Selective Androgen Receptor Modulator RAD140 Inhibits the Growth of Androgen/Estrogen Receptor–Positive Breast Cancer Models with a Distinct Mechanism of Action

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Abstract

Purpose: Steroidal androgens suppress androgen receptor and estrogen receptor positive (AR/ER⁺) breast cancer cells and were used to treat breast cancer, eliciting favorable response. The current study evaluates the activity and efficacy of the oral selective AR modulator RAD140 in in vivo and in vitro models of AR/ER⁺ breast cancer.

Experimental Design: A series of in vitro assays were used to determine the affinity of RAD140 to 4 nuclear receptors and evaluate its tissue-selective AR activity. The efficacy and pharmacodynamics of RAD140 as monotherapy or in combination with palbociclib were evaluated in AR/ER⁺ breast cancer xenograft models.

Results: RAD140 bound AR with high affinity and specificity and activated AR in breast cancer but not prostate cancer cells. Oral administration of RAD140 substantially inhibited the growth of AR/ER⁺ breast cancer patient-derived xenografts (PDX). Activation of AR and suppression of ER pathway, including the ESR1 gene, were seen with RAD140 treatment. Coadministration of RAD140 and palbociclib showed improved efficacy in the AR/ER⁺ PDX models. In line with efficacy, a subset of AR-repressed genes associated with DNA replication was suppressed with RAD140 treatment, an effect apparently enhanced by concurrent administration of palbociclib.

Conclusions: RAD140 is a potent AR agonist in breast cancer cells with a distinct mechanism of action, including the AR-mediated repression of ESR1. It inhibits the growth of multiple AR/ER⁺ breast cancer PDX models as a single agent, and in combination with palbociclib. The preclinical data presented here support further clinical investigation of RAD140 in AR/ER⁺ breast cancer patients.

Introduction

Breast cancer is the second leading cause of cancer-related death in women, with an estimated 246,660 newly diagnosed cases and 40,450 deaths in the United States alone in 2016 (1). Breast cancer is a heterogeneous disease categorized into several histopathologic subtypes based on the status of estrogen receptor α (ER), progesterone receptor (PR), and HER2 receptor. Although ER-positive (ER⁺) breast cancers are now treated with standard-of-care agents targeting the ER axis, including tamoxifen, fulvestrant, and aromatase inhibitors (AI), and HER2-positive tumors are treated with HER2 inhibitors, such as trastuzumab, novel therapeutic approaches are still needed to address resistance emerging from these established regimens (2, 3). More recently, combined administration of ER-targeted therapies with the inhibitors of cyclin-dependent kinase (CDK) 4/6 (4) or mTOR (5) have yielded improved therapeutic efficacy in ER⁺ breast cancer, and these combination therapies now represent a new generation of standard of care for this indication.

Recent histopathologic studies revealed that the androgen receptor (AR) is the most commonly expressed hormone receptor in breast cancer, with 75% to 90% of ER⁺ and approximately 30% of ER⁻ breast cancers expressing AR (6, 7). Although AR is widely present in breast cancer tissue, accumulating evidence has demonstrated that the role of AR in these tumors is subtype-dependent (8–11). AR antagonists including bicalutamide and enzalutamide have been shown to reduce the growth of AR-positive but ER⁻, including triple-negative, breast cancer in preclinical models (12–14) and in patients (11). In contrast, in ER⁻ breast cancers, AR has been considered antiproliferative and has been associated with a favorable prognosis. Clinically, until the 1970s, breast cancers were often treated with nonselective steroidal androgens including testosterone derivatives and danazol with response rates of 20% to 25% (9, 15–18). In line with these clinical experiences, preclinical studies have also shown treatment with classic androgens, including DHT, reduced the growth of AR and ER⁻ (AR/ER⁻) breast cancer cells in vitro (19–21) and in vivo (22). Classic androgen-based therapy for breast cancer declined due to its virilizing effects, potential risk of further aromatization to estrogens, and the emergence of ER-targeted agents including tamoxifen. However, it is worth noting that more recent clinical evidence showed androgen treatment also led to remission in patients who were progressing after ER-targeted therapy with objective response rate around 17% to 39% (23, 24). Fulvestrant...
The expression of the androgen receptor (AR) in estrogen receptor (ER)-positive breast cancer has been well described. Androgens inhibit the proliferation of AR and ER-positive (AR/ER+) breast cancer cells, and androgen-based treatment showed clinical benefit in breast cancer patients. However, the clinical utility of the classic steroidal androgen-based therapies has been limited. We report here that RAD140, an oral, tissue selective androgen receptor modulator, exhibited potent activity in inhibiting the growth of multiple AR/ER+ breast cancer xenograft models. The coadministration of RAD140 with CDK4/6 inhibitor elicited enhanced efficacy in these AR/ER+ models. Mechanistically, RAD140 potently regulates AR target genes, while suppressing the ER targets and the ESR1 mRNA. These suggest a distinct AR-mediated mechanism of action compared with the traditional ER-targeted agents. RAD140 may present a novel therapeutic approach for AR/ER+ breast cancer. The first-in-human study of RAD140 in recurrent breast cancer has been initiated.

Materials and Methods

Nuclear receptor binding assay

The PolarScreen nuclear receptor competitor assays (Thermo Fisher Scientific) for AR, ER, PR, and glucocorticoid receptor (GR) were used to determine the binding affinity and specificity of RAD140. Briefly, RAD140 and fluorescence-labeled ligands (Fluormones) for AR, ER, PR, or GR, respectively, were added to each reaction well, and the plate was incubated for 4 hours at room temperature in the dark. Polarization values for each well were measured and plotted against the concentration of RAD140. As a reference, the binding affinity of the standard ligands DHT, 17β-estradiol, progesterone, and dexamethasone (for AR, ER, PR, and GR, respectively) was also measured. The relative binding affinity of RAD140 versus the 4 standard ligands is reflected by the ratio of IC50 (standard ligand)/IC50 (RAD140). A commercially available spectrum screen was performed for RAD140 (MDS Pharma Services). The binding of RAD140 (1 μmol/L) to a panel of cellular targets was assessed (Supplementary Table S1). Applicable binding was defined as a larger than 50% inhibition of the reference radio-ligand.

In vivo efficacy study

All study protocols were reviewed by Institutional Animal Care and Use Committees and Radius Health, Inc., and conducted in compliance with U.S. and European regulations of protection of laboratory animals. The HBCx-22, HBCx-21, and HBCx-3 breast cancer patient-derived xenograft (PDX) models were established and evaluated at XenTech by implanting tumor fragments subcutaneously in the flank female athymic nude mice (Foxn1nu, Envigo RMS). The ST897 breast cancer PDX model was established in female athymic nude mice (Foxn1nu, Charles River Laboratories) and studies conducted at South Texas Accelerated Research Therapeutics. These models were characterized as ER and PR positive and negative for HER2 overexpression based on IHC.

Cell culture and treatment

The ZR-75-1, HCC1428, and T47D breast cancer cells and LNCaP prostate cancer cells were purchased from ATCC and maintained in medium and condition as recommended by the vendor. All cell lines involved in the study were tested negative for mycoplasma contamination (MycoAlert Detection Kit) and subjected to annual authentication using STR profiling (Thermo Fisher Scientific). All the cells were under passage 15 at the time of experiments. HCC1428 long-term estrogen-depleted (LTED) cells were established by culturing these cells in medium with 10% charcoal dextran-stripped serum (CSS) for 16 weeks, and the expressions of AR, ER, and PR were confirmed by Western blotting (Supplementary Fig. S1A). For proliferation assay, cells were seeded in medium with 10% CSS at 30,000 cells per well in 24-well plates and subjected to treatment with RAD140, DIHT, or DMSO for 14 days with treatments being renewed every 3 days. At the end of the treatment period, trypsinized cells were stained with Trypan blue before being subjected to live cell counting in a Nexcelom Cellometer (Nexcelom Bioscience). The PC3-AR cell line, described previously (29), was a generous gift from Dr. Steve Balk of Beth Israel Deaconess Medical Center (Boston, MA) and Dr. Changemeng Cai of University of Massachusetts (Boston, MA). For ZR-75-1 cell-based assays, cells were seeded and incubated in medium with 5% CSS for 48 hours. Cells were then treated with 17β-estradiol at 1 nmol/L final concentration or vehicle (ethanol) before being incubated with DMSO, RAD140 (Radius Health, Inc., as described previously; ref. 30), or DHT (Sigma Aldrich) in the presence or absence of enzalutamide or ARN-509 (Selleckchem) for 24 hours. The experiments were performed in duplicate. The nuclear and cytoplasmic fractions of the cells were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific) following the manufacturer's instructions.

AR reporter gene assays

AR reporter gene assay was carried out using a Cignal Androgen Receptor Reporter (luc) Kit (Qiagen). ZR-75-1 breast cancer cells, LNCaP, and PC3-AR prostate cancer cells were transfected with a tandem androgen-responsive element (ARE)-driven firefly luciferase construct and a Renilla luciferase construct in RPMI1640 media containing 5% CSS. Forty-eight hours later, transfected cells were treated with DMSO, RAD140, or DHT. The luciferase activity was determined using the Dual-Luciferase Reporter Assay (Promega) as a representation of AR transcription activity and normalized to the activity of Renilla luciferase. Each set was carried out in triplicate. The values were presented as average fold changes ± SD over the vehicle control.

Translational Relevance

The high prevalence of AR observed in ER+ breast cancers, along with the prior clinical efficacy demonstrated with classic androgens, provides a strong rationale for the development of a new generation of oral, selective AR agonists and to further exploit their therapeutic benefits in AR/ER+ breast cancer. Here, we describe the tissue-selective AR agonist activity of RAD140, an oral nonsteroidal selective AR modulator (SARM), and its distinct AR-mediated mechanism of action including the suppression of ESR1. RAD140 exhibited potent antitumor activity in the in vivo models of AR/ER+ breast cancer both as a single agent and in combination with the CDK4/6 inhibitor palbociclib. RAD140 represents a new generation of tissue-selective AR agonists and potentially a novel therapeutic option for AR/ER+ breast cancer.
and later confirmed using Western blot analysis and qPCR (Supplementary Table S2; Supplementary Fig. S1). The efficacy study in T-47D breast cancer cell line–derived xenograft model, established in NOD/Shi-scid/IL-2Rγnull mice, was conducted at WuXi AppTec. These xenograft models were supplemented with exogenous estradiol in drinking water (HBcx-3, HBcx-21, HBcx-22, and ST897) or subcutaneous pellet (0.18 mg/90-day release, Innovative Research America) implanted 3 days prior to tumor cell inoculation (T-47D). The PDX-bearing mice were randomized once their tumor volumes reached 60 to 200 mm³, as described previously (31). The cell line–derived xenografts with volumes between 125 and 250 mm³ were enrolled in treatment groups. RAD140, DHT, or fulvestrant were administered as single agent or in combination with palbociclib. RAD140, palbociclib, and the vehicle (0.5% carboxymethyl cellulose) were given orally. Fulvestrant (clinical formulation, AstraZeneca) was given as subcutaneously cutaneous injection. DHT was administered as subcutaneously implanted pellets (12.5 mg/60-day release, Innovative Research America) from the day of randomization to the end of treatment (14). Tumor volume and body weight were measured twice a week. Tumor growth inhibition (TGI %) was calculated on the basis of the change of mean tumor volume from treatment day 1 to the last day of assessment and presented as a percentage of that of the vehicle control group [100 × (1 - ΔTVtreatment/ΔTVvehicle)], as an indication of antitumor efficacy. Statistical analyses were performed for the changes in individual tumor volume (ΔTV, from treatment day 1 to the last day of assessment) between the groups compared.

IHC

Formalin-fixed paraffin-embedded sections of xenograft tumors underwent antigen retrieval and were then blocked using 5% goat serum and avidin blocking solution. IHC was performed in a Leica Bond III auto-stainer (Leica Biosystems) according to the manufacturer's instructions. The primary rabbit mAbs against ER (SP1) and PR (SP42) were obtained from Dianova. Rabbit mAb against Ki67 (D2H10) and phospho-retinoblastoma protein (phospho-Rb) were from Cell Signaling Technology. Mouse antibody against AR (441) was obtained from Dako. The AR IHC staining protocol included the use of Immunodma Background Blocker (Bio SB). Sections shown in each figure were stained and photographed under identical conditions. Staining and scoring of the samples were performed by a pathologist blinded to the treatment groups.

Western blot analysis

Tumor lysates from the in vivo studies were prepared using Cell Lysis Buffer with protease and phosphatase inhibitors (Cell Signaling Technology) in a FastPrep Sample Preparation System (MP Biomedicals). Cell lysates were prepared using Cell Lysis Buffer as described above. Total protein was quantitated and subjected to Western blot analysis using antibodies against AR (PG-21, EMD Millipore; Clone 441, Thermo Fisher Scientific), Eρα, PR, GAPDH, β-tubulin, and HDAC2 (Cell Signaling Technology).

Gene expression analysis

Total RNA from xenograft samples and cells was extracted using the RNeasy Mini Kit (Qiagen), with a DNase incubation step included to ensure complete removal of genomic DNA. The expression of genes was evaluated using qPCR and RNA sequencing (RNA-seq). For qPCR, TaqMan primer and probe sets for AR, KLK2, FKBP5, ZBTB16, ERα, PGR, TFF1, GREB1, BLM, FANCI, LMB1, MCM2, MCM4, MCM7, 18S, and GAPDH were purchased from Thermo Fisher Scientific. The PCR reaction was set up with TaqMan Fast Virus 1-Step Master Mix in a Quant Studio 6 Flex qPCR system (Thermo Fisher Scientific). Samples from a minimum of 3 tumors from each treatment group or those from a minimum of 2 replicated cell-based assays were included for the analysis. The expression of genes of interest were normalized to that of GAPDH or 18S and presented as the mean fold change compared with the vehicle control group. For RNA-seq analysis, total RNA samples were converted into cDNA libraries using the TrueSeq Stranded mRNA Sample Preparation Kit (illumina). Libraries are quantitated, normalized, and pooled before being subjected to HiSeq 2 × 50 bp paired end sequencing on an Illumina sequencing platform. De-multiplexed FASTQ files were aligned to HG19 human genome using the STAR aligner version 2.4 and genetic features were quantified using RSEM version 1.2.14. The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE104177. The AR and ER target genes with significant changes in expression (FDR < 0.05) were analyzed and log2 fold changes presented in heatmap. Global pathway analysis was performed for genes with significant changes in expression (FDR < 0.001) using the functional annotation tools (Gene Ontology) available on the National Health Institute DAVID Bioinformatics Resources platform (https://david.ncifcrf.gov).

Results

RAD140 binds to AR with high affinity and high specificity

The binding affinity of RAD140 was assessed using fluorescence polarization-based competitive binding assays for AR, ER, PR, and GR and compared against the standard ligands for each nuclear receptor. As positive controls, assays evaluating the binding of the standard ligands, including DHT, 17β-estradiol (E2), progesterone, and dexamethasone to AR, ER, PR, and GR, respectively, were performed. In the competitive binding assay against the AR–Fluoromone, RAD140 exhibited 1.6-fold lower AR affinity than DHT (Fig. 1A). This suggests binding affinity of RAD140 to AR is slightly lower but comparable with that of natural androgen. RAD140 exhibited 33-fold lower affinity to PR compared with progesterone. No binding of RAD140 to ER or GR was detected. In addition, a cellular target spectrum screen was performed to determine the binding selectivity of RAD140 against a broad panel of 165 molecular targets that included many pharmacologically relevant molecules including ion channels, G-protein–coupled receptors, enzymes, and nuclear hormone receptors (Supplementary Table S1). No appreciable interaction of RAD140 with any targets screened, other than AR and PR, was detected. These results indicate RAD140 binds to AR with high affinity and specificity.

RAD140 is a potent tissue-selective AR agonist

SARMs are selective AR ligands that have been shown to act as agonists or antagonists in a tissue-context–dependent manner (32). We previously demonstrated that RAD140 exhibits differential activity in prostate versus in the muscle (30). To further assess the activity of RAD140 on AR transcription in tissue context, an AR reporter assay was performed in ZR-75-1, a human breast cancer cell line expressing endogenous AR and ER (21), and the
AR-positive prostate cancer cell line LNCaP (29). Dose responses by RAD140 were compared with the known AR agonist DHT. Treatment of steroid-depleted ZR-75-1 cells with 1, 10, and 100 nmol/L RAD140 induced AR transcription activity by 1.9- to 2.9-fold, comparable with the induction seen with DHT at the same concentrations (Fig. 1B, left). In contrast, in the low-passage, androgen-sensitive LNCaP cells treated with RAD140 at 0.1 to 10 nmol/L, no induction of AR activity was observed while DHT at 1 or 10 nmol/L led to more than a 10-fold induction of AR transcription activity (Fig. 1B, right). To rule out the possibility that the attenuated AR activity of RAD140 is due to the expression of a mutant AR T877A in LNCaP cells, we also performed AR reporter assay in PC3-AR cells, which express ectopic wild-type AR (29). Similar to the finding in LNCaP cells, RAD140 exhibited much attenuated activity on ectopic wild-type AR in prostate cancer cells compared with DHT (Supplementary Fig. S2).
results indicated that RAD140 is a tissue-specific AR agonist, with selective activity in breast cancer cells but not in prostate cancer cells.

Classic AR agonists are known to induce AR conformation change and subsequent localization to the nuclei, while pure AR antagonists, such as enzalutamide and ARN-509 act partially via blocking AR nuclear localization (33, 34). We then examined whether RAD140 induces the nuclear expression of AR in breast cancer cells. Treatment of ZR-75-1 cells with RAD140 led to increased AR expression in the nuclear fraction while the estrogen (E2) showed no apparent effect (Fig. 1C). The positive control, DHT, substantially increased the nuclear expression of AR. ARN-509 substantially reduced the nuclear expression induced by DHT and RAD140, suggesting a direct antagonism against these two AR agonists. This provided further evidence that RAD140 is an AR agonist in breast cancer cells.

RAD140 monotherapy suppresses the growth of AR/ER+ breast cancer PDX models

It has been previously reported that DHT inhibits the proliferation of ER+ breast cancer cells driven by supplemental estradiol (21). We evaluated the activity of RAD140 and DHT in an estrogen-independent AR/ER+ breast cancer line HCC1428 LTED, in which ER signaling appears to be active as judged by PR expression (Supplementary Fig. S1). RAD140 exhibited inhibitory effect on the proliferation of these endocrine-resistant breast cancer cells at concentrations as low as 10 nmol/L, with an apparent maximal effect seen at 100 nmol/L (Fig. 1D). This was comparable with the inhibitory effect seen with DHT in the 0.1 to 10 nmol/L range. To further assess how the AR agonist activity of RAD140 translates into antitumor activity in vivo, RAD140 was evaluated as a single agent in AR/ER+ breast cancer PDX models. Oral administration of RAD140 induced significant TGI of 76%, 59%, and 58% when compared with the vehicle control groups in HBCx-22, HBCx-3, and HBCx-21, respectively (Fig. 2A, Supplementary Fig. S3A and S3B). In the HBCx-22 model, fulvestrant, a selective ER downregulator (SERD) and standard of care for ER+ breast cancer dosed at 1 mg weekly, exhibited a statistically significant antitumor activity, as judged by a TGI of 59%, comparable with a previous report of this model and indicative of its dependency on ER (31). It has been previously determined that the 1 mg weekly administration of fulvestrant achieves exposure equivalent to the 500 mg monthly dose in human (35). The changes in tumor volume of the RAD140- or fulvestrant-treated HBCx-22 xenografts appeared to be lower compared with those treated with vehicle (Fig. 2B). To further verify the concept of inhibiting the growth of AR/ER+ breast cancer tumors with AR agonists, T-47D cell line–derived xenografts were treated with DHT, RAD140, or fulvestrant (Supplementary Fig. S3C). Treatment with DHT or RAD140 inhibited the growth of these T-47D tumors to a comparable extent. Fulvestrant also elicited potent growth inhibition. In addition, the activity of the combination of fulvestrant with RAD140 was evaluated in HBCx-22 model, but no appreciable improvement in tumor growth inhibition was observed, suggesting a potential overlap in the pathway inhibition induced by these two agents in this model (Supplementary Fig. S3D). This observation is consistent with previous clinical experience with anastrozole–fulvestrant combination and preclinical data of fulvestrant–RAD1901 combination, both of which showed such intense ER blockade failed to yield additional benefit (35, 36). Together, these results indicated that RAD140 as a single agent is effective in inhibiting the growth of AR/ER+ breast cancer xenografts.

We then examined the changes in common breast cancer biomarkers in the HBCx-22 xenografts treated with RAD140 or fulvestrant using IHC. As shown in Fig. 2C, HBCx-22 tumors in the control group were positive for AR, ER, and PR, consistent with prior characterization of this model (Supplementary Fig. S1). An apparently increased AR expression, predominantly localized in the nuclei, was seen in RAD140-treated tumors. This demonstrated that RAD140 possesses AR agonist properties, such as stabilizing AR and promoting its nuclear localization, which are commonly seen with classic androgens (37, 38). Interestingly, RAD140 treatment led to substantially decreased expression of ER and its downstream target PR. A profound decrease in Ki67 expression was also seen in these RAD140-treated tumors, indicating a suppressive effect on the proliferation of these tumor cells. Treatment with fulvestrant also led to substantial decreases in ER, PR, and Ki67 expression, to levels seemingly comparable with those seen with RAD140. Furthermore, the epithelial nature of the terminal tumor mass was confirmed using IHC assays for cytokeratin (CK) 18 and vimentin (Supplementary Fig. S4). This suggests SARM and SERD both inhibit the ER pathway and breast cancer cell proliferation.

Pharmacodynamic analysis reveals RAD140-mediated regulation of AR and ER target genes and ESR1

It has been proposed that androgens suppress ER signaling in AR/ER+ breast cancer cells, which may contribute to the anti- proliferative activity (19, 21). To better understand the mechanism of action of the SARM RAD140 in AR/ER+ breast cancer models, we examined the pharmacodynamic changes with a focus on the AR and ER pathways. In the HBCx-22 model, a profound induction of AR target genes, including FKBP5, KLK2, and ZBTB16, was seen in RAD140-treated tumors (Fig. 3A, top) while the expression of the AR gene was not affected. Fulvestrant, as expected, did not induce the expression of these AR target genes, although a slight increase in AR message was seen. The ER target genes PGR, TFF1, and GREB1 were found to be substantially suppressed in RAD140-treated tumors to levels comparable or even lower than that seen with fulvestrant (Fig. 3A, bottom). Interestingly, in these RAD140-treated tumors, the mRNA expression of ESR1, the coding gene of ER, was profoundly suppressed. In contrast, the fulvestrant treatment did not lead to appreciable change in ESR1 gene expression. At protein level, both RAD140 and fulvestrant exhibited a profound effect in decreasing the expression of ER and PR in HBCx-22 xenografts (Fig. 3B, left), consistent with the IHC findings described above. Similar decrease in ER and PR expression was seen in HBCx-3 AR/ER+ xenografts treated with RAD140 (Fig. 3B, middle). In the T-47D xenografts, treatment with RAD140, DHT, or fulvestrant also led to decreased ER and PR expression (Fig. 3B, right).

Next, the modulation of AR and ER target genes by RAD140 was further examined in vitro. As determined in ARE-luc reporter assay and proliferation assay (Fig. 1B and D), RAD140 at 100 nmol/L exhibited maximal effects, comparable with DHT at 10 nmol/L; therefore, 100 nmol/L was selected as the RAD140 concentration for further in vitro assays. In ZR-75-1 cells treated with RAD140, substantial induction of FKBP5 and ZBTB16 messages was seen (Fig. 3C), which was comparable with that seen with DHT. The induction of these AR targets by RAD140 or DHT was completely blocked by the antagonist enzalutamide. The expression of the AR
gene did not seem to be affected by RAD140 or DHT treatment. The ER target genes \textit{PGR} and \textit{TFF1} were induced by 1 nmol/L of E2. Treatment with either RAD140 or DHT was found to repress the E2-induced expression of these genes. Similar to the effects seen in xenograft models, treatment with RAD140 or DHT decreased \textit{ESR1} mRNA. The effects of RAD140 and DHT on AR pathway and ER pathway genes appear to be comparable, suggesting an AR-specific effect. Furthermore, competitive blockade of AR activation by enzalutamide effectively reversed the AR and ER pathway modulation by RAD140 or DHT. The incomplete reversal of \textit{ESR1} suppression by enzalutamide may be due to the relatively lower affinity of this compound compared with AR
Figure 3.
Expression of AR and ER pathway genes and ESR1 in RAD140-treated AR/ER⁺ breast cancer cells and xenografts. A, Tumor samples from the HBCx-22 collected 6 hours after last treatment were subjected to qPCR analysis for the expression of AR, ER, and the indicated downstream target genes. B, Samples from HBCx-22, HBCx-3, and T-47D xenograft models were collected 6 hours after last treatment and subjected to Western blot analysis for the expression of AR, ER, and PR. C, Steroid-deprived ZR-75-1 cells were pretreated with 1 nmol/L of E2 for 2 hours and then treated with RAD140 (100 nmol/L) or DHT (10 nmol/L) in the presence or absence of enzalutamide (10 μmol/L) for 24 hours. RNA of the treated cells was isolated and subjected to qPCR analysis for the expression of AR, ESR1, and the indicated downstream targets.
agonists RAD140 and DHT. These *in vivo* and *in vitro* observations further demonstrated the AR agonist activity of RAD140 in AR/ER⁺ breast cancer models. More importantly, RAD140 and DHT both inhibited ER downstream targets, as well as the ESR1 gene, suggesting a novel mechanism of action of AR agonists in inhibiting the ER pathway in breast cancer cells.

**Global modulation of signaling pathways by SARM in AR/ER⁺ breast cancer xenografts**

To further understand the global effect of RAD140 on signaling cascades in AR/ER⁺ breast cancer cells, RNA-seq analysis of HBCx-22 xenografts was performed. We examined a broader range of AR and ER target genes with significantly altered expression in RAD140-treated tumors (Fig. 4A). The results indicated that in addition to the targets examined by qPCR, RAD140-treated tumors had higher levels of AR-activated genes in addition to those shown in qPCR. A subset of the ER target genes was found to be suppressed in RAD140-treated tumors, consistent with the qPCR findings. Using a stringent filtering criteria (FDR < 0.001), we found that globally 86 genes were significantly upregulated and 77 were downregulated by at least 2-fold in RAD140-treated HBCx-22 xenografts (Fig. 4B; Supplementary Tables S3 and S4). In the Gene Ontology analysis, these upregulated genes were found to be most significantly enriched in the metabolic process. This was consistent with the previous report describing the role of AR as a master regulator of central metabolism and biosynthesis in prostate cancer cells (39). The downregulated genes in RAD140-treated tumors were found to be enriched in categories...
associated with cell division, DNA replication, and cell-cycle progression. This negative regulation of gene transcription was in line with previous reports on the transcription suppressor role of AR (40, 41). These data further confirmed the regulation of AR and ER pathways by RAD140 and suggested a unique mechanism of action of RAD140 via the AR-mediated transcription repression. These data offer evidence for further uncovering the underlying mechanism of RAD140 as a single agent in AR/ER+ breast cancer models.

Enhanced antitumor activity of combined administrations of RAD140 with CDK4/6 inhibitor

Given the observed effect of RAD140 on cell cycle and DNA replication–related genes, we hypothesized that combined administration of this SARM and a CDK4/6 inhibitor, which inhibits the phosphorylation and subsequent degradation of Rb, thus blocking cell-cycle entry (42–44), may produce improved antitumor activity in AR/ER+ breast cancer models. Palbociclib, a CDK4/6 inhibitor recently approved for breast cancer treatment in combination with ER-targeted agents in as early as first line of therapy (45–47), was selected for this study. Two AR/ER+ PDX models, HBCx-3 (Fig. 5A) and ST897 (Fig. 5B), were treated with RAD140, palbociclib, or a combination of RAD140 with palbociclib. Compared with the vehicle-treated group, RAD140 as monotherapy produced statistically significant inhibition of tumor growth in these models compared with vehicle control. Palbociclib as a single agent also inhibited the growth of these xenografts to a comparable level. Importantly, RAD140 and palbociclib when given together elicited improved efficacy over palbociclib or RAD140 monotherapies. Similarly, RAD140 and palbociclib combination produced improved efficacy compared with palbociclib alone in an additional AR/ER+ PDX model, HBCx-22 (Supplementary Fig. S5A). An additional TGI analysis performed for HBCx-3 xenografts treated with RAD140 and palbociclib with lower starting volume (≤108 mm3) versus those with higher starting volume (>108 mm3) did not reveal appreciable difference between the two subgroups, suggesting the combination treatment had similar effect in tumors of different starting size. As the combination of RAD140 with palbociclib induced apparent regression in ST897 tumors, we examined the expression of apoptotic marker cleaved caspase-3 in ST897 and HBCx-3 tumors treated with RAD140, palbociclib, or combination but did not observe clear signs of apoptosis (Supplementary Fig. S5B), suggesting a cytostatic but not cytotoxic effect. Together, these results indicate that the combination of RAD140 with palbociclib led to improved antitumor activity compared with each of these agents given as single agent.

The expressions of AR, ER, PR, Ki67, and phospho-Rb in the HBCx-3 PDX were assessed using IHC (Supplementary Fig. S6). Similar to that seen in HBCx-22 (Fig. 2C), RAD140 treatment led to apparent enrichment of AR in the nuclei, suggesting AR activation. ER and PR expression was profoundly decreased in tumors treated with RAD140–palbociclib combination. It has been recently reported that in castration-resistant prostate cancer cells, activated AR recruits hypophosphorylated Rb to the loci of genes implicated in DNA replication and suppress their transcription (41). We hypothesized that AR activated by RAD140 inhibits DNA replication–related genes in breast cancer cells, and this effect is enhanced by coadministration of palbociclib. Indeed, the HBCx-3 tumors treated with RAD140 alone had decreased expression of the DNA replication–related genes including ESR1 mRNA level was also observed in these RAD140-treated HBCx-3 xenografts. Palbociclib alone, in contrast, did not have an apparent effect on AR target genes but did seem to repress PGR and induce TFF1 and ESR1 to a relatively modest degree compared with the changes induced by RAD140 treatment. We next explored the underlying mechanism for the improved efficacy and enhanced inhibition of cell proliferation seen with the RAD140–palbociclib combination. It has been recently reported that in castration-resistant prostate cancer cells, activated AR recruits hypophosphorylated Rb to the loci of genes implicated in DNA replication and suppress their transcription (41). We hypothesized that AR activated by RAD140 inhibits DNA replication–related genes in breast cancer cells, and this effect is enhanced by coadministration of palbociclib. Indeed, the HBCx-3 tumors treated with RAD140 alone had decreased expression of the DNA replication–related genes including ESR1 mRNA level was also observed in these RAD140-treated HBCx-3 xenografts. Palbociclib alone, in contrast, did not have an apparent effect on AR target genes but did seem to repress PGR and induce TFF1 and ESR1 to a relatively modest degree compared with the changes induced by RAD140 treatment.

Discussion

This study demonstrates for the first time that RAD140, an orally available SARM, is an AR agonist in breast cancer cells and suppresses the growth and proliferation of multiple AR/ER+ breast cancer cell line and xenograft models. The AR pathway was found to be activated in RAD140-treated breast cancer cells and xenografts, while genes within the ER pathway, including ESR1, were suppressed. In addition, RAD140 treatment was found to decrease the expression of DNA replication–related genes in breast cancer cells, consistent with previous report that these genes were suppressed in androgen-treated prostate cancer cells. Combined administration of RAD140 with the CDK4/6 inhibitor palbociclib was more efficacious compared with either of the agents used alone. These findings suggest a distinct mechanism of action of RAD140, which includes the AR-mediated suppression of ESR1 in inhibiting AR/ER+ breast cancer growth. Accumulating evidence in the recent years has further defined the role of AR in breast cancer and has led to renewed interests in evaluating AR-targeted agents for breast cancer. The high prevalence of AR in the predominant ER+ subtype of breast cancers (6), clinical benefit rates as high as 39% seen with androgen therapy in breast cancer patients progressing on ER-targeted treatments (23, 24), along with prior experience with steroidal androgens in breast cancer together lend support to the development of a
Combined administration of RAD140 with CDK4/6 inhibitor inhibited AR/ER\textsuperscript{+} PDX growth. 

A, Mean tumor volumes ± SEM of HBCx-3 breast cancer xenografts treated with vehicle, RAD140 (100 mg/kg twice daily), palbociclib (75 mg/kg once daily), or a combination of RAD140 and palbociclib for the indicated period of time. \( n = 7 \)/group. Intergroup comparisons were carried out using Student t test based on changes in tumor volume (\( \Delta TV \)) from the beginning to the end of the study. For the comparison between RAD140 and vehicle groups, \( P < 0.05 \); *; the combination group versus palbociclib, \( P < 0.05 \); the combination group versus RAD140, \( P < 0.01 \).

B, Mean tumor volumes ± SEM of ST897 breast cancer xenografts treated with vehicle, RAD140 (100 mg/kg twice daily), palbociclib (75 mg/kg once daily), or a combination of RAD140 and palbociclib for the indicated period of time. \( n = 8 \)/group. Statistical analysis was performed as in A. For the comparison between RAD140 and vehicle groups, \( P < 0.001 \); **; combination group versus RAD140 group, \( P = 0.0012 \); **; combination group versus palbociclib group, \( P = 0.0027 \).

C, Tumor samples from the HBCx-3 model, as described in A, were collected 6 hours after last treatment and subjected to qPCR analysis for the expression of AR, ESR1, and their downstream targets, and the genes implicated in DNA replication including BLM, FANCI, LMNB1, MCM2, MCM4, and MCM7. Expression of these genes was normalized to that of ribosomal RNA 18S. Mean normalized expression ± SD from three individual tumors of each group is presented.
new generation of oral, nonsteroidal AR agonists for the treatment of AR/ER \* breast cancer.

SARMs are tissue-selective AR agonists by design and may offer a novel approach to inhibit the growth of AR/ER \* breast cancers, with substantially attenuated side effects commonly seen with classic nontissue selective androgens (32, 49). The activity of RAD140 are AR specific, as evidenced by the similar effects observed with DHT and RAD140 on AR and its downstream targets, along with the reversal of these effects by AR antagonists (Figs. 1C and 3C). In addition, due to its nonsteroidal structure, the SARM RAD140 is not subject to further conversion to estrogens by CYP19 aromatase, or to DHT by 5α-reductase, thus reducing the potential risk of stimulating ER \* tumor growth or increasing virilization. As a proof-of-concept study, we report here that RAD140 treatment inhibited the growth of the breast cancer xenograft models supplemented by exogenous estrogen. Of note, RAD140 and its classic androgen comparator, DHT, also inhibited the proliferation of an endocrine-resistant breast cancer cell line model, HCC1428 LTED, suggesting AR agonists may inhibit the growth of ER \* breast cancer models with or without estrogen supplementation. Furthermore, in multiple PDX models, RAD140 inhibited tumor growth as a single agent, and this effect was enhanced by combining with palbociclib. PDX models have been demonstrated to closely recapitulate human tumors with regards to tumor heterogeneity and response to standard-of-care agents (31, 50). Indeed, heterogeneity was observed in the PDX models used in this study, as indicated by variable levels of AR, ER, and PR expression, suggesting a good representation of the patient tumors with a specificity of expression levels of these nuclear receptors. HBCx-3 xenograft model, HCC1428 LTED, suggesting AR agonists may inhibit the growth of AR \* breast cancer cells in vitro and in vivo, while suppressing a subset of ER target genes. Importantly, we found that treatment with these AR agonists also led to substantial suppression of ER at both mRNA and protein levels. This suggests that AR may negatively regulate ER signaling by two mechanisms: while AR may compete with ER for coregulators (functional interference), it may directly downregulate ER expression (direct suppression). The reduction of ER expression by AR agonists has not been extensively documented but can be seen in a study by Poulin and colleagues (57), in which DHT treatment in ZR-75-1 cells for 8 or 12 days led to decreases of ER message by 50% or 65%, respectively. Another study also showed reduced ER protein level in ZR-75-1 cells treated with DHT, along with decreased expression of classic ER targets PGR and TFF1 (56), although the mechanism for the decrease in ER expression had remained to be further elucidated. Our data show both RAD140 and DHT treatment led to decreased ESR1 expression at as early as 24 hours, which may present a novel mechanism by which RAD140 suppresses ER signaling and differentiates from traditional ER-targeted agents that act via inhibiting ER protein function or leading to its degradation. This may translate into unique therapeutic benefit of SARMs in overcoming resistance to ER-targeted therapy seen in tumors with reactivated ER signaling, either via posttranslational modification or enrichment of ESR1 alterations. The inhibitory effect of RAD140 on the proliferation of HCC1428 LTED cells shed light on its efficacy in such E2-independent, albeit ER-active, models but further studies are needed to fully evaluate the activity of SARMs in similar models in vivo. In addition to the suppression of ER signaling, our RNA-seq analysis suggests that RAD140 may inhibit tumor growth via other AR-mediated mechanisms. Of note, RAD140 treatment suppressed genes implicated in DNA replication and cell-cycle progression, which were further suppressed by concomitant CDK4/6 inhibition. Gao and colleagues (41) described the role of AR as a tumor suppressor in castration-resistant prostate cancer (CRPC) cells by inhibiting DNA replication–related genes via the recruitment of hypophosphorylated Rb. In addition, the clinical and preclinical efficacy seen with high-dose androgen therapy in CRPC was also attributed to androgen-induced...
stabilization of AR, which prevents the relicensing of DNA for replication and subsequent cell-cycle progression [58, 59]. In the current study, the AR-mediated suppression of ESR1 and its downstream pathway, along with the suppression of DNA replication-and cell-cycle–related genes seen with RAD140 treatment suggest a distinct mechanism of action of this SARM in AR/ER+ breast cancer cells. The potent antitumor activity and tissue-selective AR activity, along with overall tolerability in animal models and oral availability together lend support to further clinical investigation of RAD140 in AR/ER+ breast cancer patients.

Disclosure of Potential Conflicts of Interest
C.P. Miller and G. Hattersley hold ownership interest (including patents) in Radius Health. No potential conflicts of interest were disclosed by the other authors.

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