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PKA Knockdown Enhances Cell Killing In Response To Radiation And Androgen Deprivation

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Abstract

The therapeutic efficacy of Gem@231, a second generation antisense molecule targeted to the RI α subunit of PKA_{RI α} (AS-PKA), administered in combination with androgen deprivation (AD) and radiation therapy (RT), was examined in androgen sensitive (LNCaP) and insensitive (PC3) cell lines.

Apoptosis was assayed by Caspase 3+7 activity and AnnexinV binding. AS-PKA significantly increased apoptosis *in vitro* from RT (both lines), with further increases in LNCaP cells grown in AD medium. In LNCaP cells, AD increased phosphorylated map-kinase (pMAPK), which was reduced by AS-PKA relative to the MM controls. AS-PKA also reduced pMAPK levels in PC3 cells. Cell death was measured by clonogenic survival assays.

In vivo, LNCaP cells were grown orthotopically in nude mice. Tumor kinetics were measured by magnetic resonance imaging and serum prostate-specific antigen. PC3 cells were grown subcutaneously and tumor volume assessed by caliper measurements. In PC3 xenografts, AS-PKA caused a significant increase in tumor doubling time relative to MM controls as a monotherapy or in combination with RT. In orthotopic LNCaP tumors, AS-PKA was ineffective as a monotherapy; however it caused a statistically significant increase in tumor doubling time relative to MM controls when used in combination with AD, with or without RT. PKA_{RI α} levels in tumors were quantified via immunohistochemical (IHC) staining and image analysis. IHC measurements in LNCaP cells showed that AS-PKA reduced PKA_{RI α} levels *in vivo*.

We demonstrate for the first time that AS-PKA enhances cell killing androgen sensitive prostate cancer cells to AD $\pm$ RT and androgen insensitive cells to RT.

Keywords

Antisense; Protein kinase A; prostate cancer; radiation; androgen deprivation

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INTRODUCTION

The combination of radiotherapy (RT) and androgen deprivation (AD) is commonly used for the treatment for clinically localized intermediate-to-high risk prostate cancer¹⁻³. Even though significant gains have been realized with RT dose escalation and the addition of AD to RT, the risk of relapse remains high. Recurrence is related to both the presence of micrometastatic disease prior to treatment and local persistence of disease after treatment^{4,5}. There are few reports of targeted agents that sensitize prostate cancer cells to RT and AD⁶⁻⁸. If such sensitization were achieved, enhanced cell death locally and distantly would be expected to enhance patient survival.

The protein kinase A family of enzymes, also known as cAMP-dependent protein kinases, have activity that is dependent on the level of cyclic AMP (cAMP) in the cell. Each is a holoenzyme consisting of two regulatory and two catalytic subunits, protein kinase A type I (PKA_{RIα}) and type II (PKA_{RIIα} and PKA_{RIIβ}) being distinguished by the different regulatory subunits RI and RII⁹. The enhanced expression of PKA_{RIα} has been associated with active cell growth. Reductions in the expression of PKA_{RIα} and/or increases of PKA_{RII} are related to reduced cell growth and differentiation-maturation¹⁰⁻¹². Under low levels of cAMP, the holoenzyme remains intact and is catalytically inactive. When the concentration of cAMP rises through activation of adenylate cyclases by specific G protein-coupled receptors or inhibition of cAMP degradation by phosphodiesterases, cAMP binds to two sites on the regulatory subunits. This binding results in a conformational change that releases the catalytic subunits resulting in protein substrate serine or threonine phosphorylation⁹.

Cancer cell lines derived from a wide variety of organ sites overexpress PKA_{RIα}¹³⁻¹⁶ and overexpression is related to higher stage and poor prognosis in patients with breast cancer¹⁷, lymph node metastases in lung cancer¹⁶ and higher grade in colon cancer¹⁴, suggesting that PKA may be a potential molecular target^{15,18}. The second generation PKA_{RIα} inhibitor studied here, Gem@231, causes tumor growth delay in combination therapy with irinotecan¹⁹, and with radiation and antibodies directed against the epidermal growth factor receptor²⁰. PKA_{RIα} inhibition has also been shown to sensitize radioresistant leukemic cells to RT in culture²¹. Reported molecular effects associated with PKA_{RIα} inhibition include reductions in epidermal growth factor receptor function^{22,23} and bcl-2 anti-apoptotic activity, as well as bax upregulation²⁴.

We have recently shown that PKA_{RIα} levels are correlated with the development of distant metastases, cause specific mortality, and local failure and overall mortality in prostate cancer²⁵. Although prostate cancer often presents initially as a slow growing tumor type, delaying the transformation of these tumors to the rapidly growing, androgen insensitive phenotype would be of clear clinical importance. Since PKA_{RIα} levels have been linked to the transition from androgen sensitivity to insensitivity (related in part to the modulation of MAP-kinase activity²⁶⁻²⁸), and genomic analysis has identified a set of 22 additional genes which exhibiting differential expression from exposure to androgen (via R1881 treatment) or PKA pathway activation (via forskolin treatment) in cultured LNCaP cells²⁹, suppression of PKA_{RIα} is a logical approach to enhancing standard prostate cancer therapies. In this paper we describe the effects of a novel, mixed backbone oligonucleotide (Gem@231)³⁰ directed against the RI regulatory subunit of the cellular proliferation and anti-apoptotic agent protein kinase A (PKA). Based on these results we propose here for the first time that treatment with AS-PKA enhances the response of androgen sensitive (LNCaP) prostate cancer cells to AD and AD+RT, and the response of androgen insensitive (PC3) cells to RT, *in vitro* and *in vivo*.

MATERIALS AND METHODS

Antisense oligonucleotides

The oligonucleotides were synthesized and provided by Idera Pharmaceuticals Inc. (Cambridge, MA). AS-PKA (Gem®231) is produced as a hybrid oligonucleotide targeted against the N-terminal 8–13 codons of the RI α regulatory subunit of PKA, with the following sequence: 5'-*GCGUGCCTCCTCACUGGC*-3'. The mismatch control (MM) is a scrambled mixed-backbone oligonucleotide obtained by mixing all four nucleosides in a mixture containing all possible sequences: 5'-*UGTCACCCCTTTTCATUCAC*-3'. AS-PKA and MM oligonucleotides were stored as frozen aliquots at –20°C.

Cell culture system

The cell culture systems for LNCaP and PC3 cells have been described in previous publications^{8,31,32}. LNCaP and PC3 cells, obtained from the American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium (DMEM)–F12, containing 1% L-glutamine, 1% penicillin-streptomycin and 10% fetal bovine serum (complete medium [CM]), in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. For LNCaP cells, androgen deprivation was achieved by culturing the cells for 3 days in medium containing 10% charcoal-stripped serum (AD medium). Androgen was replaced by adding the synthetic androgen R1881 (NEN Life Science Products, Boston, MA) at 1×10^{–10}M to AD medium. We have shown previously in titration experiments that this concentration is optimal for growth promotion³³.

Western blot analyses

Levels of PKA, Androgen Receptor (AR), β -actin, and other proteins of interest were analyzed after the different treatments. LNCaP cells were cultured in complete, AD, or AD +R1881 medium for 3 days and then transfected with 200 nM of AS-PKA or MM in 5 ml culture medium for 24 h in the presence of 6 μ g/ml lipofectin (LF; Invitrogen, Carlsbad, CA). PC3 cells were cultured only in complete medium for 2 days and then treated with the same protocol presented above. Three hours after γ -irradiation to 5 Gy (RT) using a ¹³⁷Cs irradiator (Model 81-14R, J.L. Shepherd & Associates, San Fernando, CA), cells were lysed in a lysis buffer (50mM Tris-HCl, pH 7.3, 2% sodium dodecyl sulfate [SDS] with protease inhibitor cocktail set I [Calbiochem, San Diego, CA] and phosphatase inhibitor [NaF 5mM, Na₃VO₄ 5mM]) and were sonicated for 30s on ice.

Protein concentration was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL). Identical amounts of protein were fractionated by SDS-PAGE electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were then incubated in blocking buffer (phosphate-buffered saline containing 0.1% Tween 20 and 5% nonfat-milk) for 1h at room temperature and were washed twice with the washing buffer (phosphate-buffered saline containing 0.1% Tween 20) for 5min. The membranes were then incubated with the appropriate primary antibody: anti-PKA monoclonal antibody (mAb) at 1:300 dilution (BD Bioscience Pharmingen, Franklin Lakes, NJ), anti-p42/44 mAb at 1:1000, anti-AR mAb at 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA), and anti- β -actin mAb at 1:4000 dilution (Calbiochem, San Diego, CA) overnight at 4°C. Membranes were washed and then incubated with 1:2000 diluted sheep anti-mouse IgG or donkey-rabbit IgG horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech, Piscataway, NJ) for 1h at room temperature. After the washes were repeated, the proteins of interest were detected by the enhanced chemiluminescence reagents according to the manufacturer's directions (Amersham, Aylesbury, UK). Each western blot assay was repeated a minimum of three times.

Apoptosis Assays

AnnexinV staining and Caspase 3+7 activity assays were used to analyze apoptotic cell death, as described previously³². LNCaP cells were plated at 2×10^5 in 60mm culture dishes containing complete, AD, or AD+R1881 medium and cultured for 2–3 days, while PC3 cells were cultured only in complete medium. Cells were then transfected with 200 nM AS-PKA or MM in the presence of lipofectin (6 μ g/ml) for a duration of 24 hours (LNCaP) or 48 hours (PC3). Cells were then irradiated at a dose of 5-Gy. After 24 hours, all cells (floating and attached for LNCaP cells) were harvested by trypsinization.

For the AnnexinV assay, cells were labeled with AnnexinV-PE and 7-amino-actinomycin D (7-AAD) (Guava Technologies Inc., Burlingame, CA) according to the manufacturer's instructions and analyzed by flow cytometry on a GuavaPC personal flow cytometer (Guava Technologies Inc., Burlingame, CA).

Caspase 3+7 activity was measured using a fluorometric substrate, Z-DEVD-Rhodamine (The Apo-ONE Homogeneous Caspase-3/7 Assay kit; Promega, Madison, WI). Twenty-four hours after irradiation (5-Gy), 5×10^4 cells in 50 μ l culture medium were mixed with 50 μ l of Homogeneous Caspase-3/7 reagent in 96-well plates and incubated at room temperature for 24 hours. Substrate cleavage was quantified fluorometrically at 485-nm excitation and 538-nm emission with a fluorescent plate reader (LabSystems Inc., Franklin, MA).

Each assay was repeated a minimum of three times and the difference between groups assessed with one-way ANOVA, using Bonferroni adjustment for multiple comparisons.

Radiation clonogenic assays

Cells were cultured for 2–3 days in complete or AD medium and then transfected with 500 nM AS-PKA or MM in the presence of lipofectin (6 μ g/mL). After 24h for LNCaP or 48h for PC3, cells were irradiated to 2, 4, and 6 Gy and clonogenic assays were performed as described previously⁷. Briefly, immediately after irradiation, cells were trypsinized and serially diluted. Known numbers of cells were replated into 100mm dishes. The plates were incubated for 12–14 days and then stained with 0.25% methylene blue. The colonies were counted using an automated counter (Imaging Products International, Inc., Chantilly, VA). The clonogenic survival results were normalized by the levels at 0 Gy. The dilutions for clonogenic assay were done in triplicate, and the results were averaged together (intra-experimental averages), whereas the data shown in the clonogenic survival curves represent the average from multiple experiments (inter-experimental average). We assessed the difference between AS-PKA and the lipofectin control and MM groups with one-way ANOVA, using Bonferroni adjustment for multiple comparisons.

LNCaP Orthotopic Mouse model

All *in vivo* studies were performed under a protocol approved by the Institutional Animal Care and Use Committee. LNCaP cells (5×10^5) were implanted in the prostates of nude mice (Harlan Sprague Dawley Inc., Indianapolis, IN), as described previously^{8,31}. About three weeks subsequent to the implantation of cells, serum PSA was assayed weekly from samples taken from ocular or tail vein bleeding. From each blood draw, 30 μ l of serum were diluted 1:5 in PSA specimen diluent (Abbott Labs, Abbott Park, IL) and analyzed for PSA concentration (in ng/mL) on an IMX analyzer (Abbott Labs).

When the PSA level was approximately 3.0–5.0 ng/mL, mice were placed into randomly assigned treatment groups. After treatment began, tumor volumes were measured weekly with magnetic resonance imaging (MRI). PSA continued to be measured in parallel with the MRI studies. For AS-PKA treatment, the oligonucleotides (AS-PKA or MM) were

administered intraperitoneally at 25 mg/kg/day, five days per week for 15 days, unless otherwise stated. In the present study, as in prior reports⁸, intraperitoneal injection was used. Animals were sacrificed when the MRI-based tumor volume exceeded 300mm³.

Immunohistochemical Analysis

Formalin-fixed paraffin-embedded tumors were cut onto glass slides and processed for immunohistochemical staining by the labeled streptavidin-biotin method, which is described in detail in another publication by our group²⁵. The primary monoclonal PKA antibody (Cat. No. 610610, BD Biosciences; 1:100 dilution) was applied, the chromagen diaminobenzidine (DAB, Research Genetics, Huntsville, AL) was used for visualization, and commercially-prepared hematoxylin (Dako Corporation, Carpinteria, CA) was used as a counterstain. Human brain tissue served as a positive control. PKA staining intensity was quantified with the ACIS II image-analysis system (Clariant Inc., San Juan Capistrano, CA). All slides were scanned using a preset color threshold, and at least 20 areas of interest in the tumor tissue (visualized at 40× magnification) were quantified. The intensity of staining was scored on a grayscale of 0–255 (arbitrary units) and a mean intensity score was generated by the software provided with the system.

Orchiectomy

Androgen deprivation *in vivo* was accomplished via bilateral orchiectomy. The scrotum was sterilized with betadine, and the testes were withdrawn from the scrotal sac through a transscrotal incision. After clamping all of the vessels connected to the testes with black braided silk, the testes were removed and the incision was sealed with surgical clips. The procedure was performed under sterile conditions while the mouse was sedated with methoxyflurane anesthesia.

Radiation Treatment

On the fifth day following the initiation of AS-PKA treatments, animals assigned to RT groups received a single fraction of 5 Gy, a dose which in a separate study was determined to give a borderline undetectable response as monotherapy⁸. Radiation was delivered with a cesium 137 irradiator (Model 81-14, J.L. Shepherd & Associates, San Fernando, CA) to the prostate while other organs were shielded. During the procedure the animals were anesthetized with a ketamine/ace-promazine cocktail and immobilized in the supine position with surgical tape.

Tumor volume measurements using MRI

MRI scans of orthotopically grown LNCaP tumors were acquired at a field strength of 7 Tesla in a vertical wide bore (10 cm) magnet using a Bruker DRX spectrometer with micro-imaging accessory. Animals were anesthetized with a mixture of 1% isoflurane in oxygen, and placed in a 3 cm birdcage radio-frequency coil. Immediately prior to scanning, an intramuscular (i.m.) injection of 0.2ml Gd-DTPA (Magnevist, Berlex Laboratories, Hamilton, NJ), diluted 10:1 with 1× phosphate buffered saline was performed. The i.m. injection was given in the shoulder to be certain that the hyper-intense injection site would not be in the imaging field of view. An image set in the axial orientation was acquired rapidly and used for proscribing a coronal orientation image set for tumor detection and volumetric measurement. 10–14 slices were acquired depending on the tumor volume. For tumors <50mm³, parameters for the coronal scan were: slice thickness=0.5mm, repetition time (T_R)=400–600 msec, echo time (T_E)=13.2 msec, in-plane resolution=0.1mm, field of view (FOV)=2.56cm (head-foot) and 1.28cm (left-right) with four averages. FOV and number of phase encode steps in the left-right direction were reduced with the use of outer-volume suppression. For larger tumors (>50 mm³), a coronal scan with a slice thickness of

0.75mm, in-plane resolution of 0.2mm, square FOV of 2.56cm, with 2 averages provided adequate spatial resolution and reduced scan time. Including animal preparation, the total scan time was 12–15 minutes. This procedure resulted in animal mortality in fewer than 1 in 500 exams. Tumor volumes were measured by manually outlining the tumor margins using Bruker Paravision software, summing the number of voxels enclosed, and multiplying by the single voxel volume. All imaging studies were performed in the Small Animal Imaging Facility at Fox Chase Cancer Center.

Mice were euthanized by CO₂ inhalation if the MRI tumor volume reached 300mm³ and/or serum PSA exceeded 80ng/mL. Tumors were immediately excised, and tumor volume (measured via calipers) and weight recorded. The tumors were fixed in 10% formalin, 60% ethanol, and/or frozen in liquid nitrogen for further analysis. Daily monitoring ensured that mice exhibiting morbidity would be euthanized within 24 hours of the appearance of symptoms.

To confirm the accuracy of the MRI tumor volume measurements, we assessed the relationship between MR volume and both tumor volume and tumor weight at necropsy in 50 animals. Pearson Correlation Coefficients were calculated and considered significant at the 5% level.

PC3 - subcutaneous model

For PC3 studies, mice were inoculated in the flank with 3×10⁶ PC3 cells, as described previously³¹. Tumor volume was assessed weekly by performing caliper measurements in three orthogonal dimensions. AS-PKA, MM and RT treatments were performed as described above for the LNCaP model, with the exception that RT was delivered to the tumor bearing leg, not the prostate.

Tumor volume and PSA analysis and statistics

The serial PSA and tumor volume (TV) measurements of each mouse were fitted to one of two exponential models. For mice whose PSA and tumor volume time series showed an overall increase, stabilization or decrease, the data were fitted to a simple exponential function:

$$S(t) = S_0 * \exp(R * t).$$

Here S(t) is the TV/PSA at time t, S₀ is the estimated baseline TV or PSA at t = 0, and R is the rate of TV/PSA growth (R>0) or decline (R<0). The doubling time for either quantity is calculated as ln(2)/R in cases where R>0. If more complex kinetics were seen, the data were fit to a bi-exponential function:

$$S(t) = S_1 * \exp(R_1 * t) + S_2 * \exp(R_2 * t).$$

In this model S₁+S₂ (t=0) is the estimated baseline TV/PSA. The first term of the equation describes the initial decline or slow growth phase, and the second term describes the subsequent rise.

Doubling times, the estimated PSA at 6 and the TV at 10 weeks were compared using Student's t-Test in order to estimate tumor response. For the statistical comparisons described, PSA measurements from LNCaP-bearing mice were truncated at 80ng/mL and TV measurements from LNCaP and PC3 mice were truncated at 300 and 500mm³,

respectively. For tumors that exhibited size decreases following treatment, or tumors that declined to zero quickly, the doubling time was set to 100 days.

Two other calculated quantities were the freedom from tumor volume failure (FFTVF - the fraction of animals who fail to develop tumors greater than 30mm³ by the tenth week after treatment) and the freedom from biochemical failure (FFBF - the fraction of animals whose PSA did not reach 1.5 ng/mL at the sixth week after treatment)³¹. The outcomes from the experimental pairs MM and AS-PKA for each of the treatments (alone, RT, AD, AD+RT) were placed in contingency tables and tested for significance using the chi-square test.

RESULTS

AS-PKA increases tumor cell apoptosis in response to RT±AD

In Table 1, we show the results of Caspase 3+7 activity and AnnexinV staining assays in LNCaP and PC3 cells treated with MM, AS-PKA, AD and/or RT. On average there were 3 assays for LNCaP and 5 for PC3 compared. In LNCaP cells, the addition of AS-PKA increased caspase 3+7 activity by significant levels relative to the control and mismatch oligonucleotides under all culture conditions. There were also significant increases in apoptosis for AS-PKA+AD and AS-PKA+RT relative to AS-PKA. While the relative increases between AS-PKA vs MM and AS-PKA+RT vs MM+RT were similar, the absolute gains were significant. The absolute values for AS-PKA+AD+RT were larger than for the AS-PKA+RT and AS-PKA+AD treatments, with a more pronounced effect noted for the AnnexinV measurements. The addition of R1881 to AD reduced the apoptotic response relative to AD in all cases. The results were similar for AnnexinV staining in that AS-PKA uniformly increased staining relative to the lipofectin and MM controls; however, for the two groups oligonucleotide+AD+R1881 and oligonucleotide+AD+R1881+RT, the differences between the AS-PKA and MM treatments were not significant.

Since PC3 cells are androgen insensitive, the assays were not performed under the conditions of AD. There were large increases in the apoptotic response measured by both methods for AS-PKA relative to the lipofectin or MM controls, with further increases seen when RT was added. Significant increases in AnnexinV binding also resulted from a comparison between AS-PKA and AS-PKA+RT groups.

AS-PKA specifically knocks down PKA_{RIα} and reduces pMAPK

In Figure 1A, the results of western blot assays of the expression of PKA in LNCaP cells treated with lipofectin control only (C), MM, or AS-PKA, under different culture conditions are displayed. Under all conditions, AS-PKA caused a significant reduction in PKA_{RIα} levels relative to control and MM, however PKA_{RIIα} and PKA_{RIIβ} levels were unchanged. When the cells were grown in complete medium (CM), AS-PKA caused a decrease in androgen receptor (AR), and phosphorylated mitogen activated protein kinase (pMAPK), while increasing levels of p53 and p21, relative to both control and MM. The MM oligonucleotide treatment also resulted in a slight increase in p53 and p21 relative to the lipofectin control.

LNCaP cells are growth inhibited in AD medium and the growth is partially restored when the synthetic androgen R1881 is added to AD³². In general, the molecular changes from AS-PKA were similar in complete, AD, or AD+R1881 media. Androgen deprivation dramatically increased pMAPK in the lipofectin and MM controls, demonstrating that AD promotes pro-survival changes. Treatment with AS-PKA reduced pMAPK relative to the controls, thereby shifting the balance toward apoptosis. This is particularly the case when one considers that p53 levels are elevated when AS-PKA is administered, which would also predispose the cells to apoptosis in response to RT. When the synthetic androgen R1881 was

added to AD, pMAPK levels returned to that of cells grown in complete medium, with responses to AS-PKA that were analogous to those in complete medium.

For PC3 cells, the western blot results are shown in Figure 1B. PC3 cells do not express p53. p21 levels were not altered by AS-PKA treatment. Although the effect was not as strong as that observed in LNCaP cells, AS-PKA treatment resulted in a decrease in pMAPK by >20% over the MM control densitometrically.

Figure 1C shows the effect of AS-PKA administered intraperitoneally on PKA_{RIα} levels in tumors grown orthotopically. There was a statistically significant reduction in PKA_{RIα} expression in AS-PKA treated tumors relative to MM controls.

Overall cell death by clonogenic survival is enhanced by adding AS-PKA to RT±AD

The clonogenic survival assay results for LNCaP cells subjected to AS-PKA±AD and RT to 2, 4 and 6 Gy are shown in Figure 1D–F. While survival of MM treated cells was about the same as the controls at each radiation dose tested, statistically significant reductions in survival were observed with the addition of AS-PKA at all RT dose levels. The clonogenic assay measures cell death from all mechanisms (e.g. apoptotic and mitotic) and reflects effects over a longer period of time, as compared to the point of time measurement from the apoptosis assays. The cell death pattern for AS-PKA in LNCaP cells was, therefore, concordant for the clonogenic and apoptosis assays, suggesting that apoptosis was a contributing mechanism and demonstrating that cell death was greatest when AS-PKA was combined with AD and RT.

The clonogenic survival results for PC3 were also reflective of those using apoptosis as the measure of cell killing. Although statistical significance was only seen at 2 Gy, AS-PKA was consistent in reducing clonogenic survival relative to the MM controls (Fig 1E).

AS-PKA accentuates tumor growth inhibition in vivo from RT±AD

LNCaP tumors as small as 1.7mm in diameter (5mm³ in volume) were consistently identified on coronal MRI images. The coronal orientation was chosen to take advantage of the bilateral left-right anatomical symmetry that is seen when tumors grow from the dorsal region of the prostate and displace the colon to either the left or the right. Once identified, tumors were easy to follow on subsequent weekly scans. We have compared the relationship between tumor volume and tumor weight at necropsy with MR volume in over 50 tumors whose MRI-based tumor volume values ranged from 10 to 300mm³. There was good agreement between MRI-based tumor volume and tumor weight ($r^2=0.94$, $p<0.001$).

For the orthotopic LNCaP model, tumor volumes and PSAs were assessed for 95 mice divided into 8 experimental groups. The time series data collection resulted in a total of 782 MRI tumor volume and 1007 PSA measurements. On average, 9.2 (range 3–23) MRI tumor volume measurements and 10.7 (range 3–31) PSA measurements were performed per mouse. Mice with tumor volumes >300mm³ and/or PSAs >80ng/mL were sacrificed. Individual mice were monitored up to 31 weeks, depending on tumor development and growth rates. MRI measurements were collected on fewer mice due to instrument down-time. The time series were analyzed using the kinetic models described in Methods. The data from one mouse (group MM+AD) was not suitable for modeling. The average (estimated) tumor volume and PSA baseline values were 14.41mm³ and 4.41ng/mL, respectively.

Figure 2 displays the average PSA and tumor volumes of the time-related changes for each treatment group. The values were estimated over the course of 10 weeks, using the parameters from the fitted kinetic model. The greatest tumor growth delays were seen for

LNCaP tumors treated with AS-PKA+AD+RT and PC3 tumors treated with AS-PKA+RT (AD was not tested in these androgen insensitive tumors). PBS+AD treatment showed results indistinguishable from MM+AD.

The fitted tumor volume and PSA curves were used to calculate doubling times, PSA levels at 6 and TV sizes at 10 weeks (Table 2). The selection of 6 and 10 week time intervals was taken from prior studies^{8,31}. AS-PKA had little effect as a monotherapy for LNCaP tumors, or when combined singly with RT, relative to the MM control groups. However, it was effective when combined with AD, either singly or in combination with RT. LNCaP tumors treated with AS-PKA+AD±RT had significantly longer doubling times ($p<0.05$) when compared with AS-PKA or MM±RT groups. The doubling times for the AS-PKA+AD±RT groups were also longer than for the MM+AD+RT group (~60 days and ~40 days respectively), although the results did not reach statistical significance.

Treatment with AS-PKA+AD+RT translated into lower PSA levels and smaller tumor volumes; although the difference with MM+AD+RT was not statistically significant. At all time points, the lowest PSA values and TVs were in the triple therapy group (AS-PKA+AD+RT). When comparing the pooled groups of AS-PKA+AD±RT with the respective MM control groups, PSA levels were lower ($p<0.05$) and overall PSA and tumor volume doubling times longer ($p<0.05$).

We then determined FFTVF and FFBF, patterned after threshold parameters that have proven valuable clinically^{34,35} (Table 3). The overall trends were similar for both endpoints, but statistically significant improvements due to AS-PKA+AD and AS-PKA+AD+RT treatments were demonstrated more consistently using FFBF. There was no difference between AS-PKA+AD and AS-PKA+AD+RT, and these two groups were associated with significant gains ($p<0.05$) in FFBF as compared to all other AS-PKA groups. The results based on FFTVF were less conclusive, but the trends were concordant. In contrast, for MM groups there were no statistically significant improvements derived from combining MM with AD or RT.

The *in vivo* effects of AS-PKA±RT for PC3 tumors are shown in Table 4. Calculated caliper-based tumor volume doubling times were significantly longer, and calculated tumor volumes significantly smaller, for the AS-PKA+RT group, as compared to all other groups. Although AS-PKA did cause a measurable reduction in tumor growth rates, the effect was much smaller than what was achieved when combined with RT. The FFTVF data were comparable, showing that at 10 weeks only six of 12 tumors had progressed to greater than 30mm³ in the AS-PKA+RT group.

DISCUSSION

PKA_{RIα} is implicated in the activation of the androgen receptor and progression to androgen insensitivity^{36–38}. While there is evidence that PKA_{RIα} inhibition has potential as a chemoprevention agent^{39,40}, chemotherapy sensitizer^{41–43}, and radiation sensitizer^{20,21}, the results presented herein are the first concerning PKA_{RIα} knockdown and the response of androgen sensitive prostate cancer cells to androgen deprivation. Moreover, the radiosensitization of prostate cancer cells *in vitro* and *in vivo* to AS-PKA has not previously been defined. Since RT+AD is a common, albeit suboptimal, clinical treatment, AS-PKA has the potential to significantly enhance prostate tumor response and patient survival.

AS-PKA significantly induced cell killing over the MM controls *in vitro* and further enhanced the apoptotic response of LNCaP cells to AD and RT. The best results were seen with the triplet of AS-PKA+AD+RT (Table 1). A similar pattern was seen for overall cell death measured by clonogenic survival *in vitro* (Figure 1D). Likewise the best results *in vivo*

using LNCaP cells grown orthotopically was with AS-PKA+AD+RT. The results were most conclusive from PSA measurements, although the pattern from MRI-based tumor volume determinations was comparable. Biochemical (PSA-based) and tumor volume (MRI-based) growth delay were apparent when AS-PKA was combined with AD. PSA and tumor volume doubling times were prolonged and freedom from failure was greater than for all other treatment groups. The biochemical failure results were the most significant and consistent (Table 3). Under the conditions used for these studies, we did not see evidence of radiosensitization by AS-PKA in LNCaP cells grown *in vivo*. A single fraction of 5 Gy was used, as we have reported in the past in other radiosensitization experiments^{8,31}. Titration of radiation dose in this model has been performed⁸; 7.5 Gy single fraction radiation alone resulted in too great of a response for radiosensitization experiments. However, a significant interaction with radiation was evident in androgen insensitive PC3 cells both *in vitro* and *in vivo*.

When PC3 cells were exposed to AS-PKA *in vitro*, apoptosis was significantly greater than for the MM controls by both AnnexinV and Caspase 3+7 assays (Table 1). The clonogenic survival differences were significant at 2.0 Gy, but not at higher doses per single fraction treatment (Figure 1E). The *in vivo* results more conclusively demonstrated the gains from combining AS-PKA with RT. PC3 tumors experienced highly significant tumor volume growth delay (FFTVF and calculated tumor volume at 10 weeks) and prolonged tumor volume doubling times (Table 4).

AS-PKA promoted increased cell death when administered alone and in combination with RT±AD in LNCaP and PC3 cells. Since PC3 cells are p53^{null} and lack androgen receptors, the action of AS-PKA may work through both p53 dependent and independent mechanisms.

Our western blot analyses demonstrate that knockdown of PKA_{RIα} in the androgen sensitive LNCaP cell line affects a number of proteins important in apoptosis and cellular proliferation; there were higher expression levels of p53, as well as increasing levels of the cyclin dependent kinase inhibitor p21. Chin et al²¹ have shown that PKA suppression enhances the induction of p53 caused by RT in RT-resistant leukemic cells²¹ and Fujino et al⁴⁴ described an increase in p21 in a variety of cancer cell lines (not including from prostate cancer). Ras/MAPK signaling has been extensively studied in prostate cancer, and has been implicated as a mechanism via which prostate cells may acquire an androgen independent phenotype^{27,45}. The MAPK and PKA signaling pathways are involved in activation of AR^{46,47}, and androgen receptor knockdown results in increased MAPK production⁴⁸. The stimulation of the PKA_{RIα} pathway also increases MAPK phosphorylation^{26,49}, whereas PKA_{RIα} suppression increases phosphorylation of MAPK (p42/p44) in androgen independent DU-145 cells²⁸. This is consistent with our observation that phosphorylated MAPK levels were reduced when used with or without AD. We show here for the first time that AS-PKA directly counteracts this anti-apoptotic aspect of AD treatment. Phosphorylated MAPK was also reduced in androgen insensitive PC3 cells by AS-PKA (Fig 1B). The MAPK pathway has been associated with radioresistance⁵⁰; inhibition of this pathway by AS-PKA may have contributed to increased radiation responses observed.

In conclusion, AS-PKA enhances prostate cancer cell apoptosis in response to RT and/or AD in cell culture. The predisposition for increased cell killing from these treatments occurred with increases in p53 and p21 (LNCaP) and reduced phosphorylated MAPK (LNCaP and PC3) levels. The *in vitro* results were, in general, reflective of the *in vivo* findings and argue strongly for the use of a PKA_{RIα} knockdown strategy clinically.

STATEMENT OF NOVELTY AND IMPACT

In this report we show both *in vitro* and *in vivo* that the knockdown of PKA_{RIα} results in significant enhancement of prostate cancer cell killing in response to androgen deprivation and radiation. The clinical implications of these findings is that patients with tumors overexpressing PKA_{RIα} may be ideal candidates for combination of PKA_{RIα} knockdown, androgen ablation and radiation, and this therapy potentially would be of particular value in patients with apparently clinically localized disease, but at a high risk of microscopic distant metastasis.

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Abbreviations

(PKA)	Protein Kinase A
(PKA _{RIα})RIα	subunit of PKA
(AS-PKA)	Antisense molecule targeted to the RIα subunit of PKA _{RIα}
(AD)	Androgen Deprivation
(RT)	Radiation Therapy

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Figure 1.

Western blot analysis of protein levels in LNCaP cells (A); protein levels in PC3 cells (B); immunohistochemical staining of PKA expression from LNCaP tumors grown *in vivo* (C; the error bars indicate the standard deviation); and clonogenic survival assay results (D–F). The p -values on the charts in D–F are for the AS-PKA groups vs controls. The AS-PKA vs MM comparisons were: 2 Gy, $p = 0.063$; 4 Gy, $p = 0.005$; 6 Gy, $p = 0.0183$ (D); 2 Gy, $p = 0.063$; 4 Gy, $p = 0.05$; 6 Gy, $p = 0.049$ (E); 2 Gy, $p = 0.102$; 4 Gy, $p = 0.884$; 6 Gy, $p = 0.356$ (F). The Bonferroni test was used for all comparisons.

Figure 2.

Growth curves for LNCaP (**A**) and PC3 (**B**) tumors in nude mice. The curves represent the treatment group average of values fitted by a kinetic model for individual animals ($n=8-13$). For LNCaP tumors (**A**), combination therapies were performed with mismatch control (MM - left), and AS-PKA oligos (right), and tumor size estimated by MRI measurements (top) and serum PSA levels (bottom). For PC3 tumors (**B**), tumor size was estimated by caliper measurements.

Table 1

Apoptosis assays in LNCaP and PC3 cells under various growth conditions

LNCaP	Caspase 3+7 activity			% AnnexinV positive		
	Mean	SEM	p*	Mean	SEM	p*
Control	192	16	-	1.30	0.14	-
AS-PKA	599	21	<.0001	3.46	0.08	.041
MM	216	6	<.0001	2.10	0.11	1.000
AD	341	19	.019	2.96	0.27	1.000
AS-PKA+AD	1114	14	<.0001	5.96	0.46	.0001
MM+AD	419	28	<.0001	3.26	0.14	.002
AD+R1881	208	11	<.0001	1.60	0.11	.494
AS-PKA+AD+R1881	681	25	<.0001	4.30	0.36	.002
MM+AD+R1881	334	25	<.0001	2.53	0.27	.296
RT	197	4	.006	2.10	0.23	1.000
AS-PKA+RT	896	21	<.0001	5.90	0.55	<.0001
MM+RT	270	28	<.0001	3.30	0.05	.003
AD+RT	384	29	.06	3.76	0.38	1.000
AS-PKA+AD+RT	1271	19	<.0001	10.06	0.67	.0001
MM+AD+RT	450	24	<.0001	5.63	0.48	.0001
AD+R1881+RT	273	24	<.0001	3.56	0.43	.059
AS-PKA+AD+R1881+RT	827	11	<.0001	5.96	0.66	.009
MM+AD+R1881+RT	337	13	<.0001	4.06	0.31	.146
PC3						
Control	96	22	-	0.92	0.14	-
AS-PKA	305	29	<.0001	4.34	0.42	<.0001
MM	137	20	.003	1.94	0.28	<.0001
RT	114	21	1.000	1.36	0.32	1.000
AS-PKA+RT	369	43	<.0001	6.04	0.17	<.0001
MM+RT	177	13	.001	2.14	0.18	<.0001

Lipofectin was included in all groups.

SEM = standard error of the mean.

Other comparisons showing significant increases (p<0.01):

For **LNCaP**, both Caspase and Annexin V assays: AS-PKA vs. AS-PKA+AD; AS-PKA+RT; AS-PKA+AD+RT; AS-PKA+RT vs. AS-PKA+AD+RT.

For **PC3**, Annexin V assay: AS-PKA vs. AS-PKA+RT

* p Compared to the group above; one-way ANOVA, Bonferroni Test. The average of 3 experiments is shown for LNCaP, 5 for PC3.

Table 2

Biochemical (PSA) and tumor volume (MRI) results for orthotopic LNCaP tumors.

Group	*PSA at 6 wks Mean ±SEM (ng/mL)	*Tumor Volume at 10 wks Mean±SEM (mm ³)	**PSA Doubling time Mean±SEM (days)	**Tumor Doubling time Mean±SEM (days)
MM	44±10	177±39	27±10	25±9
AS-PKA	40±10	193±43	21±6	23±9
MM+RT	33±9	170±42	23±10	18±5
AS-PKA+RT	49±10	217±41	17±8	13±4
MM+AD	29±9	182±39	22±9	34±14
AS-PKA+AD	13±6 [#]	150±45	50±14 ^{†Ω}	61±14 ^{†Ω}
MM+AD+RT	19±8	108±34	39±12	43±11
AS-PKA+AD+RT	6±2 ^{##†}	82±34 ^{##}	56±11 [#]	63±10 ^Ω
Pooled MM+AD ±RT	23±6	143±27	31±8	39±9
Pooled AS-PKA+AD±RT	9±3 ^{††}	116±33	53±9 ^{††}	62±9 ^{††}

All statistical comparisons were performed with Student's t-Test

* Values are calculated from fitted data. PSA=80 ng/mL and tumor volume=300 mm³ are assumed for tumors with larger than these values.

** Serum PSA and MRI-based tumor volume doubling times were calculated from fitted data for mice where the curves exhibited an upward trend after treatment. For the rest of the animals 100 days was assumed.

[#] p<0.10 for all pairwise comparisons with each of the following groups: MM, AS-PKA, MM+RT, AS-PKA+RT and MM+AD.

^{##} p<0.05 for all pairwise comparisons with each of the following groups: MM, AS-PKA, MM+RT, AS-PKA+RT and MM+AD.

[†] p<0.10, compared to the group above

^{††} p<0.05, compared to the group above

[‡] p<0.10 for all pairwise comparisons with each of the following groups: MM, MM+RT and AS-PKA+RT.

^Ω Statistical significant difference (p<0.05), compared to all monotherapy ±RT groups.

Table 3

Freedom from biochemical (FFBF) and tumor volume (FFTVF) failure results.

	MM Groups	MM	MM+RT	MM+AD	AS Groups	AS	AS+RT	AS+AD
#FFBF	MM 8% (1/12)				AS 0% (0/11)			
	MM+RT 9% (1/11)	0.95			AS+RT 9% (1/11)	0.31		
	MM+AD 17% (2/12)	0.54	0.59		AS+AD 55% (6/11) [‡]	<0.01	0.02	
	MM+AD+RT 31% (4/13)	0.16	0.19	0.41	AS+AD+RT 64% (9/14) [‡]	<0.01	<0.01	0.62
#FFTVF	MM 25% (3/12)				AS 30% (3/10)			
	MM+RT 27% (3/11)	0.90			AS+RT 0% (0/8)	0.09		
	MM+AD 33% (4/12)	0.65	0.75		AS+AD 40% (4/10)	0.64	0.04	
	MM+AD+RT 46% (6/13)	0.27	0.34	0.51	AS+AD+RT 67% (6/9)	0.11	<0.01	0.24

The table entries represent p-values determined using the chi-square test.

Other comparisons: Statistically significant difference ($p < 0.05$) between pooled data (MM+AD and MM+AD+RT) vs (AS-PKA +AD and AS-PKA+AD+RT) for FFBF. All statistical comparisons were performed with the chi-square test.

[#] FFBF = Freedom from biochemical failure (PSA less than 1.5 ng/mL at 6 weeks)

^{##} FFBF = Freedom from tumor volume failure (tumor volume less than 30 mm³ at 10 weeks)

^{*} $p = 0.08$, compared to MM+AD.

[‡] $p = 0.06$ compared to MM+AD+RT.

Table 4

Caliper-based tumor volume results for PC3 cells grown *in vivo* in the hind legs of nude mice.

Group	*Doubling time Mean±SEM (days)	Tumor Volume at 10 weeks Mean±SEM (mm ³)	FFTVF (10 weeks)
MM	11±2	438±41	0%(0/11)
AS-PKA	30±7 [†]	347±47 [†]	8%(1/12)
MM+RT	23±8	443±44	9%(1/11)
AS-PKA+RT	88±18 ^{†+}	116±32 ^{†,+}	50%(6/12) ^{†,+}

Abbreviations: RT, radiotherapy; FFTVF, freedom from tumor volume failure (<100 mm³).

All statistical comparisons were performed with Student's t-Test

* Tumor doubling times were calculated from fitted data for mice where tumor volume exhibits an upward trend after treatment. For the rest of the animals 100 days is assumed.

[†] Statistically significant difference (p<0.05), compared to the group above

⁺ Statistically significant difference (p<0.05), compared to both groups of oligonucleotide treatment alone (MM and AS).