCF7 tumor cells). At present, the degree of heterogeneity of PRLR expression within these PDX tumor models has not been defined. Combined with the lower expression of PRLR in these PDX tumors, the limited efficacy with REGN2878-DM1 may be partially due to the use of an ADC with a stable linker, which only kills cells that internalize and catabolize sufficient levels of the ADC. In contrast, ADCs employing cleavable linker drugs, where the released active drug is able to permeate from targeted cells to neighboring antigen-negative cells, can induce so-called bystander killing. Accordingly, as expression of PRLR in breast tumors may be modest and heterogeneous, the exploration of REGN2878 with linker-drugs with greater propensity for bystander effect is being investigated.

A pharmacodynamic assessment of REGN2878-DM1 in T47Dv11 tumor-bearing mice demonstrated significantly increased levels of pHH3 in treated tumors. This effect is consistent with the expected induction of mitotic arrest resulting from the targeted delivery of DM1 following specific internalization of REGN2878-DM1. Induction of pHH3 has also been observed following treatment with the anti-tubulin agent vinblastine and following treatment with other ADCs employing either maytansinoid- or auristatin-based drugs [33, 44]. Further assessment and optimization of pHH3 detection may determine if this biomarker generally correlates with antitumor efficacy of tubulin-targeting ADCs [33].

Although REGN2878 and REGN2878-DM1 potently block PRLR signaling, the unconjugated antibody shows little antitumor activity on its own. Because murine PRL fails to activate human PRLR [45, 46], growth of xenograft tumors in mice is independent of PRLR signaling, and thus PRLR blockade has little effect. In contrast, estrogen receptor signaling is active in vivo and has been implicated in regulating the expression of PRLR [35, 36]. We therefore assessed the effect of estrogen inhibition on the activity of REGN2878-DM1 in vivo using the SERD fulvestrant. In the T47Dv11 model assessed here, combined treatment of REGN2878-DM1 and fulvestrant had superior antitumor efficacy relative to the single agents. Combined with preliminary in vitro experiments that demonstrated that culture of cells with cytostatic concentrations of fulvestrant or tamoxifen did not significantly reduce PRLR expression, the in vivo results suggest that REGN2878-DM1 may remain active in patients undergoing treatment with anti-estrogens and that combination strategies should be further investigated [47, 48]. Further, it has been reported that signaling through the PRL–PRLR axis can confer resistance to multiple chemotherapies used in the treatment of breast cancer including paclitaxel and doxorubicin [49, 50]. Accordingly, it is possible that the signal-blocking activity of REGN2878-DM1 may combine with chemotherapies for additive efficacy and that further combination studies may be warranted [47].

ADCs may have utility in targeting receptors and other targets expressed preferentially in cancer where signaling-blocking or ADCC-inducing antibodies may have shown limited clinical efficacy. For example, an antibody toward CD30 antigen, which is expressed in Hodgkin’s lymphoma, was active in preclinical models but inactive clinically [37, 51]. Subsequently, this antibody was conjugated to the auristatin MMAE to form the ADC Adcetris (brentuximab vedotin) that has demonstrated significant antitumor activity in Hodgkin’s lymphoma and Anaplastic Large Cell Lymphoma clinically [52]. Perhaps similarly, the anti-PRLR antibody LFA102 was recently observed not to have sufficient activity in clinical testing against PRLR-positive cancers despite its inhibition of PRLR signaling [24]. Doses of LFA102 up to 60 mg/kg did not show dose-limiting toxicities, suggesting that blocking PRLR in vitro is well tolerated, although the tolerability of anti-PRLR ADCs will have to be assessed in appropriate toxicology studies. Targeting PRLR-positive cancers with REGN2878-DM1 may provide greater antitumor efficacy than agents that only disrupt active PRLR signaling.

Despite the success of anti-HER2 therapies in breast cancer, significant populations of patients remain ineligible for these treatments due to limited expression of HER2 target. Further, for patients who have progressed through other therapies including hormonal therapy, options for effective treatment in advanced disease are limited and overall survival remains poor. Thus, the need for the development of novel and effective therapies in advanced breast cancer remains. In this study, we have demonstrated that PRLR shares the prerequisites for targeting with an ADC including overexpression in breast cancer, limited normal tissue expression, and rapid intracellular internalization [25]. REGN2878-DM1 was effective in vitro and demonstrates significant antitumor activity in vivo. These results support the continued exploration of ADCs targeting PRLR in advanced breast cancers with overexpression of the receptor.
Disclosure of Potential Conflicts of Interest

M.P. Kelly has ownership interest (including patents and stock ownership) in Regeneron Pharmaceuticals. S. Coetzee is a manager and has ownership interest (including patents) in Regeneron Pharmaceuticals. F. Delfino has ownership interest (including patents and stock ownership) in Regeneron Pharmaceuticals. T.B. Potocky has ownership interest (including patents and stock ownership) in Regeneron Pharmaceuticals. G. Thurston has ownership interest (including patents) in Regeneron Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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