**ORIGINAL ARTICLE**

Bicalutamide-activated oncolytic adenovirus for the adjuvant therapy of high-risk prostate cancer

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Conditionally replicating adenoviruses (CRAds) utilize tissue-specific promoters to control the expression of the early genes, E1A and E1B, to preferentially replicate and lyse tumor cells (oncolysis). Previous CRAds used in prostate cancer (PCa) gene therapy require androgens to activate prostate-specific promoters and induce viral replication. Unfortunately, these CRAds have reduced activity in patients on androgen-suppressive therapy. We describe a novel prostate-specific CRAd generated by fusing the E1A gene to the androgen receptor (AR) cDNA with a point mutation in codon 685 (C685Y). The E1A-AR fusion neutralizes the previously described mutual inhibition of E1A and AR, and the C685Y point mutation alters specificity of steroid ligand binding to the AR, such that both androgens and nonsteroidal anti-androgens can activate viral replication. We demonstrate that the mutated E1A-AR retained the ability to function in regulating AR-responsive genes and E1A-responsive viral genes. In combination therapy of virus, bicalutamide (anti-androgen) and radiation, a profound impact on cell death by viral oncolysis was seen both in vitro and tumor xenografts. To our knowledge, this is the first gene therapy engineered to be enhanced by anti-androgens and a particularly attractive adjuvant strategy for intensity-modulated radiation therapy of high-risk PCas.

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INTRODUCTION

Despite intense research, advanced prostate cancer (PCa) remains an incurable disease. Traditional chemotherapeutic strategies have only modestly extended life expectancy by a few months in hormone refractory disease.¹⁻³ This drug resistance can be associated with the unusually slow growth rate seen in PCa compared with other cancers.⁴⁻⁵ Prostate-specific conditionally replicating adenoviral gene therapy offers an alternative approach to traditional therapy due to the ability to infect quiescent and dividing cells.⁶⁻⁷ Several groups have developed gene therapy vectors for PCa treatment, however, for a variety of reasons only a few have been translated clinically.⁸⁻¹⁰ Prostate-specific conditionally replicating adenoviruses (CRAds) work by placing the replication control genes under the control of a prostate-specific promoter, resulting in a selectively replication-competent adenovirus (RCA). Early clinical experience has demonstrated that CRAds offer a safe, auxiliary platform for treating PCa; however, monotherapy with these agents has been associated with only modest clinical activity. Our group has been integrally involved in the development of prostate-specific CRAds for clinical translation.¹¹,¹² In order to optimize the oncolytic activity of these agents, we have focused on understanding the biology of action in PCa cells. Our analyses reveal that the early adenoviral genes (E1A) interact with the androgen receptor (AR) in PCa cells, thus limiting the activity of both E1A and AR. This mutual inhibition led to decreased potency of the adenoviral vectors. Recently, we were able to overcome this deficiency by fusing E1A with AR, such that the chimeric fusion now allows augmentation of activity, rather than inhibition.¹³

As the standard of care for high-risk patients (Gleason 7 or higher, T2 or higher) is to combine androgen-suppressive hormone therapy with radiation therapy,¹⁴,¹⁵ we sought to develop a virus that could be activated by anti-androgens. By incorporating a single-point mutation in codon 685 changing the amino acid from cysteine to tyrosine (C→Y) in the AR ligand-binding domain (LBD)¹⁶ of the E1A-AR fusion, we were able to construct a virus that is activated for replication by both androgens and nonsteroidal anti-androgens. This novel virus is an ideal construct for combination with intensity-modulated radiation therapy external beam radiation therapy for the treatment of high-risk PCAs.

MATERIALS AND METHODS

Cell culture and reagents

LNCaP, DU145, 293HEK and OVCAR3 cancer cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured as per the supplier’s protocol. C4-2 was obtained from Leland Chung, who derived the cell line from LnCaP. They were cultured in RPMI (Cellgro Mediatech, Herndon, VA, USA) supplemented with 10% fetal bovine serum (FBS) and maintained at passages 50–60. Virus packaging cell line DPL-S11 derived the cell line from LNCaP. They were cultured in RPMI (Cellgro Mediatech, Herndon, VA, USA) and 50 µg l⁻¹ G418. It was maintained in DMEM with 10% FBS. All media were supplemented with 5 µg ml⁻¹ ciprofloxacin hydrochloride (US Biological, Swampscott, MA, USA) and 50 µg ml⁻¹ Gentamicin (Quality Biological, Gaithersburg, MD, USA). All cells were cultured at 37 °C in an atmosphere containing 5% CO₂. All the restriction enzymes used are obtained from New England Biolabs (Ipswich, MA, USA). Primary mouse monoclonal antibodies for AR (AR 441) and E1A (M73) were bought from

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Santa Cruz Biotechnology (Santa Cruz, CA). Adenoviral DNA-binding protein (DBP) mouse monoclonal 86-8 was a kind gift from Dr Arnold J Levine (Cancer Institute of New Jersey). Most of the chemicals and reagents used in this study were ordered from Sigma-Aldrich (St Louis, MO, USA) unless otherwise specified.

Generation of recombinant adenoviruses
AdS-prostate-specific enhancer (PSE)/probasin promoter (PBN) E1A-AR and AdS PSE/PBN-E1A-ARC685Y were generated using AdEasy system (Stratagene, La Jolla, CA, USA). The E1A-AR chimera includes wild-type (WT) AR, whereas the E1A-ARC685Y includes a mutated AR in the LBD (C685Y) introduced by site-directed mutagenesis, and fused with the C terminus of adenovirus E1A gene. Briefly, shuttle plasmids RpsToad-PSE/PBN-E1A-AR or RpsToad-PSE/PBN-E1A-ARC685Y that carries prostate-specific enhancer and rat PBN driving E1A-AR or E1A-ARC685Y were linearized with PmeI restriction endonuclease. After gel purification, the linearized vectors were separately transformed into the electro-competent DPL-S11 cells for homologous recombination. The desired clones (pAdS-PSE/PBN-E1A-AR and pAdS-PSE/PBN-E1A-ARC685Y) after screening were transformed into DH10B cells for large-scale DNA amplification. For viral propagation, the recombinant plasmids (pAdS-PSE/PBN-E1A-AR and pAdS-PSE/PBN-E1A-ARC685Y) were linearized with EcoRI and transfected into adenovirus packaging cell line DPL-S11 to generate recombinant adenoviruses.

CN702 is a WT E3-deleted serotype-5 recombinant adenovirus described previously. Adeno-X-LacZ (Clontech, Mountain View, CA, USA) was used as a control virus. All viruses were used at two multiplicities of infection (MOI). FFIG, a replication-defective reporter virus that encodes green fluorescent protein (GFP) under the control of the major late promoter was used at 30 MOI. Large-scale viral purification was performed using either CsCl gradient ultracentrifugation or commercial adenovirus purification kit (Adenopure, Puresyn, PA, USA) and kept in dialysis buffer containing 15 mM Tris (pH 7.8), 2 mM MgCl2, and 5% sucrose. The titers of the viral stocks were determined using the Adeno-XTM Rapid Titer Kit (BD Biosciences, Palo Alto, CA, USA) and 293 cells. All viral stocks were tested for WT RCA background generated by homologous recombination using quantitative PCR with primers spanning WT E1A promoter and E1A gene. The RCA content of all the viruses amplified in DPL-S11 cells were undetectable.

Western blot analysis
Cells were washed with 1 x PBS and resuspended with five volumes of cold lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF and 0.5% NP-40) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The cell lysate was incubated on ice for 30 min and then centrifuged for 10 min at 4 °C. Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis, and the resolved proteins were then transferred to a nitrocellulose membrane. After blocking with 5% nonfat milk in tris buffered saline overnight at 4 °C, the blot was incubated with primary antibody at 1 h at room temperature. The membrane was then probed with horse radish peroxidase conjugated secondary antibody for 1 h and developed (ECL-Plus system, Amersham Pharmacia, Piscataway, NJ, USA) using the manufacturer’s protocol.

Reporter-based viral replication assay
A reporter FFIG virus was utilized for viral replication assay. As described previously, FFIG is a replication-defective reporter virus that was made by linking GFP to the viral major late fiber gene through an internal ribosome entry site. It expresses GFP in a replication-dependent manner when infected with a replicating adenovirus. Cancer cells plated at the density of 2 x 10⁴ cells per well in 48-well plates were infected with two MOI of virus (AdS PSE/PBN-E1A-ARC685Y AdS PSE/PBN-E1A-AR, CN702 or Adeno-LacZ) together with 30 MOI of reporter FFIG virus. Viral replication was monitored by GFP expression using fluorescence microscopy. Each saved image from the fluorescence microscope was blindly scored by two individuals. Data from these experiments were plotted as an average number of GFP cells per field.

In vitro radiation and cell viability
Acute single-HDR radiation was performed 24 h prior to viral infection at a dose 6 Gy (0.67 Gy/min); Gammarcell 40 ¹³⁷Cs irradiator, Atomic Energy Commission of Canada); nonirradiated control cells were seeded and infected at the same time. Cell viability was measured using (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium assay kit (American Type Culture Collection) over a period of 9 or 12 days post treatment. Briefly, growth media was removed and replaced with MIT solution that was tenfold diluted with fresh media; after incubation for 3 h at 37 °C, detergent reagent provided in the kit was added to lyse the purple precipitates. At each time point, percentage of cell survival after respective treatment was calculated by normalizing to the growth of untreated cells.

Luciferase assay
For luciferase assay PC3 cells plated at the density of 1 x 10⁴ cells per well in 96-well plate were transfected with 100 ng of the AR reporter pBK-PSE/PBN-Luc plasmid, together with equal molar concentrations of either pBK-CMV-AR or pBK-CMV-E1A-AR or pBK-CMV-E1A-ARCG85Y using Lipofectamine Plus Reagent (Invitrogen, Carlsbad, CA, USA). The transfection media was replaced with media containing 10% dextran–charcoal-stripped serum in the presence or absence of 5 nM synthetic androgen R1881 (Methyltrienolone, Sigma) and 5 μM to 40 μM bicalutamide (Sigma-Aldrich) after 4 h post transfection. Luciferase activity was measured 24 h post transfection using the dual-luciferase reporter system (Promega, Fitchburg, WI, USA). pRL-CMV (10 ng per well) was used as an internal control in all wells. All transfection assays were performed in triplicate and normalized to internal control pRL-CMV reporter. Luciferase activity is reported as relative light forming units to reflect the renilla normalization.

Statistical analysis
Statistical analysis was performed on Graph Pad Prism 5.0 (La Jolla, CA, USA), running on an IBM compatible computer, using the Windows operating system. Comparisons for paired data were analyzed using the Student’s t-test. Statistical significance was defined as a P-value < 0.05 and was denoted in each of the figures by an asterisk.

RESULTS
Functional evaluation of an activating mutation of ARCG85Y in the E1A-ARCG85Y chimera and construction of a novel CRAd
Mutation from cysteine to tyrosine in the LBD of AR at codon 685 (C685Y) induces a significant conformational change, resulting in the inability of the AR to be activated by nonsteroidal antiandrogens such as hydroxy-flutamide and bicalutamide. We introduced the C685Y gene mutation into an E1A-AR gene fusion product to produce the E1A-ARCG85Y chimera (Figure 1A). In order to confirm whether the mutant C685Y AR was transcriptionally active in the E1A-ARCG85Y chimeric fusion, a reporter assay was performed as previously described using the AR-dependent PSE/PBN driving the firefly luciferase gene. AR-negative PCa cells (for example) are unable to activate the prostate-specific promoters utilized in our constructs, as they all require active AR for maximal activity. However, reconstitution of AR transiently does allow full activation of our prostate-specific constructs. Hence, PC3 cells (AR negative) co-transfected with the dual-luciferase reporter plasmids (pBK-PSE/PBN-F.Luc and pBK-CMV-R.Luc) together with the AR-expression plasmids pDNA3.1-hAR, pBK-CMV-E1A-AR, or pBK-CMV-E1A-ARCG85Y induced expression of luciferase in the presence of the synthetic androgen R1881 (5nM) when compared with charcoal-stripped media (Figure 1B). However, in the presence of anti-androgen (bicalutamide), the reporter expression was...
Ad5-PSE/PBN-E1A-AR\textsuperscript{G685Y} retains androgen requirement and AR specificity for replication

We have previously reported that the replication of the parental virus Ad5-PSE/PBN-E1A-AR was strictly dependent on the presence of androgen and was highly specific to AR-positive prostate cell lines.\textsuperscript{13} The addition of the point mutation to E1A-AR\textsuperscript{G685Y} within the context of the intact CRAd was assessed to ensure that Ad5-PSE/PBN-E1A-AR\textsuperscript{G685Y} virus retains the same features. A replication-incompetent reporter virus (FFIG) was used to follow viral replication by linking expression of GFP to the major late promoter, which is only active during the terminal phases of viral replication as previously described. Cells (LNCaP, C4-2, DU145 and OVCAR3) were coinfected with two MOI of the Ad5-PSE/PBN-E1A-AR\textsuperscript{G685Y}, Ad5-PSE/PBN-E1A-AR, CN702 (E3-deleted WT Ad5 virus, positive control) or Ad-Lac-Z (replication-deficient virus, negative control) virus together with FFIG (30 MOI). All cells were incubated in media without FBS during infection. Two hours post infection (p.i.), the media was supplemented with 5 nm R1881 in 10% charcoal-stripped FBS. Ad5-PSE/PBN-E1A-AR\textsuperscript{G685Y} successfully replicated in LNCaP and C4-2 cells (both AR-positive prostate cell lines) and had significantly higher amounts of GFP-infected cells compared with AR-negative control DU145 and non-prostate cell lines) and had significantly higher amounts of GFP-infected cells compared with AR-negative control DU145 and non-prostate cell lines. The replication of Ad5-PSE/PBN-E1A-AR\textsuperscript{G685Y} was strictly dependent on the pre-

Previously, we demonstrated that pretreatment of cells with radiation enhances viral replication and cytotoxicity.\textsuperscript{18} The addition of radiation to Ad5-PSE/PBN-E1A-AR\textsuperscript{G685Y} infection was assessed to evaluate whether any benefit to viral replication also occurs with Ad5-PSE/PBN-E1A-AR\textsuperscript{G685Y}. Various cell lines (LNCaP, C4-2, DU145 and OVCAR3) were pretreated with 6 Gy of acute single-dose ionizing radiation followed by coinfection with the viruses, Ad5-PSE/PBN-E1A-AR\textsuperscript{G685Y}, Ad5-PSE/PBN-E1A-AR, CN702 (E3-deleted Ad5 WT control)\textsuperscript{19} and reporter virus FFIG (which turns cells green in proportion to viral replication). The replication of Ad5-PSE/PBN-E1A-AR\textsuperscript{G685Y} was enhanced by radiation in LNCaP and C4-2 cells, as significantly higher amounts introduced to a higher extent by the chimeric mutant E1A-AR\textsuperscript{G685Y} compared when E1A-AR and AR at various concentrations of bicalutamide (Figure 1b). To confirm that viral E1A is functional in the E1A-AR\textsuperscript{G685Y} construct, we looked at the ability of the E1A-AR\textsuperscript{G685Y} chimera to activate the transcription of the immediate downstream E1B gene of adenovirus. Using a reporter plasmid of the E1B promoter-driving firefly luciferase, PC3 cells were transfected with equal amount of plasmids (pBK-CMV-E1A-AR\textsuperscript{G685Y} or pBK-CMV-E1A-AR or pUC19 and pBK-CMV-R.Luc for normalization). As shown in Figure 1c, the induction of the E1B promoter by E1A was not compromised by the addition of the mutant AR\textsuperscript{G685Y} and was induced to higher levels with different concentrations of bicalutamide similar to E1A alone and was statistically higher compared with E1A-AR (Figure 1c), consistent with an augmentation of activity by the mutated AR.

Next, the overall expression of the E1A-AR\textsuperscript{G685Y} fusion construct was assessed. Lysates collected from the PC3 cells transfected with the plasmids pBK-CMV-E1A-AR\textsuperscript{G685Y} or pcDNA-E1A were subjected to western blot analysis. Fusion E1A-AR\textsuperscript{G685Y} protein was detected with two different antibodies (anti-AR and anti-E1A) and was expressed as a single-intact protein at the predicted size of ~155 kDa (45 kDa for E1A and 110 kDa for AR) compared with the pcDNA3.1-transfected PC3 cells (Supplementary Figure 1). On the basis of these results, we concluded that both E1A and AR\textsuperscript{G685Y} components of the fusion were able to express and function as transcriptional activators in the presence of androgen or anti-androgens (bicalutamide) and, as a result, are suitable for use in the construction of prostate-specific CRAds for high-risk PCA.

Figure 1. E1A-AR\textsuperscript{G685Y} activates the expression of an androgen receptor (AR) and viral promoter reporter: schematic of E1A/AR fusion construct. The large (13S) E1A protein is fused on the C-terminal end to the amino acid 2 of the AR (DBD, DNA-binding domain, LBD, ligand-binding domain). A single-point mutation was introduced to convert cysteine 685 to tyrosine in the LBD of AR (a). Firefly luciferase assay was performed using the AR reporter pBK-PSE/probasin promoter (PBN)-F.Luc co-transfected with a renilla luciferase transfection control (pRL-CMV) along with expression plasmids: AR, E1A-AR or E1A-AR\textsuperscript{G685Y}. Transfections were performed in PC3 (AR negative) cell lines in the presence of 5 nm R1881 and 1, 10 or 20\textmu M bicalutamide. Significant difference between E1A-AR and AR in R1881 and E1A-AR\textsuperscript{G685Y} vs AR and E1A-AR in bicalutamide is represented by * (P<0.05) (b). Similarly, the E1A reporter plasmid pE18-F.Luc was co-transfected with expression plasmids, encoding E1A, E1A-AR and E1A-AR\textsuperscript{G685Y} or vector alone (pUC19) in PC3 cells in the presence of 5 nm R1881, 1, 10 or 20\textmu M bicalutamide. The addition of the point mutation to E1A-AR\textsuperscript{G685Y} did not hamper E1A’s ability to activate transcription of the viral promoter E1B in the presence of R1881 and in the presence of bicalutamide. Significant difference of E1A and E1A-AR\textsuperscript{G685Y} vs E1A-AR at all concentrations of bicalutamide is represented by * (P<0.05) (c). Error bars represent mean ± s.e.
of GFP-infected cells were seen in radiation-treated cells compared with nonirradiated cells (Figure 2a). Although it appears there is a decrease in CN702-infected C4-2 cells with radiation compared with nonradiation (Figure 2b), there is more cell kill seen in the MTT cytotoxicity assay when irradiated cells are compared with nonirradiated cells (Figure 2b); therefore, this decrease in GFP-infected cells is likely to be due to viral-mediated cell lysis. To further evaluate the effect of radiation on viral oncolysis, we pretreated the cells (LNCaP, C4-2 and DU145) with 6 Gy of acute single-dose ionizing radiation followed by viral infection (two MOI). Cell viability was observed over a period of 12 days using MTT assay. Pretreatment of radiation significantly enhanced viral kill compared with the virus alone in both AR-positive PCa cell lines (Figures 2b and c). Interestingly, in C4-2 cells in the absence of radiation both viruses exerted little to no cytotoxicity, however, in combination with radiation, the Ad5-PSE/PBN-E1A-ARC685Y had killed over 78% of the C4-2 cells and Ad5-PSE/PBN-E1A-AR had 68% cell kill at the end of 12 days (Figure 2c). As expected, no viral-derived cytotoxicity was observed in the control cell line DU145 (Figure 2d). Therefore, Ad5-PSE/PBN-E1A-ARC685Y retains the ability to cause tissue-specific cytotoxicity, which is enhanced by radiation in hormone-sensitive and AR-positive PCa cells similar to the parental virus.

Replication and cytotoxicity of Ad5-PSE/PBN-E1A-ARC685Y in combination with radiation in the presence of anti-androgen (bicalutamide)

Testosterone suppression through androgen deprivation therapy is standard treatment for patients, presenting with advanced PCa.20 However, a large fraction of patients treated with androgen deprivation therapy develop androgen-resistant cancer, which is exemplified by the C4-2 cell line model.21 This cell line was chosen as a model of androgen-independent disease to test the potency of Ad5-PSE/PBN-E1A-ARC685Y in the presence of the nonsteroidal anti-androgen bicalutamide. First, replication of Ad5-PSE/PBN-E1A-ARC685Y in C4-2 was monitored by FFIG assay as previously described.22 C4-2 cells plated in charcoal-stripped media were irradiated (6 Gy) 24 h prior to Ad5-PSE/PBN-E1A-ARC685Y viral infection (2 MOI) together with FFIG (30 MOI) in the presence of bicalutamide (5–40 μM). Viral replication was monitored using fluorescent microscopy (through GFP expression in the FFIG reporter virus) and quantitated by scoring of GFP-positive-infected cells. Ad5-PSE/PBN-E1A-ARC685Y was able to replicate in the presence of bicalutamide in irradiated C4-2 cells; furthermore, there was a dose-dependent response of the Ad5-PSE/PBN-E1A-ARC685Y replication with increasing concentration of bicalutamide (Figure 3a and Supplementary Figure 3).

Next, we looked at replication-mediated cytotoxicity of Ad5-PSE/PBN-E1A-ARC685Y in the presence of radiation and bicalutamide. C4-2 cells were irradiated with 6 Gy of acute single-dose radiation, then infected with the Ad5-PSE/PBN-E1A-ARC685Y or mock treated in the presence of bicalutamide ranging from 5 to 40 μM. Cell viability was observed by MTT over a period of 12 days. The virus caused minimal cell death in nonirradiated cells in the presence of bicalutamide; however, when both treatments were combined together, the viability of the C4-2 cells significantly decreased in a dose-dependent manner (Figure 3b). These results demonstrate that Ad5-PSE/PBN-E1A-ARC685Y virus is not only able to replicate in the presence of the bicalutamide in androgen-independent cells, but it can also markedly increase the therapeutic impact of neo-adjuvant radiation therapy.
Ad5-PSE-PBN-E1A-AR or CN702 (positive control) or Ad-Lac-Z viral-derived cytotoxicity, we chose to focus on 10 μM PBN-E1A-ARC685Y replication in the presence of bicalutamide is cell lines tested in order to ensure that the specificity of Ad5-PSE/PBN-E1A-AR replication in 20 and 40 μM bicalutamide (vs 5 and 10 μM; *P < 0.05) (a). Growth inhibition and cytotoxicity of Ad5-PSE/PBN-E1A-ARC685Y was assessed by MTT assay 12 days after treatment in the presence and absence of 6 Gy of ionizing radiation, and increasing concentrations of bicalutamide from 5 to 40 μM. Significant higher cell kill in radiation-treated cells vs mock-treated cells in all concentrations of bicalutamide (*P < 0.001) (b). Error bars represent mean ± s.e.

Comparison of the effect of bicalutamide and radiation on viral replication in vitro

Although Ad5-PSE/PBN-E1A-ARC685Y virus in combination with radiation and 40 μM bicalutamide caused the greatest extent of viral-derived cytotoxicity, we chose to focus on 10 μM bicalutamide in the following experiments, as this is the calculated expected concentration corresponding to a 70-kg patient taking 100–150 mg bicalutamide orally per day. Next, we expanded the cell lines tested in order to ensure that the specificity of Ad5-PSE/PBN-E1A-AR replication in the presence of bicalutamide is limited to AR-positive PCa cells. C4-2, DU145 and OVCAR3 cells were first pretreated with 6 Gy of acute single-dose ionizing radiation, followed by coinfections of Ad5-PSE/PBN-E1A-ARC685Y or Ad5-PSE-PBN-E1A-AR or CN702 (positive control) or Ad-Lac-Z (negative control) with reporter FFIG virus in the presence of 10 μM bicalutamide and GFP-positive cells were scored. The presence of bicalutamide severely hampered the ability of the parental Ad5-PSE-PBN-E1A-AR virus to replicate due to the androgen blockade, as very low levels of GFP-infected cells were seen (Figure 4 and Supplementary Figure 4). In comparison, Ad5-PSE/PBN-E1A-ARC685Y replication was restricted to androgen-insensitive C4-2 PCa cells in the presence of bicalutamide, and replication was significantly induced when combined with a single acute dose of irradiation (6 Gy).

Next, we compared the cytotoxic effect of the parental Ad5-PSE-PBN-E1A-AR virus to Ad5-PSE/PBN-E1A-ARC685Y virus. The triple therapy of two MOI of virus, 6 Gy of radiation and 10 μM bicalutamide was tested on C4-2, DU145 and OVCAR3 cells, respectively, and the resulting cytotoxicity was measured using MTT assay over 12 days. The viruses were not cytotoxic to any of the bicalutamide-treated cell lines in the absence of radiation (Figure 5). However, when radiation (6 Gy) was combined together with viruses and bicalutamide (10 μM), a significant therapeutic effect was observed in Ad5-PSE/PBN-E1A-ARC685Y-infected C4-2 cells. Approximately 71% (P < 0.05) of cells were killed when compared with 39% in parental Ad5-PSE/PBN-E1A-AR-infected C4-2 cells (Figure 5a). This finding of radiation and bicalutamide-induced viral cell death was not observed in the other two control cell lines (DU145 and OVCAR3). These in vitro results demonstrate the utility of Ad5-PSE/PBN-E1A-ARC685Y in gene therapy of advance prostate disease in combination with radiation.

Antitumor effect of Ad5-PSE/PBN-E1A-ARC685Y in combination with radiation in androgen-ablated C4-2 xenograft mouse model

Lastly, the therapeutic efficacy of Ad5-PSE/PBN-E1A-AR virus in combination with radiation and bicalutamide was tested in vivo.
Androgen-independent C4-2 xenograft tumors were established in nude mice. Once the tumor volume reached to ~220 mm³, animals were randomized into three different groups (virus alone, radiation alone and virus plus radiation). One week prior to radiation and viral infection, all mice were started on the bicalutamide regimen (20 mg kg⁻¹) given i.p. three times a week throughout the duration of the study. The treatment schedule consisted of three separate doses of 2 Gy of local radiation for a total of 6 Gy of radiation and subsequently followed by three separate intratumoral injections of 1 × 10⁷ PFU of Ad5-PSE/PBN-E1A-ARC₆₈₅Y in the virus plus radiation group. We selected large tumor burden in these experiments in order to maximize the chance of demonstrating an additive effect between the two therapies, which may have been missed due to eradication of the tumors by either virus or radiation alone. Owing to the variation in size of the tumors in each group the fold tumor growth averages were plotted.

Fold growth of the tumor volume of the virus plus radiation group was statistically significantly lower than the virus alone group between 10 to 28 days post start of treatment. The virus plus radiation group compared with radiation alone was statistically significantly lower from 24 days post start of treatment through the duration of the study (Figure 6a). Residual tumor cells that have recovered from radiation treatment and begin to proliferate would cause the increase in fold tumor growth seen in the radiation alone group toward the end of the study. In the virus plus radiation-treated tumors, the growth stays approximately the same as the radiation alone tumors increase and this appears to be a long-lasting virus effect against tumor growth. This may be why this group becomes significantly lower than radiation at these later time points, but loses the earlier seen significance compared with the virus alone group. Although the low amount of virus used was not able to overcome the tumor burden used in the study, it is possible that given at a much higher MOI, with the addition of radiation and the presence of bicalutamide, the combination may have been able to eradicate these tumors completely. These results indicate that even at a low dosage of virus, the cumulative effective of radiation and virotherapy was able to keep the tumor size much smaller compared with single-treatment groups.

To further confirm that the differential therapeutic effect observed in the virus plus radiation group was due to active viral replication and oncolysis, tumors harvested at the end of the study from each treatment group were subjected to immunohis-tochemistry (IHC). We used the adenovirus E2 72 K DNA-binding protein (DBP) for IHC staining because it has been previously shown as a marker for viral replication and is expressed in abundance during viral infection.²² By IHC, we were able to detect the expression of DBP in both Ad5-PSE/PBN-E1A-ARC₆₈₅Y alone and the Ad5-PSE/PBN-E1A-ARC₆₈₅Y plus radiation groups; however, the amount of expression of the DBP was significantly higher in the virus plus radiation group, indicating that the active-replicating virus still persisted long after (35 days p.i.) the initial infection and contributed to the therapeutic effect of the tumors seen in these animals (Figure 6b). This data demonstrate the persistence of Ad5-PSE/PBN-E1A-ARC₆₈₅Y virus long after initial virus injection, and has the potential to enhance both the therapeutic efficacy of radiation and viral replication in the presence of hormone therapy to be used for the treatment of high-risk and androgen-refractory PCas.

DISCUSSION

Early clinical experience has demonstrated that CRAds offer a safe, auxiliary platform for treating PCa; however, monotherapy with these agents has been associated with only modest clinical activity.¹² More recently, we have found that the combination of these adeno-viral vectors with chemotherapy or radiation therapy

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Figure 6. Oncolytic activity of Ad5-prostate-specific enhancer (PSE)/probasin promoter (PBN)-E1A-AR C685Y in combination with radiation and bicalutamide treatment in vivo tumor xenograft model. C4-2 prostate cancer xenografts were established by injecting $1 \times 10^6$ cells into the dorsal rear flank region of athymic nude mice ($n = 6$ per group) to examine the antitumor activity of Ad5-PSE/PBN-E1A-AR C685Y virus. All animals were put on bicalutamide therapy of 20 mg kg$^{-1}$ given three times a week i.p. Radiation group tumors were pretreated with 2 Gy of ionizing radiation given on 3 separate days for a total of 6 Gy prior to virus infection. Ad5-PSE/PBN-E1A-AR C685Y virus was injected three times intratumorally at $1 \times 10^7$ plaque-forming units on the day of the last dose of radiation, day 1 post radiation and day 4 post radiation, and tumors were measured three times a week along with bicalutamide treatment for the duration of the study. Average fold tumor growth was plotted for each animal group. There was a long-lasting antitumor growth activity seen in Ad5-PSE/PBN-E1A-AR C685Y plus radiation group that was significant compared with virus treatment alone from 10–28 days post treatment ($P < 0.05$). Ad5-PSE/PBN-E1A-AR C685Y-treated tumors compared with radiation alone were significantly lower starting 24 days post treatment and continued throughout the study ($P < 0.05$). Error bars represent fold mean ± s.e. (a). After 35 days, mice were euthanized, and tumors harvested from all groups for immunohistochemistry (IHC) were sectioned and stained with anti-adenoviral DNA-binding protein (DBP) to assess active viral replication. H&E staining of radiation + AdC685Y (b), radiation alone (c) and AdC685Y alone (d). IHC of adenovirus 72 K DBP × 20 magnification radiation + AdC685Y (e), radiation alone (f) and AdC685Y Alone (g). IHC of Adenovirus 72 K DBP × 40 magnification radiation + AdC685Y (h), radiation alone (i) and AdC685Y alone (j).
results in significant enhancement of activity.18–23 In this study, we sought to augment the activity of radiation therapy by the incorporation of a conditionally RCA, designed specifically for the intermediate to high-risk PCs patient, who normally would receive neo-adjuvant androgen suppression.

Patients with high-grade cancer (Gleason 7 or higher) are typically treated with either radiation therapy or radical surgery. Which approach is used depends on the clinical situation, the patient’s preference and the likelihood of success. Patients who ultimately elect to proceed forward with radiation therapy most typically are treated with a combination of hormone therapy (androgen suppression) and intensity-modulated radiation therapy, particularly if the cancer is Gleason 8 or higher and the disease is palpable. Unfortunately, these relatively high-risk patients have a significant treatment failure rate (in excess of 20% at 5 years)24 despite advanced imaging technology and escalating radiation doses. Hence, there is a necessity for improvement in outcomes for this group. Using an anti-androgen inducible prostate-specific CRAd in combination with radiation therapy, we were able to engineer a gene therapy vector specifically for these high-risk prostate patients.

Mutations in AR, especially in the LBD, are one of the mechanisms proposed to explain how advanced PCs may escape androgen deprivation.25–27 These mutations expand the specificity and affinity of the AR to other hormones, resulting in inappropriate receptor activation.28 In order to use these genes in prostate cancer, adenoviral gene therapy to activate the vectors for therapeutic applications, we fused the N terminus of an ARC685Y mutant, which has been shown to become activated by nonsteroidal anti-androgens. Studying the biology of this point mutation, we found that the mutated E1A-AR685Y fusion retained ability to function both in regulating AR-responsive genes (for example, the PBN we use to direct replication) and found that it replicated more slowly than our previous constructs (data not shown) and had minimal activity in terms of killing PCa cells (Figure 1a). However, when this virus was combined with increasing concentration of bicalutamide and high-dose rate radiation, it exerts a profound impact on viral replication, which was shown to be bicalutamide dose dependent. The replication of Ad5 PSE/PBN-E1A-AR685Y virus in the presence of androgens and anti-androgens (bicalutamide) was shown in the context of the replication-deficient reporter virus (FFIG), for which GFP expression is linked to the viral major late promoter and can only replicate when coinfected with RCA (Figures 2 and 3). We believe that the therapeutic effect observed in C4-2 cells is because of the viral oncolysis that was induced by bicalutamide and radiation treatment, as no such effect was observed in AR-negative prostate DU145 or control OVCAR3 ovarian cancer cell lines (Figure 4). To further support our in vitro results, we measured the activity of this CRAd in the in vivo androgen-insensitive C4-2 tumors xenograft model, which have been treated with radiation alone, virus alone or combination with radiation and virus in the presence of bicalutamide (20 mg kg−1). As expected, virus in combination with radiation was able to significantly reduce the size of the established tumors compared with the virus or radiation alone. We also evaluated and compared the presence of replication foci by IHC in virus-treated tumors vs tumors treated with virus irradiation. Tumors harvested at the end of the experiment were stained with adenoviral DBP. A robust staining pattern with larger foci was only observed in the virus and radiation combination group compared with the virus alone.

In summary, we describe a novel prostate-specific CRAd engineered to replicate specifically in patients receiving hormone therapy via nonsteroidal anti-androgens. These findings highlight a novel therapeutic strategy for augmenting radiation therapy in high-risk PCa.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES
1 Petrylak DP. Chemotherapy for advanced hormone refractory prostate cancer. Urology 1999; 54: 30–35.
2 Petrylak DP, Tannen GM, Hussain MH, Lara Jr PN, Jones JA, Taplin ME et al. Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 2004; 351: 1513–1520.
3 Kantoff P. Recent progress in management of advanced prostate cancer. Oncology 2005; 19: 631–636.
4 Berenguer RR, Vukanic, J Epstein JI, CarMichel M, Cisek L, Johnson DE et al. Implication of cell kinetic changes during the progression of human prostatic cancer. Clin Cancer Res 1995; 1: 473–480.
5 Visakorpi T, Kallioniemi OP, Parhonen JY, Isola JJ, Heikkinen AI, Koivula TA. Flow cytometric analysis of DNA ploidy and S-phase fraction from prostatic carcinomas: implications for prognosis and response to endocrine therapy. Br J Cancer 1991; 64: 578–582.
6 Spindler KL, Ceng CY, Berk AJ. An adenovirus early region 1A protein is required for maximal viral DNA replication in growth-arrested human cells. J Virol 1995; 53: 742–750.
7 Greber UF, Willett W, Webster P, Helenius A. Stepwise dismantling of adenovirus replication, which was shown to be bicalutamide dose dependent. The replication of Ad5 PSE/PBN-E1A-AR685Y virus in the presence of androgens and anti-androgens (bicalutamide) was shown in the context of the replication-deficient reporter virus (FFIG), for which GFP expression is linked to the viral major late promoter and can only replicate when coinfected with RCA (Figures 2 and 3). We believe that the therapeutic effect observed in C4-2 cells is because of the viral oncolysis that was induced by bicalutamide and radiation treatment, as no such effect was observed in AR-negative prostate DU145 or control OVCAR3 ovarian cancer cell lines (Figure 4). To further support our in vitro results, we measured the activity of this CRAd in the in vivo androgen-insensitive C4-2 tumors xenograft model, which have been treated with radiation alone, virus alone or combination with radiation and virus in the presence of bicalutamide (20 mg kg−1). As expected, virus in combination with radiation was able to significantly reduce the size of the established tumors compared with the virus or radiation alone. We also evaluated and compared the presence of replication foci by IHC in virus-treated tumors vs tumors treated with virus irradiation. Tumors harvested at the end of the experiment were stained with adenoviral DBP. A robust staining pattern with larger foci was only observed in the virus and radiation combination group compared with the virus alone.

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19 Rodriguez R, Schuur ER, Lim HY, Henderson GA, Simons JW, Henderson DR. Prostate attenuated replication competent adenovirus (ARCA) CN706: a selective cytotoxic for prostate-specific antigen-positive prostate cancer cells. Cancer Res 1997; 57: 2559–2563.

20 Ryan CJ, Small EJ. The selection of hormonal therapy in prostate cancer: who, when, and for how long? J Natl Compr Canc Netw 2004; 2: 261–268.

21 Wu HC, Hsieh JT, Gleave ME, Brown NM, Pathak S, Chung LW. Derivation of androgen-independent human LNCaP prostatic cancer cell sublines: role of bone stromal cells. Int J Cancer 1994; 57: 406–412.

22 Reich NC, Sarnow P, Duprey E, Levine AJ. Monoclonal antibodies which recognize native and denatured forms of the adenovirus DNA-binding protein. Virology 1983; 128: 480–484.

23 Hoti N, Chowdhury WH, Mustafa S, Ribas J, Castanares M, Johnson T et al. Armoring CRAds with p21/Waf-1 shRNAs: the next generation of oncolytic adenoviruses. Cancer Gene Ther 2010; 17: 585–597.

24 Bolla M, Fourneret P, Beneyton V, Tessier A, Jover F, Verry C. Combination of external irradiation and androgen suppression for prostate cancer: facts and questions. Cancer Radiother 2010; 14: 510–514.

25 Han G, Buchanan G, Ittmann M, Harris JM, Yu X, Demayo FJ et al. Mutation of the androgen receptor causes oncogenic transformation of the prostate. Proc Natl Acad Sci USA 2005; 102: 1151–1156.

26 Waltering KK, Urbanucci A, Visakorpi T. Androgen receptor (AR) aberrations in castration-resistant prostate cancer. Mol Cell Endocrinol 2012; 360: 38–43.

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