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Harnessing Androgen Receptor Pathway Activation for Targeted Alpha Particle Radioimmunotherapy of Breast Cancer

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Disclosure of Potential Conflicts of Interest

D.L.J. Thorek is a consultant/advisory board member for and holds ownership interest (including patents) in Diaprost AB. S. Strand is listed as a co-inventor on a patent regarding this humanized form of 11B6, which will be owned by Diaprost, and holds ownership interest in Diaprost. H.G. Lilja holds ownership interest (including patents) in Diaprost AB and OPKO Health, and reports other remuneration from OPKO Health. S.M. Larson reports receiving commercial research grants from Regeneron and Telix, holds ownership interest (including patents) in Voreyda, Imaginab, and Elucida, and is a consultant/advisory board member for Johnson and Johnson. D. Ulmert holds ownership interest (including patents) in Diaprost AB. No potential conflicts of interest were disclosed by the other authors.

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Abstract

Purpose: The impact of androgen receptor (AR) activity in breast cancer biology is unclear. We characterized and tested a novel therapy to an AR-governed target in breast cancer.

Experimental Design: We evaluated the expression of prototypical AR gene products human kallikrein 2 (hK2) and PSA in breast cancer models. We screened 13 well- characterized breast cancer cell lines for hK2 and PSA production upon *in vitro* hormone stimulation by testosterone [dihydrotestosterone (DHT)]. AR-positive lines were further evaluated by exposure to estrogen (17b-Estradiol) and the synthetic progestin D-Norgestrel. We then evaluated an anti-hK2–targeted radiotherapy platform (hu11B6), labeled with alpha (a)-particle emitting Actinium-225, to specifically treat AR-expressing breast cancer xenografts under hormone stimulation.

Results: D-Norgestrel and DHT activated the AR pathway, while 17b-Estradiol did not. Competitive binding for AR protein showed similar affinity between DHT and D-Norgestrel, indicating direct AR–ligand interaction. *In vivo* production of hK2 was sufficient to achieve site-specific delivery of therapeutic radionuclide to tumor tissue at >20-fold over background muscle uptake; effecting long-term local tumor control.

Conclusions: [^{225}Ac]hu11B6 targeted radiotherapy was potentiated by DHT and by D-Norgestrel in murine xenograft models of breast cancer. AR activity in breast cancer correlates

with kallikrein-related peptidase-2 and can be activated by D-Norgestrel, a common contraceptive, and AR induction can be harnessed for hK2- targeted breast cancer a-emitter radiotherapy.

Introduction

The ovarian steroid hormones estrogen and progesterone orchestrate mammary gland development during puberty and pregnancy. The estrogen receptor (ER) and progesterone receptor (PR) have been implicated as drivers of malignant transformation of breast tissue and induction of breast cancer. ER and PR are used as a means of classifying breast cancer subtypes of the disease and when expressed, may be effective therapeutic targets. Reduction of hormone receptors expression and ablation of receptor-positive cells usually confers some degree of response. Patients lacking ER, PR, or HER2 amplification (termed triple-negative breast cancer or TNBC) generally have more aggressive and more lethal disease (1). The lack of expression of these three target receptors eliminates immediate molecularly targeted options for therapy.

Recent studies have shown that approximately 60%–70% of breast cancer expresses androgen receptor (AR; refs. 2, 3), with variations between clinical subtypes and ethnic backgrounds (4, 5). Although some studies have shown that AR expression is associated with a lower recurrence rate (6), there remains considerable debate about the prognostic value of AR expression in patients with breast cancer (7–10). AR is the archetypal male steroid hormone receptor; it is central for male sex reproductive organ development and is the key molecular driver of prostate cancer. Clinically, the AR-controlled expression of the kallikrein-related peptidases *KLK2* and *KLK3*, which encode human kallikrein 2 (hK2) and PSA, respectively, are useful for prostate cancer risk stratification and disease monitoring. In males, abundant expression of these proteins is specific to luminal cells of the prostate and prostate cancer tissues. The physiologic enzymatic function of hK2 is to convert pro-PSA to catalytically active PSA, which functions to degrade Semenogelin 1/2 in the seminal fluids to free spermatozoa (11).

As in prostatic tissue, the presence of functional AR correlates with hK2 and PSA expression in breast cancer cell lines (12–14). Despite these findings, the use of PSA and hK2 as biomarkers for breast cancer has been controversial (15–18) and is not clinically utilized to inform patient management. Preclinical studies of hu11B6, a humanized antibody specific for the catalytic cleft of hK2, have shown sensitive and specific *In vivo* targeting of prostate cancer and breast cancer (19–21). hu11B6 is specifically internalized by hK2-expressing cells through a neonatal Fc-receptor (FcRn)–driven process. We reasoned that ^{225}Ac-DOTA-hu11B6 ([^{225}Ac]hu11B6), an alpha-particle emitting radio-immunotherapeutic, could override traditional molecular resistance mechanisms commonly associated with breast cancer due to the cell-specific, high energy, and short path length of the delivered radiation. In this study, we first screened breast cancer cell lines for the expression of prostate kallikreins after stimulation with steroid hormones. We then evaluated this production capacity as a novel strategy to target models of breast cancer using the [^{225}Ac]hu11B6 construct. Under the appropriate hormone stimulation, we are able to site specifically deliver therapeutic radionuclide to multiple models of human disease and achieve local tumor control. Further- more, we demonstrate that activation of AR-positive

breast cancer lines by the widely prescribed progestin contraceptive and known AR agonist D-Norgestrel (22–28) can facilitate kallikrein-targeted RIT^{225}Ac-DOTA-hu11B6.

Materials and Methods

Reagents and cell culture

Reagents and hormones were purchased from Sigma-Aldrich unless otherwise noted. Cell growth media were obtained from the Media Preparation Core Facility at Memorial Sloan Kettering Cancer Center (MSKCC, New York, NY). Breast cancer cell lines AU-565, BT-20, HCC-1806, MDA-MB-415, MDA-MB-435, MDA- MB-468, T-47D, and ZR-75–01 were kind gifts from Dr. Gabriella Chiosis (MSKCC, New York, NY). BT-474, LNCaP, MCF-7, MDA-MB-231, VCaP, and ZR-75–30 were purchased from ATCC. MFM-223 was purchased from Sigma. All cell lines were cultured in the conditions recommended by their respective sources. AU-565, HCC-1806, LNCaP, T-47D, ZR-75–01, and ZR- 75–30 were cultured in RPMI1640 medium. BT-20 and MFM-223 were cultured in MEM. BT-474, MDA-MB-231, MDA-MB-415, MDA-MB-435, and MDA-MB-468, and VCaP were cultured in DMEM. All media were supplemented with 10% FCS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. For competitive binding and AR activation experiments, cells were cultured in medium with 10% CSS (charcoal-treated dextran-treated FBS) with 100 U/mL of penicillin and 100mg/mL of streptomycin for 24 hours prior to use.

Androgen activity *in vitro* measurements

Cells were seeded between 1–5 10^5 cells in tissue culture plates and exposed to 0.1, 1, or 100 nmol/L of dihydrotestosterone (DHT), estrogen, D-Norgestrel, or vehicle (ethanol) control for 168 hours in triplicate. VCaP cells were treated with 1 mmol/L enzalutamide in combination with the other hormones above, or with 200 nmol/L of abiraterone in combination with D-Norgestrel. Conditioned media were collected, centrifuged to remove cellular debris, and analyzed by a time-resolved ELISA for free and total PSA and hK2 (see below).

cDNA and qPCR

qRT-PCR was performed on mRNA harvested using Commercial Kit (79656 and 74104, Qiagen). Reverse transcription was performed using a Commercial Kit (4368813, Applied Biosystems). KLK2, KLK3, ESR1, ESR2, NR3C3 (PR), NR3C4 (AR), NR3C1 (GR), FCGRT, EGFR, HER2, ACTB, and FOLH1 primers for qPCR were purchased from SAbiosciences. SYBR Green qPCR Mastermix for qPCR were obtained commercially (330529, Qiagen).

Immunoblotting

Cell pellets were collected by trypsin dissociation and lysed by RIPA buffer. Electrophoresis and transfer was performed using standard protocol. Anti-AR antibody (C-19, Santa Cruz Biotechnology) and anti-PR antibody (3176S, Cell Signaling Technology Inc.) were incubated at 1: 3,000 and 1:1,000 dilution, respectively. Anti-rabbit secondary antibody was diluted at 1:3,000 for both blots.

Proliferation assay

MFM-223 was seeded at 5 10^5 cells per well in a 6-well tissue culture plate and treated with DHT, estrogen, or D-Norgestrel in CSS medium for 144 hours. Viable cells were counted on Vi-Cell Cell Counter (Beckman Coulter) in triplicate at 196 hours.

Competitive binding assay

Equilibrium binding affinities of D-Norgestrel and 5α-DHT were measured in VCaP cells (grown in CSS medium for 72 hours) by displacing 16b-fluoro-5α-DHT ([^{18}F]FDHT; produced by the MSKCC Radiochemistry Core) as reported previously (29). Samples were incubated at room temperature for 1 hour followed by whole-cell harvesting (M-24T, Brandel Inc.) by Glass Microfiber Filter (Whatman 934-AH, GE Life Sciences) and washed three times with ice-cold TRIS-buffered saline. Immobilized radioactivity on the filter papers was counted on a gamma counter (dynamic window centered at 511 keV, Wizard2, PerkinElmer Inc.).

PSA and hK2 immunoassays

Total PSA and hK2 were measured using an in-house research assay, which has previously been described by Vaisänen and colleagues (30). Briefly, streptavidin-coated microtiter plates were incubated with the biotinylated catcher antibody H1117 (for PSA) or 6H10 (for hK2), followed by washing and incubation with samples and standards. After another round of washing, the $Eu^{3þ}$-labeled tracer antibody H50 (for PSA) or 7G1 (for hK2) was added. After incubation and washing steps, enhancement solution was added before reading the plates. Both assays have a functional detection limit of 0.04 ng/mL.

Animal studies

All animal experiments were conducted in compliance with institutional guidelines at MSKCC and under supervision by the MSKCC Research Animal Resource Center. For xenograft studies, female athymic BALB/c nude mice (6–8-weeks old, 20–25 g) were obtained from Charles River. Three to 4 days prior to breast cancer tumor cell inoculation, mice were implanted with a 60-day extended release subcutaneous 17b- estradiol releasing pellet via trocar on the lateral side of the neck between the ear and the shoulder (0.72 mg/pellet, SE-121, Innovative Research of America, Inc.). Flank tumors from MFM- 223 and BT-474 cells, cultured as above, were inoculated by subcutaneous injection of 1–5 × 10^6 cells in a 200-μL cell suspension of a 1:1 vol/vol mixture of medium with Matrigel (Collaborative Biomedical Products, Inc.). Tumors of 100–200 mm^3 developed after approximately 6 weeks. Animals in treatment groups as specified were implanted with either DHT (12.5 mg, 60-day release) or D-Norgestrel (10 mg, 60-day release) following inoculation.

Pharmacokinetic tissue distribution

Biodistribution studies were conducted to evaluate the uptake and pharmacokinetic distribution of [^{225}Ac]hu11B6 or the noninternalizing control [^{225}Ac]hu11B6^{H435A} in breast cancer xenograft models. Mice received a single 11.1 kBq activity dose of [^{225}Ac]hu11B6 (300 nCi on 5 μg antibody) for injection via intravenous tail-vein injection (t = 0

hour)Animals (n = 4–5 per group) were euthanized by CO_2 asphyxiation at indicated time points postinjection of the radiopharmaceuticals. Blood was immediately harvested by cardiac puncture. Tissues (including the tumor) were removed, weighed, and counted using a gamma counter with a 360–480 keV window at secular equilibrium (Packard Cobra, Packard Instrument Co., Inc.). Aliquots (0.020 mL) of the injected activities were used as decay correction standards and background signal was subtracted from each sample. The percentage of injected activity (%IA) was normalized to the weight per gram of each tissue for each animal and data plotted as mean %IA/g ± SD.

Anti-hK2 antibody and radiochemistry

Humanized 11B6 (hu11B6) and recombinant mutant hu11B6^{H435A} (modified at Histidine 435 to Alanine, to abrogate FcRn binding; ref. 15) were developed by DiaProst AB. hu11B6 and hu11B6^{H435A} used for Actinium-225 labeling was produced by Innovagen AB.^{225}Ac (Oak Ridge National Laboratory) was labeled to the hu11B6 antibody or control antibody (huIgG_1) and purified using a 2-step labeling procedure; protocol details are as previously published for this antibody(31). Radioactivity was measured at secular equilibrium with a Squibb CRC-17 Radioisotope Calibrator (E.R. Squibb and Sons, Inc.) set at 775, and multiplying the displayed activity value by 5. The radiochemical reaction commenced by adding37 MBq (1 mCi) of 225Ac nitrate dissolved in 0.2 mol/L hydrochloric acid (Thermo Fisher Scientific) to a solution of 1.0 mg of S-2-(4-Isothiocyanatobenzyl)-1,4,7,10-tetraazacyclo-dodecane tetraacetic acid (DOTA-Bz-SCN, Macrocyclics, Inc.) in 0.10 mL metal-free water. The pH was adjusted with the addition of 0.1 mL of 2 mol/L tetramethylammonium acetate (Aldrich) and 0.02 mL of 150 g/L L-ascorbic acid (Aldrich) to yield a pH 5.5 reaction mixture. The reaction mixture was heated at 55–60 C for 30 minutes. The antibody solution was added to the [^{225}Ac]DOTA-Bz-SCN reaction mixture and the pH adjusted to 9.5 with the addition of 0.15 mL of 1 mol/L carbonate/bicarbonate buffer solution. The reaction was held at 37 C for 30 minutes and subsequently quenched with0.020 mL of 50 mmol/L diethylenetriaminepentaacetic acid (DTPA, Aldrich). The conjugation reaction mixture was purified by size exclusion chromatography using a P6 resin (Bio-Rad) as the stationary phase and 1% human serum albumin (HSA, Swiss Red Cross) in 0.9% NaCl (Abbott Laboratories) as the mobile phase. A 0.001 mL aliquot of the final product, [^{225}Ac]11B6, or controls (labeled with the same procedure), was used to determine the radiochemical purity using instant thin-layer chromatography with a stationary phase of silica gel impregnated paper (Gelman Science Inc.) and two different mobile phases. Mobile phase I is 10 mmol/L ethylenediaminetetraacetic acid and II is 9% sodium chloride/10 mmol/L sodium hydroxide. The strips were cut and the pieces counted in a Packard Cobra g-counter (Packard Instrument Co., Inc.) using a 370–510 KeV window. The purified radioimmunoconstruct was formulated for intravenous injection in a solution of 1% HSA (Swiss Red Cross) and 0.9% sodium chloride (Normal Saline Solution, Abbott Laboratories).

Therapy studies

A single 11.1 kBq (300 nCi) activity dose of [^{225}Ac]hu11B6, [^{225}Ac]hu11B6^{H435A}, or^{225}Ac-labeled nonspecific huIgG_1 was injected into the tail vein in 10 female BALB/c athymic mice bearing either MFM-223 or BT-474 flank xenografts. Tumor sizes were 100–

200 mm^3 at the initiation of study and animals were randomized into treatment groups. Length (L) and width (W) of the tumors were measured by caliper and the volume (V) for a rotated ellipsoid [V = (W × 2L)/2] was calculated. Weight loss of 20% or a tumor diameter exceeding 15 mm was considered the endpoint. Tumor volumes were measured twice per week, or greater, and overall survival was monitored for as long as 1 year. Hormonal modulation of AR was begun 7–10 days prior to therapeutic interventions; either 17b-Estradiol or DHT pellets were implanted or D-Norgestrel administration was begun.

Statistical analysis

Androgen activity measurements were analyzed by one-way ANOVA and competitive binding data were fitted using nonlinear one site, $\log IC_{50}$ model in GraphPad Prism. Analysis of survival data was performed by the log-rank test (Mantel– Cox) and presented to four significant digits, again in GraphPad Prism.

Results

Breast cancer cell line kallikrein production

We first investigated expression of the prostate kallikreins in a panel of 13 human breast cancer cell lines under androgen (DHT) stimulation. Supernatant was analyzed to measure secreted PSA and hK2 levels that were significantly above the detection limit of the assay. The androgen-sensitive LNCaP prostate cancer line was used as a positive control (Fig. 1A). Induction of PSA and hK2 secretion by DHT and D-Norgestrel were only found in AR-positive cell lines (BT-474, MFM-223, and T-47D). Subsequently, we tested all 13 cell lines under estrogen, D-Norgestrel, and vehicle control stimulation and assayed for secreted hK2 and PSA. The presence of the progestin D-Norgestrel resulted in hK2 levels comparable with non- treated LNCaP (Fig. 1B and C). These findings correlated well with the transcriptional activity of KLK2 and KLK3 obtained by qRT-PCR analysis of the 13 cell lines, which also included a panel of genes relevant for breast cancer biology and hormone status (Supplementary Fig. S1A–S1C). After excluding the negative cell lines, gene expression correlation analysis between DHT and either progestin or estrogen stimulation was performed; DHT was highly correlated with D-Norgestrel in all three AR-positive breast cancer cell lines (Pearson $P < 0.0001$), but not with estrogen (Supplementary Fig. S1D–S1G). To confirm previously published results, immunoblotting of AR and PRa/PRb was performed on BT-474, MFM-223, and T-47D lysate using LNCaP cell line as a positive control. The results demonstrate variable degrees of AR and PR activity; notably, MFM-223 is devoid of PR (Fig. 1D).

Direct AR–D-Norgestrel interaction

KLK2/3 expression and hK2 and PSA production in PR- negative MFM-223 cells under the stimulation of D-Norgestrel was observed (Fig. 1E; Supplementary Fig. S1B). Experiments were conducted in charcoal-stripped media to remove background steroids; however, other steroids and progestins can be converted to AR substrates in culture and *In vivo*. To ensure that the activation of AR by D-Norgestrel was direct, we conducted additional kallikrein secretion assays with a metabolic inhibitor of AR. We used the MFM-223 cell line under hormone stimulation, and in the presence of abiraterone, a mechanistic inhibitor of steroid

biosynthesis, which blocks 17a-hydroxylase/17,20-lyase (CYP17A1). In combination treatment (D-Norgestrel plus abiraterone), no statistically significant difference in the production of total hK2 or PSA was noted, when compared with D-Norgestrel alone (Fig. 1E). These results indicate that D-Norgestrel can directly activate AR signaling. To further elucidate this mechanism, we used a radio-fluorine-labeled DHT ([^{18}F]FDHT) to measure AR binding in a heterologous competitive radioligand binding assay. For this study, we utilized the prostate cancer cell line VCaP, which has been extensively characterized and expresses wild-type AR (32, 33). D-Norgestrel and the natural AR ligand, DHT, effectively displaced [^{18}F]FDHT with nearly identical IC_{50}s of 2.16 ± 0.04 nmol/L and 2.71 ± 0.03 nmol/L, respectively. Further-more, in the presence of 1 μmol/L enzalutamide (a direct AR antagonist), no specific binding of [^{18}F]FDHT was observed (Supplementary Fig. S2A). VCaP cells in culture were exposed to enzalutamide (1 μmol/L) with either DHT or D-Norgestrel (100 nmol/L). At low concentrations of D-Norgestrel, the presence of enzalutamide inhibited hK2 production. However, at the higher enzalutamide concentration (1.0 nmol/L), the levels of hK2 produced were equivalent to DHT stimulated production in the presence of the inhibitor (Supplementary Fig. S2B).

Radiochemistry

The radiochemical yield of [^{225}Ac]hu11B6 is 3.7% ± 2.1% (mean ± SD, n = 13) and the radiochemical purity is 99.3% ± 0.5% (n = 13); specific activity is 0.79 Ci/g ± 0.055 Ci/g (n = 13).

Binding affinity

The K_d of hu11B6 antibody was 15.0 nmol/L in binding to recombinant hK2. DOTA-functionalized 11B6 bound with16.3 nmol/L affinity. [^{225}Ac]hu11B6 bound with 16.6 nmol/L affinity (average of two measurements). These data demonstrate that neither DOTA functionalization nor subsequent radio-labeling with^{225}Ac affects the immunoreactivity of the antibody for its cognate target.

In vivo expression and targeting of hK2

For the purpose of utilizing hK2 expression in a directed radio-immunotherapeutic strategy, we next tested whether KLK2 expression could be induced in AR-positive breast cancer tumors *In vivo*. Mice bearing MFM-223 and BT-474 xenografts were implanted with pellets releasing D-Norgestrel, DHT, DHT plus 17b-estradiol, or vehicle. The tumors were harvested when they reached approximately 100 mm^3. Analyses of MFM-223 and BT-474 tumor lysates showed that both D-Norgestrel and DHT induced high levels of hK2 (Fig. 2A). This encouraged us to investigate a recently developed hK2-targeting antibody (hu11B6) in AR-positive breast cancer in conjunction with steroid hormone induction of KLK2 (21, 34). We tested the most potent therapeutic variant of this agent; hu11B6 labeled with the cytotoxic a particle emitting radionuclide, [^{225}Ac]hu11B6.

In MFM-223 xenografts, DHT stimulation of hK2 production leads to specific uptake at the site of disease at 120 hours (Fig. 2B). The clearance of the antibody in the liver is also noted. A mutated version of the antibody (hu11B6^{H435A}), which does not bind to FcRn, but retains binding affinity to the hK2 protein, was also tested to determine the contribution of

internalization to the overall therapeutic effect. Here we do not observe specific uptake at the disease site (Fig. 2C and D) indicating that breast cancer cell internalization leads to accumulation of the radiolabeled antibody. Tissue uptake, retention, and clearance kinetics of [^{225}Ac]hu11B6 in androgen-stimulated animals was measured using biodistributions over 4, 48, 120, and 360 hours (Fig. 2E). MFM-223 uptake appears to plateau after 120 hours and nonspecific organs largely clear by 360 hours with the exception of the liver and spleen.

To confirm the specific homing and accumulation of the [^{225}Ac]hu11B6 drug in a second breast cancer model, the longitudinal biodistribution in mice bearing BT-474 xenografts was evaluated. Radiotherapeutic organ and tumor distribution after simulation with DHT and D-Norgestrel are shown in Fig. 3A and B, respectively. As with the MFM-223 biodistribution, we see significant accumulation of the [^{225}Ac]hu11B6 at time points after administration with low uptake in background organs. Normal immunoglobulin clearance by the liver and spleen lead to some uptake that clears with time. No significant accumulation in the tumors are noted when the animals are treated with estrogen pellet supplementation alone (Fig. 3A), as hK2 production is not active. [^{225}Ac]huIgG$_1$, a nonspecific isotype control radiolabled antibody, does not localize to the BT-474 lesions, with accumulation less than 1/10th of the hormonestimulated tumor localization (Fig. 3B). Hormone treatment alone does not have an effect on tumor cell proliferation *in vitro*, out to 8 days (Supplementary Fig. S3).

Antitumor treatment effect in models of aggressive breast cancer

Encouraging *In vivo* pharmacokinetic data indicating selective targeting and retention of an hK2-targeted agent in multiple models justified the evaluation of [^{225}Ac]hu11B6 as a radiotherapeutic. Triple-negative MFM-223 and HER2-overexpressing BT-474 breast cancer xenograft growth or regression were monitored to assess the antitumor effect of the a particle emitting [^{225}Ac]hu11B6 with concomitant hormone stimulation. In animals supplemented with either DHT or D-Norgestrel, treatment with a single 300 nCi dose of [^{225}Ac]hu11B6 resulted in a significant antitumor effect; growth was delayed in both BT-474 and MFM-223 bearing animals by 120 and 365 days, respectively (Fig. 4). The internalizing hK2-targeting antibody is required for local tumor control (Fig. 4A and B), as is the amplification of hK2 production by appropriate hormone stimulation (estrogen with radiotherapeutic is not effective). Minimal residual disease, including undetectable tumor masses, was observed in 67% (8/12) of the D-Norgestrel þ RIT arm and 75% (9/12) of the DHT + RIT arm.

Treatment efficacy in aggressive breast cancer

The efficacy of hK2-targeted radiotherapy with respect to survival benefit was also examined in models of aggressive breast cancer. MFM-223- and BT-474 xenograft–bearing animals were randomized and treated with [^{225}Ac]hu11B6, non- internalizing [^{225}Ac]hu11B6^{H435A} or [^{225}Ac]huIgG$_1$ control and were assessed longitudinally. Figure 5 shows the Kaplan–Meier analysis for MFM-223 and BT-474 xenograft models following these single administration treatments. Notably, the median survival time of animals treated with [^{225}Ac]hu11B6 alone was 121 days, which was not statistically significantly different than [^{225}Ac]hu11B6^{H435A} plus DHT (132 days, P 0.4927) or D-Norgestrel (191.5 days, P = 0.0893). In contrast, a significant survival benefit (P > 0.0001) was observed in tumor bearing animals treated with internalizing [^{225}Ac]hu11B6 plus hK2- inducing DHT or D-

Norgestrel stimulation when compared with [^{225}Ac]hu11B6 alone (without hormone) or with internalization-deficient [^{225}Ac]hu11B6^{H435A} plus hormone-induced hK2 amplification. The median survival time for [^{225}Ac]hu11B6 with DHT was 315.5 days and was not defined for the [^{225}Ac]hu11B6 with D-Norgestrel due to treatment effect.

None of the BT-474 mice treated with hormones-alone survived past day 103, while single dose administration of targeted, internalizing a RIT resulted in an overall survival of greater than 4 months (Fig. 5B). The addition of the targeted a particle radiotherapy provided a potent survival advantage over hormone monotherapy for both DHT (P =.0006) and D-Norgestrel (P =.0004). The median survival of DHT and D-Norgestrel alone treated groups were 63 and 59.5 days, respectively; median survival values for the [^{225}Ac]hu11B6 groups was not defined due to local tumor control.

Discussion

A majority of primary and metastatic breast cancer cases express AR (2). This discovery offers an alternative means to detect, characterize, and potentially treat breast cancer that is complementary to existing approaches. In this context, understanding the role of AR and its activity in this disease is vital. *KLK2* (hK2) and *KLK3* (PSA) are two prototypical AR-governed prostate kallikreins whose expression in breast cancer tissues may provide insight to the AR in breast cancer (35, 36). Although the etiologic function of AR remains elusive, its activation as determined by either PSA or hK2 secretion can be used as an indication of disease (11, 14).

In this study, we screened 13 breast cancer cells lines with varying expression of estrogen, androgen, PRs, and HER2 for hK2 secretion upon hormone stimulation. We found that only the AR-positive breast cancer cell lines produce detectable levels of this protease upon DHT and D-Norgestrel stimulation (Fig. 1A and B). Production of hK2 protein after DHT or D-Norgestrel treatment of 3 days was dramatically higher, by three-to-four orders of magnitude, in AR-positive BT-474, MFM-223, and T-47D when compared with hormone-naÏ ve controls (Fig. 1A and C). Gene expression profiles of BT-474, MFM-223, and T-47D are asymmetric with respect to the level of protein secretion (Supplementary Fig. S1). In addition, the AR content as revealed by Western blot analysis for BT-474 and especially T47-D is low (Fig. 1D), suggesting that hormone-induced kallikrein expression and secretion is a viable target even in low-expressing lesions.

D(–)-Norgestrel (levonorgestrel) is a synthetic progestin derived from testosterone, via removal of the methyl group at position C-19 and addition of an ethinyl group at position C-17 and a methyl group at position C-18. It is a potent agonist for the PR and possesses significant capacity to activate the AR (22–28). It has been extensively used as contraceptive in oral formulations, subcutaneous rod implants, and in intrauterine devices. Also, it has been a component of hormone replacement regimens to counteract the effects of estrogen on the endometrium. Its safety and widespread availability make it highly attractive if it can be leveraged as an adjuvant therapeutic for breast cancer radioimmunotherapy. We evaluated the capacity of D-Norgestrel, as an alternative to the canonical AR ligand, DHT, to activate AR and induce KLK2 expression to activate hK2-targeted radioimmunotherapy. We found

that D-Norgestrel induced hK2 and PSA expression and that this effect was not attenuated by abiraterone, a steroid biosynthesis inhibitor. It should be noted that D-Norgestrel is a direct AR agonist that does not require enzymatic conversion and that uncontrolled/ background (trace level) steroids converted to androgens are not significant to these observations.

To date, there are no approved AR-targeted therapies for patients with breast cancer. Trials with enzalutamide, a second-generation antiandrogen are ongoing, but still remain in the early-stages (NCT01597193, NCT02007512). Hormone stimulated expression of disease-specific targetable proteins such as the kallikreins is an intriguing therapeutic paradigm for AR activity mono- or combination therapy. This is especially relevant in TNBC, which often retain AR expression (7, 37). To that end, we investigated directed, molecularly specific alpha-particle therapy of breast cancer cells under androgen and progestin stimulation with an antibody targeted to active hK2. Here, the tissue-specific expression of hK2 is exploited to selectively deliver highly energetic a particle emissions from Actinium-225 and its daughter radionuclides, while sparing adjacent and distant (healthy) tissues. Alpha particles are high-energy helium nuclei that possess exquisite cytotoxic potential (38). In fact, one single alpha-particle traversal of a cell nucleus will result in severe genotoxic insult and is often lethal to the cell (39). Under appropriate hormone stimulation (either D-Norgestrel or DHT), adequate levels of hK2 were induced in both AR-positive breast cancer cell lines evaluated (BT-474 and MFM-223) to facilitate specific and sustained uptake as analyzed by the [^{225}Ac]hu11B6- labeled construct (Figs. 2 and 3). Tumor volume changes following therapy with the wild-type internalizing Actinium- 225–labeled antibody were statistically significant and sustained in both models, resulting in profound advantages in overall survival for animals treated with radiotherapeutic and hormone stimulation in combination. FcRn-mediated antibody internalization is important for efficacy, as we show that the noninternalizing mutant antibody (hu11B6^{H435A}) has unremarkable performance characteristics, which resemble the untargeted radiolabeled isotype control (huIgG_1). Finally, we evaluated this strategy for antitumor effect in the same two AR-positive breast cancer models. Alone, the hormones used to stimulate hK2 production had no effect on MFM-223 cell growth (Supplementary Fig. S3).

Here, we survey the expression of kallikreins in breast cancer cell lines and report our initial experience with [^{225}Ac]hu11B6, a molecularly specific radioimmunotherapeutic, in models of advanced breast cancer. Highly promising results in multiple, aggressive, AR-positive breast cancer cell lines were obtained *In vivo*, following a single dose of [^{225}Ac]hu11B6. With the recent approval of the first alpha-particle emitting radiotherapy (Radium-223 dichloride), which demonstrates improved survival for patients with bone metastatic castrate-resistant prostate cancer (40) there is great interest in targeted alpha- particle–emitting agents. PSMA-targeted alpha-particle– emitting agents are under evaluation for disseminated castrate-resistant prostate cancer under compassionate use guidelines in several European countries. The novel work presented here demonstrates that direct AR activation (via DHT or an androgenic progestin) can lead to sufficient hK2 production to be targeted with radioimmunotherapy. We posit that hK2-targeted alpha-particle–emitting radioimmunotherapy may have capacity to localize to and eradicate disseminate foci of breast cancer. The use of an antibody-targeting vector should preclude toxic absorbed dose

levels to the kidney (as seen with small-molecule radiotherapy agents); however, detailed toxicologic assessment in relevant models will be conducted as we pursue clinical translation of this novel therapy. We also recognize that the AR activation step will require regulatory approval through separate evaluations of dose and timing. Induction of AR in the tumor would be required only during the treatment period, which also generates a potential possibility for the treating physician to terminate the RIT treatment by discontinuing dosing AR-activating DHT or D-Norgestrel. Also, treating patients with breast cancer with testosterone has been shown to have beneficial therapeutic effects (41).

A variety of clinical applications for this therapeutic strategy can be envisioned, which uniquely serve patients with breast cancer with disease resistant to targeted therapy (for example, trastuzumab-refractory metastatic breast cancer) or those without recourse to targeted therapy (for example, triple-negative AR-positive disease). Molecularly targeted RIT has tremendous potential to address diffuse disease, micrometastases, as well as sites that are traditionally difficult to access in patients with breast cancer including the brain and bone compartments. Future work will continue to investigate the translational potential of [^{225}Ac]hu11B6 for the benefit of women with advanced breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

We have developed and applied a strategy that enables cell-targeted radiopharmaceutical therapy of aggressive breast cancers. This strategy exploits the production of what are canonically human male protein products, the prostate kallikreins, in androgen receptor (AR)-positive breast cancers. The AR is widely expressed in breast cancer tissues, including in aggressive disease that is considered triple-negative (negative for estrogen, progesterone, and EGFRs). Using hormones (both androgens and progestins), we show that the production of the prostate kallikreins occurs *in vitro* and *in vivo*. Furthermore, the production of human kallikrein–related peptidase 2 (hK2) can be therapeutically targeted by a mAB functionalized with an alpha particle–emitting radionuclide. This has the potential to significantly change the way that targeted therapy is conceptualized for difficult to treat triplenegative AR-positive breast cancer.

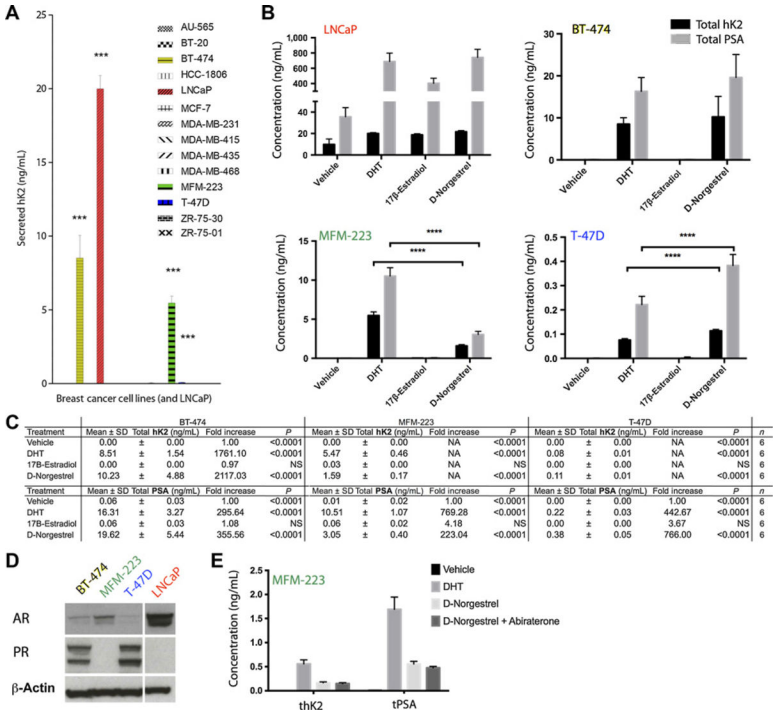

Figure 1.

Breast cancer cell hormone stimulated production of prostate kallikreins. **A**, Concentration of secreted hK2 for a panel of human breast cancer lines, and positive control LNCaP prostate adenocarcinoma, following DHT treatment as measured by immunoassay (>0.04 ng/mL). **B**, Total PSA and total hK2 concentration in culture media of LNCaP (control), BT-474, MFM-223, and T-47D after 172 hours of incubation with 100 nmol/L DHT, 17b-Estradiol, or D-Norgestrel. The levels of hK2 and PSA differs significantly in MFM-223 and T-47D cell lines. **C**, Total PSA and total hK2 secretion (which account for different isoforms of the proteins) in the three PSA- and hK2-positive breast cancer cell lines. **D**, Western blot analysis of androgen receptor (AR) and progesterone a/b receptor (PGR) with b-actin as a loading control. The three cell lines show varying degree of AR expression with MFM-223 being the highest and T-47D the lowest. PGRs are expressed in BT-474 and T-47D only. LNCaP and T-47D are AR and PGR controls, respectively.**E**, thK2 and tPSA expression in MFM-223 cell line treated with a panel of hormones and inhibitors. The D-Norgestrel alone, and D-Norgestrel with abiraterone (200 nmol/L) treated groups are not statistically different.

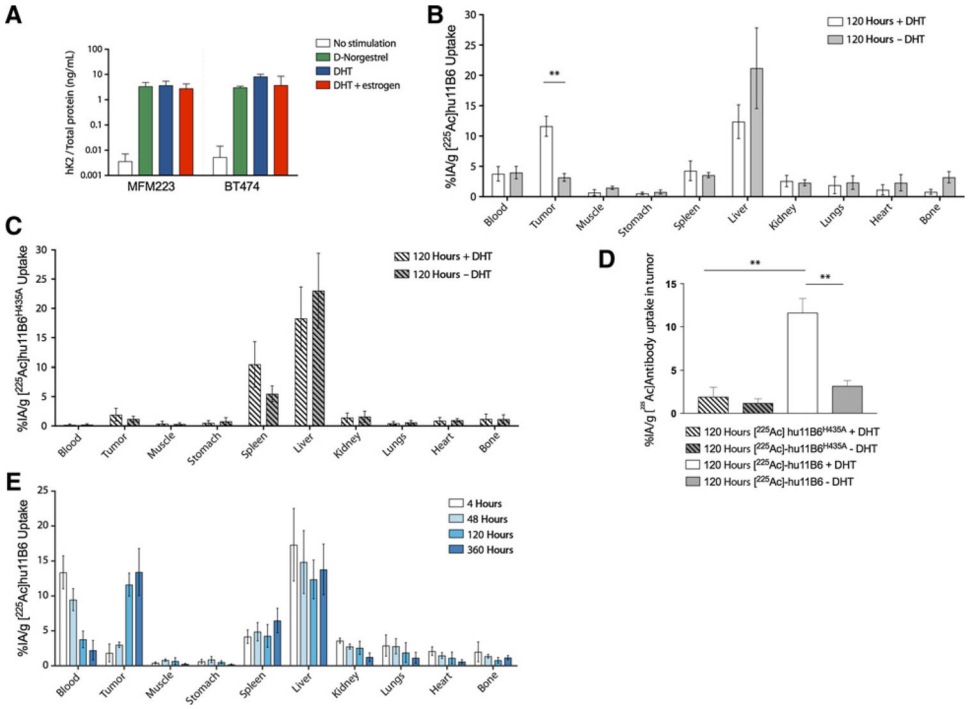

Figure 2.

In vivo production and targeting of hK2 under hormone stimulation. **A**, Breast cancer lesions were evaluated by fluorescence lifetime ELISA with hormone stimulation as noted. D-Norgestrel, DHT, and DHTþ17b-estradiol were able to produce significant increases in hK2 enzyme relative to tumor protein content. Organ and tumor distribution of tracer [^{225}Ac]hu11B6 (**B**) and noninternalizing [^{225}Ac]hu11B6^{H435A} in the MFM-223 breast cancer model (both at an 11.1 kBq activity dose; **C**). Both internalizing antibody and hormone stimulation provided to produce hK2 are required for tumoral uptake, compared in **D**. **E**, Kinetic biodistribution of the [^{225}Ac]hu11B6 at 4, 48, 120, and 360 hours in DHT-stimulated MFM-223 bearing mice for major organs of interest and tumor.

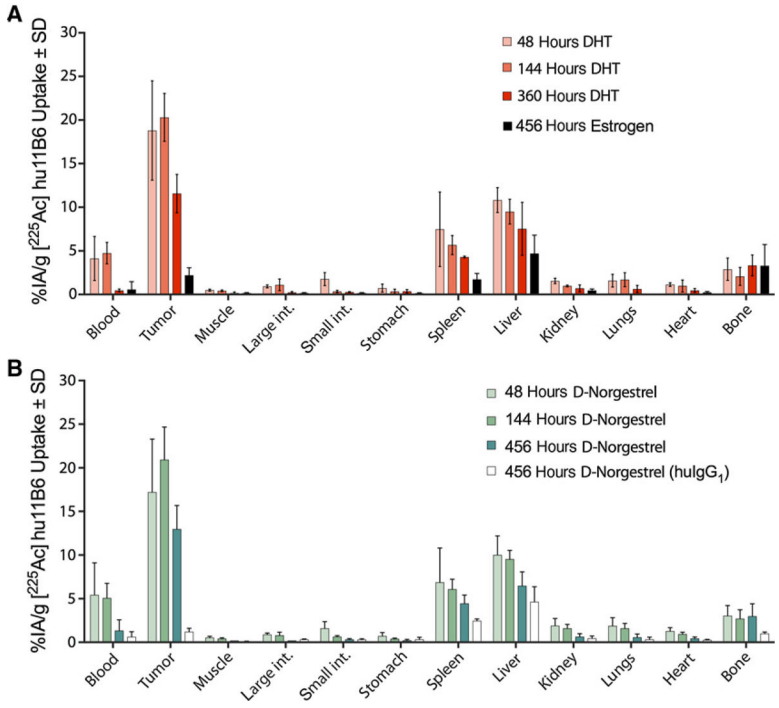

Figure 3.

Pharmacokinetics of [^{225}Ac]hu11B6 in BT-474 breast cancer model. Mice bearing BT-474 xenografts (HER2-overexpressing and ER-negative) were tested to confirm tumor targeting and specificity. (**A**) DHT and (**B**) D-Norgestrel–stimulated animals have comparable radiolabeled antibody uptake (not statistically significant different). Estrogen alone does not induce hK2 secretion and therefore uptake in the tumor is abrogated. Furthermore, isotype control [^{225}Ac]huIgG$_1$ is not taken up or retained in the tumors *in vivo*.

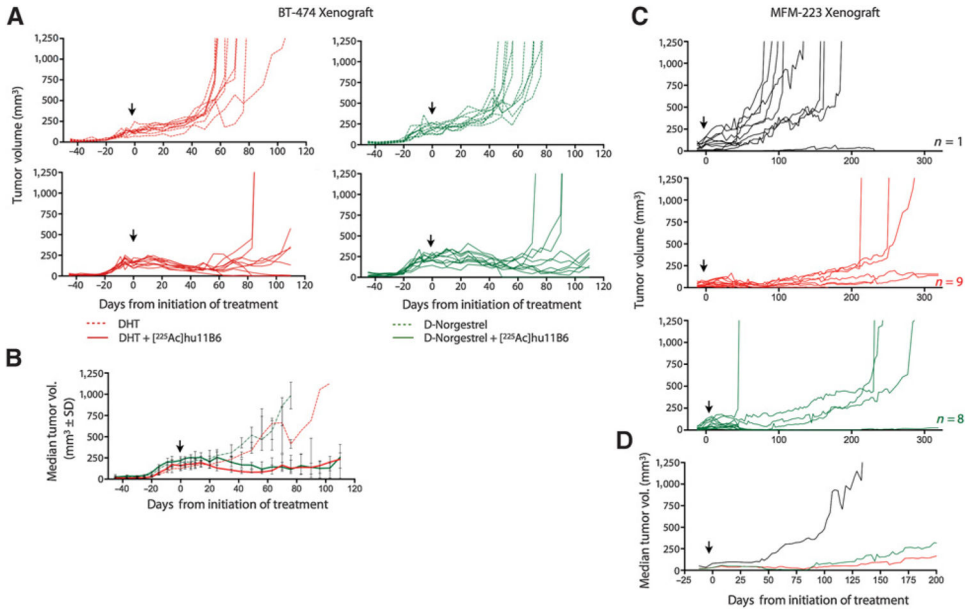

Figure 4.

Treatment efficacy in aggressive breast cancer models. **A**, Individual replicates of tumor volumes of BT-474 xenograft bearing mice were monitored following treatment with hormones alone (DHT or D-Norgestrel) or either hormone supplemented with a single 11.1 kBq (300 nCi) intravenous injection of [^{225}Ac]hu11B6. **B**, Median tumor volume (± SD) of these treatment groups shown. C, MFM-223 bearing animals were treated with[^{225}Ac]hu11B6 with estrogen, plus DHT or D-Norgestrel stimulation. Regression and minimal residual disease were realized in a significant proportion of the animals treated with the radiotherapeutic and hK2-stimulating hormones. D, Median tumor volume measures from the MFM-223 treated groups.

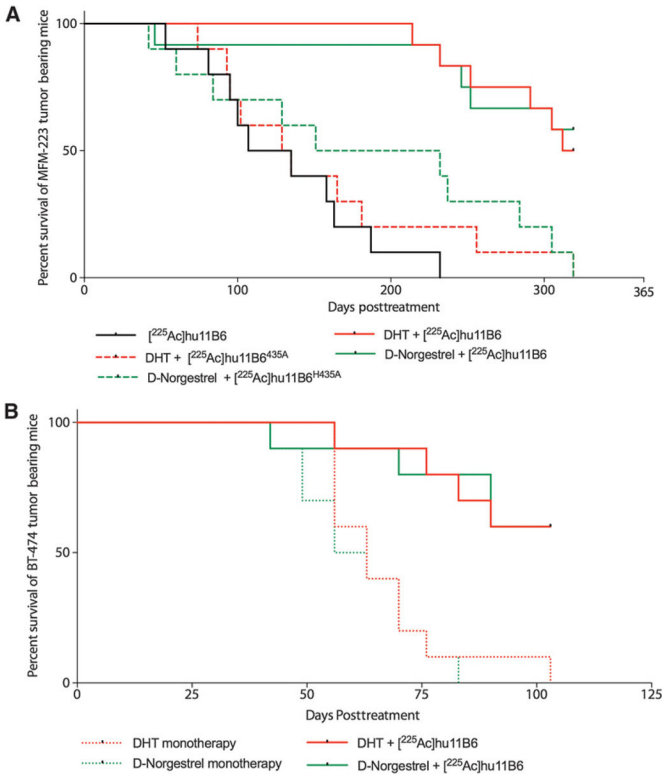

Figure 5.

Survival measures in aggressive breast cancer models. Kaplan–Meier plot of animals treated with targeted radiotherapeutical and hormone stimulation. A, MFM-223 xenograft–bearing animals that received the internalizing wild-type [^{225}Ac]hu11B6 (11.1 kBq) and hK2-producting stimulation by DHT or D-Norgestrel had significantly greater ($P < 0.001$) survival than animals treated with the same hormone regimen with a noninternalizing variant of the radioimmunotherapy, [^{225}Ac]hu11B6^{H435A}, or the antibody without stimulation ([^{225}Ac]hu11B6 alone). B, Likewise, in a triple-negative breast cancer model (BT-474) mice that received [^{225}Ac]hu11B6 in combination with DHT or D-Norgestrel fared significantly better than those on hormone treatment alone ($P < 0.001$).