

Enhanced antiproliferative and proapoptotic effects on prostate cancer cells by simultaneously inhibiting androgen receptor and cAMP-dependent protein kinase A

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The androgen-signaling pathway with the androgen receptor (AR) as its key molecule is widely understood to influence prostate tumor growth significantly even after androgen ablation. Under androgen-deprived conditions, the AR may be activated inappropriately through interaction with other molecules, including cyclic AMP-dependent protein kinase A (PKA). In a previous study, we have shown that knocking down the AR significantly inhibits prostate tumor growth. In this study, we show that combined inhibition of the AR and the regulatory subunit I alpha of PKA (RI α) with small interference RNAs significantly increased the growth-inhibitory and proapoptotic effects of AR knockdown. This treatment strategy was effective in androgen-sensitive and in androgen ablation-resistant prostate cancer cells. In addition, we report that downregulating PKA RI α was sufficient to inhibit PKA signaling and interestingly also impaired AR expression and activation. Vice versa, AR knockdown induced a decline in PKA RI α , associated with reduced PKA activity. This mutual influence on expression level was specific, because siRNAs against the AR did not affect expression of PKA RI α in AR negative DU-145 cells and a siRNA control did not affect protein expression. Another important finding of our study was that depletion of PKA RI α also potentiated the antiproliferative effect of the antiandrogen bicalutamide in androgen-sensitive LNCaP. We therefore concluded that combined inhibition of PKA RI α and AR may be a promising new therapeutic option for prostate cancer patients and might be superior to solely preventing AR expression.

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Key words: androgen receptor; cAMP-dependent protein kinase A; prostate cancer; siRNA

Prostate cancer is a very common malignancy with significant complexity concerning the mechanisms that regulate its manifestation and progression. Development of normal prostate and early-stage prostate cancer depends on circulating androgens that elicit their effects through the androgen receptor (AR).¹ The AR is a transcription factor, which—upon activation in the cytoplasm where it undergoes a cascade of activation processes—translocates to the nucleus where it finally binds to androgen-responsive elements of androgen-regulated genes such as the prostate-specific antigen (PSA). In prostate tumor cells, the activation of the androgen-signaling cascade is critical for survival and progression.² Therefore, blocking AR activation by androgen ablation still represents 1 of the preferential treatment options for advanced prostate cancer.³ However, tumor regression as a response to androgen ablation is only palliative, because tumors eventually relapse due to transition of the malignancy from androgen-dependent to a hormone-refractory state.¹ This transition results in poor prognostic outcome and limited treatment options, which are usually insufficient for tumor elimination.⁴ Consequently, after treatment failure, hormone-refractory prostate cancer becomes lethal.

The development of hormone-refractory prostate cancer is likely triggered by several different mechanisms. Apart from androgen-dependent prostate cancer, significance of AR as a key molecule is also evident in the hormone-refractory stage of the disease.^{5–7} Possible mechanisms by which the AR may contribute to prostate tumor growth include increased expression of AR^{6,8,9} or AR mutations that render the receptor promiscuous, allowing it to accept a broad spectrum of ligands that can bind to and activate the receptor.^{10–14} Under castrated conditions, it is also considered that low concentrations of androgens remaining in the tissue may contribute to the activation of AR.¹⁵ Besides, AR can be activated in a so-called ligand-independent manner through cross-talk with

other cell-survival molecules such as cyclic AMP (cAMP)-dependent protein kinase A (PKA).^{2,16–18} In particular, it was shown that PKA, as activated by forskolin, can phosphorylate the AR and thereby stimulate the expression of PSA.¹⁹

cAMP plays a critical role in the regulation of cell growth and differentiation thereby exerting a dual function *via* 2 different receptor subtypes, PKA-I and PKA-II, each consisting of 2 catalytic and 2 regulatory subunits, thereby forming a heterotetrameric enzyme. The 2 PKA subtypes have different regulatory subunits (RI and RII), whereas the catalytic subunits are identical for both.²⁰ In addition, they differ in expression and function. Although PKA-II is found in normal nonproliferating tissues and in growth-arrested cells, PKA-I is overexpressed in growth-stimulated cells.²⁰ In particular, the regulatory subunit RI α (PKA RI α) of PKA-I has been reported to be constitutively overexpressed in several tumor types, including colorectal, breast and lung cancers, in which it was associated with a poor prognosis.^{20,21} Likewise, prostate tumor growth was arrested by blocking PKA with site-selective cAMP analogs^{20,21} and downregulation of PKA-RI α with an antisense oligonucleotide.^{21,22} These studies have shown that sequence-specifically knocking down the regulatory subunit RI α is sufficient to effectively reduce PKA-RI α mRNA and protein levels, resulting in growth inhibition and induction of apoptosis.

One of the valuable strategies to prevent inappropriate AR activation is by inhibiting AR itself. In fact, we have previously shown that the inhibition of AR expression by antisense molecules results in significant regression of prostate tumor growth *in vitro* and *in vivo*.^{23,24} The aim of this study was to increase the growth inhibitory potential of AR knockdown by simultaneously inhibiting PKA-I signaling. We show that the combined use of small interference RNAs (siRNAs) against AR and PKA RI α -enhanced growth arrest of androgen-dependent and androgen ablation-resistant prostate cancer cells compared to single treatment. Targeting the regulatory subunit RI α of PKA-I is sufficient in effectively reducing PKA RI α protein levels as well as activity of PKA-I and interestingly also impaired AR signaling, indicating a possible cross-talk between the 2 pathways.

Material and methods

Cell culture and chemicals

LNCaP and DU-145 prostate cancer cells (obtained from the American Type Culture Collection, USA) were cultured in RPMI

Additional supporting information may be found in the online version of this article.

Abbreviations: AR, androgen receptor; CREB, cAMP response element-binding protein; PKA RI α , protein kinase A regulatory subunit type I alpha; PKA RII β , protein kinase A regulatory subunit type II beta; PKA C α , protein kinase A catalytic subunit alpha; PSA, prostate-specific antigen; siRNA, small interference RNA; pVASP, phosphorylated vasodilator stimulated phosphoprotein; TBP, TATA-binding protein.

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the addition of an acid stop solution. Absorbance was measured at 450 nm. PKA activity values were finally normalized to protein content.

Immunohistochemistry

Immunohistochemistry was performed with 3- μ m paraffin tissue sections using the Ventana Discovery—XT-staining automate (Roche). Tris–EDTA pretreatment for pVASP and α -methylacyl-CoA racemase (AMACR), and citrate buffer pretreatment for AR was followed by incubation with primary antibody solution for 1 hr. No pretreatment was applied for PSA staining. The following antibodies were used: AR (1:80, Biogenex, USA), pVASP (1:35, Cell Signaling Technology, USA) and PSA (1:5,000, DAKO, Denmark), AMACR (1:300, DAKO, Denmark). Specificity of staining was controlled by including an unspecific control antibody (DAKO, Denmark).

For manual immunohistochemistry of PKA RI α , antigen retrieval was achieved by autoclaving the tissue samples in citrate buffer at 115°C for 3 min. After blocking with TBST-M (TBST containing 5% nonfat dry milk), slides were incubated with primary antibody overnight at 4°C (PKA RI α 1:400). The signal was developed with the Zymed Polymer Detection System (Invitrogen) according to the manufacturer's instructions. For double staining of AR and PKA RI α , we first stained for PKA RI α manually and then performed AR staining using the Ventana Discovery—XT staining automate. AMACR was used to stain tumor epithelial cells.

Statistics

All values were expressed as mean \pm standard deviation. Each value is the mean of at least 3 independent experiments in each group. Significant differences among groups were analyzed by 1-way ANOVA and Dunnett *t*-tests. *p* values below 0.05 were defined as statistically significant (**p* < 0.05; ***p* < 0.001 for comparison between single-targeting treatment and control and +*p* < 0.05; ++*p* < 0.001 for comparison between binary targeting treatment and combination of each siRNA with siLUC).

Results

Progression of prostate cancer to an androgen-independent state does not involve loss of AR expression, hence allowing the AR to be activated inappropriately. In a previous study, we therefore proposed that inhibiting AR expression with short antisense oligonucleotides or siRNA might be a very efficient method to inhibit androgen-sensitive as well as castration-resistant prostate cancer and provide a treatment advantage over standard androgen ablation. In this study, we aimed at potentiating the effect of this AR knockdown by simultaneously targeting an additional survival pathway in prostate cancer. For this purpose, we chose PKA-I, which is not only considered to be an important survival molecule in prostate cancer, but also as 1 of the proteins that are able to activate the AR in a ligand-independent manner. We therefore hypothesized that this multitargeting approach would not only have a stronger overall antitumor effect but also exclude the possibility that cells, which weakly express AR, could escape therapy.

Expression and localization of AR and PKA RI α in prostate cancer tissue

Because the efficacy of targeted therapies is dependent on sufficient expression of the target molecules, we first assessed the expression pattern of AR and PKA RI α in human prostate cancer. For this purpose, we performed immunohistochemical staining of PKA RI α and AR on paraffin-embedded tissue sections. As shown in Figure 1, both target molecules were found to be significantly expressed throughout tumor cells (Fig. 1*b*), which were verified by staining with the tumor marker AMACR (Fig. 1*a*). Besides, staining of both targets was found in benign areas as well as in stroma. The staining pattern of all 4 molecules, in general, was

heterogeneous, a feature that is characteristic for prostate tissue. The AR was localized in the nucleus as well as in the cytoplasm (Fig. 1*c*). Because the AR translocates from the cytoplasm to the nucleus upon ligand binding, this expression pattern is expected in tumors that had not undergone prior androgen ablation treatment. Likewise, significant amounts of PKA RI α (Fig. 1*e*) were detected in the cytoplasm of tumor cells. In addition, we revealed expression of the downstream effector molecules of AR and PKA-I, PSA and pVASP (Figs. 1*d* and 1*f*), indicating that AR and PKA-I pathways are activated in prostate cancer cells *in vivo*. Hence, simultaneous targeting of these 2 pathways seems to be a reliable approach.

Inhibition of AR and PKA RI α by siRNA-mediated knockdown

To block target expression sequence-specifically as well as efficiently, we tested the use of short siRNAs and assessed their efficacy in androgen-sensitive LNCaP and VCaP and the androgen ablation-resistant subline LNCaPabl. This subline has been previously established in our laboratory by long-term culture in an androgen-deprived medium, thereby gaining characteristic features of androgen ablation-resistant prostate cancer. Downregulation of the AR was achieved with 3 different siRNAs (siAR-1, siAR-2 and siAR-3). For siRNA-mediated knockdown of PKA RI α , we used a target sequence, which corresponds to a previously described antisense oligonucleotide that has already reached clinical evaluation.^{22,28,29} Concentrations of 20 nM of each siRNA were sufficient to significantly reduce protein expression of AR and PKA RI α , respectively, in all 3 cell lines, LNCaP (Fig. 2*a*), LNCaPabl (Fig. 2*b*) and VCaP (Fig. 2*c*). Hence, target knockdown worked in androgen-sensitive as well as in androgen ablation-resistant prostate cancer cells irrelevantly of AR expression levels. The siLUC negative control did not affect AR expression, suggesting the specificity of the siRNAs.

Downregulation of PKA RI α is sufficient to inhibit PKA-I signaling

Because PKA-I forms a tetramer but the siRNA in this study was only directed against the regulatory subunit of the protein, we further assessed whether depletion of PKA RI α is sufficient to inhibit PKA signaling. To do so, we determined phosphorylation of VASP (pVASP), a specific downstream substrate molecule of PKA and phosphorylation of cAMP response element-binding protein (pCREB) another main target of activated PKA in LNCaP cells. As summarized in Figure 3, pVASP was significantly diminished by siRNA-mediated knockdown of PKA RI α by 75% versus control (Fig. 3*a*, *p* < 0.001). Similarly, levels of pCREB were decreased in response to PKA RI α downregulation (Fig. 3*b*). Moreover, PKA activity was significantly reduced to 53% in comparison with mock control (Fig. 3*d*, *p* = 0.001). In contrast to siPKA, the siLUC control had only a minor effect on pVASP expression or pCREB and did not affect PKA activity. Within the same setting, forskolin (5 μ M for 48 hr) was used as a positive control showing induction of PKA activity up to 157% (Fig. 3*d*, *p* < 0.001).

We next examined expression levels of another PKA regulatory subunit, PKA RII β , that has been previously associated with differentiation and growth arrest phenotype.^{20–22} As shown in Figure 3*b*, PKA RII β levels did not decrease but even slightly increased in response to siRNA treatment. In addition, nuclear expression of the catalytic α subunit of PKA (PKA C α) was found to be even higher in response to downregulating PKA RI α when compared with controls (Fig. 3*c*).

Downregulation of PKA RI α results in reduction of AR expression and vice versa

To our surprise, we observed that PKA RI α and AR expression levels were correspondingly inhibited after treatment of the cells with siRNAs against the AR (siAR-1, siAR-2 and siAR-3). In addition, AR was shown to be downregulated in the presence of

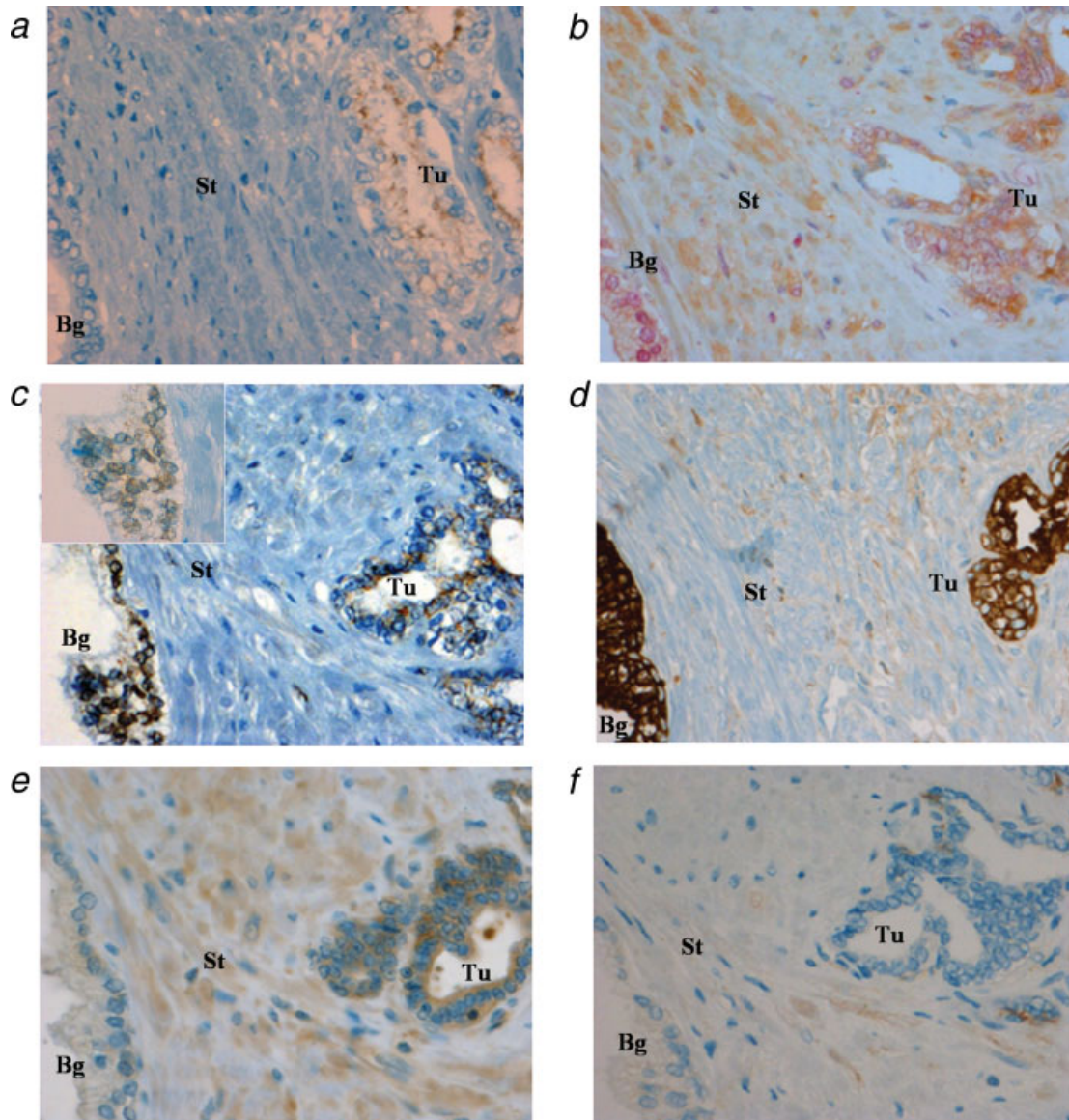


FIGURE 1 – Expression and activation pattern of AR and PKA RI α in prostate cancer *in vivo*. (a) Prostate tumor epithelial cells were identified by staining for the tumor marker alpha-methylacyl-CoA racemase (AMACR). (b) Dual staining of both, AR (red) and PKA RI α (brown), shows strong and ubiquitous expression of both targets in prostate cancer cells. Single stainings were performed to confirm the expression pattern. Figure c depicts nuclear as well as cytoplasmic expression of the AR (small inset: AR staining at $\times 1000$ magnification) (c). PKA RI α was primarily detected in the cytoplasm (e). Moreover, we detected the downstream effector molecules prostate specific antigen (PSA) (d) and phosphorylated vasodilator stimulated phosphoprotein (pVASP) (f). Slides were counterstained with hematoxylin (all magnifications $\times 400$; Bg, benign; Tu, tumor; St, stroma).

siPKA. This phenomenon was observed in LNCaP, LNCaPabl (Figs. 2a and 2b) and VCaP, which express higher levels of AR than LNCaP and LNCaPabl (Fig. 2c). siLUC did not affect the expression levels of AR or PKA RI α in any cell line. In contrast, PKA RI α expression was not affected by the siRNAs directed against the AR in AR-negative DU145 cells (Fig. 2d). Because of the absence of AR in DU145, we concluded that the siRNAs against AR were able to decrease PKA RI α levels only in the presence of AR, as shown in LNCaP, LNCaPabl and VCaP, thus signifying a potential impact between AR and PKA pathways at the protein expression level.

Concurrently, it was revealed that AR knockdown in LNCaP also resulted in reduced pVASP by about 60% in comparison with mock control (Fig. 3a). Likewise, pCREB and PKA activity were significantly reduced upon depletion of AR, whereas PKA RI β and nuclear PKA C α expression was not affected (Figs. 3b and

3d). In particular, PKA activity was reduced by 30% with siAR-1 ($p = 0.043$) and by 42% with siAR-2 ($p = 0.002$). These data further supported the notion that there is a mutual influence of AR and PKA pathways on expression of the proteins.

We next investigated the effect on the AR target molecule PSA. For this, we transiently transfected LNCaP cells with the respective siRNAs in the presence of 1 nM R1881 and measured PSA levels in the cell-culture supernatant 72 hr afterward. As shown in Figure 3e, knockdown of the AR with siAR-1, siAR-2 or siAR-3 induced a 60%, 65% and 56% reduction of PSA compared to mock control ($p < 0.001$), respectively (Fig. 3e). Furthermore, inhibition of PKA RI α resulted in a 42% decrease of PSA ($p < 0.05$). Again, PSA levels were not affected by the siLUC control, indicating that these effects were specific.

To further strengthen our outcome that there might be a mutual influence of the 2 molecular pathways, we activated AR and PKA

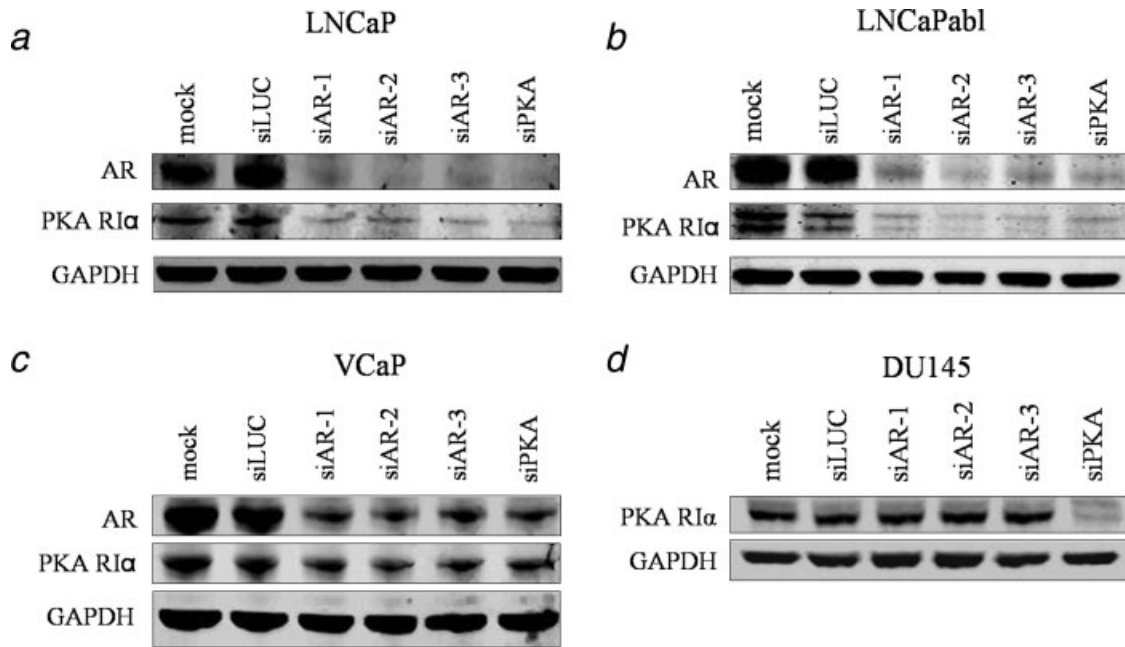


FIGURE 2 – Knockdown of AR and PKA RI α using siRNAs (20 nM) against AR (siAR-1, siAR-2 and siAR-3) or PKA RI α (siPKA) in AR positive LNCaP (a), LNCaPabl (b), VCaP (c) and AR negative DU-145 (d). AR and PKA RI α protein levels were determined by Western blotting and compared with the housekeeping gene GAPDH. siLUC was used as negative control.

in LNCaP cells by different concentrations of androgen or forskolin and dbcAMP for 72 hr, respectively. Cells were treated with R1881 in 3% (CS-FCS) RPMI 1640, whereas forskolin treatment was done in 10% FCS RPMI 1640. PKA RI α expression levels should be upregulated by stimulation of the cells with androgens, thus resulting in increased levels and activity of PKA and AR. In fact, after treatment of LNCaP with increasing concentrations of R1881, not only AR but also PKA RI α protein levels raised (Fig. 4a). Moreover, when forskolin or dbcAMP was added to the cells, PKA RI α and AR protein levels were similarly increased (Fig. 4b). We then investigated the activity status of the 2 pathways in LNCaP after addition of R1881, forskolin or dbcAMP. As shown in Figures 4c–4f, PSA levels were increased and, similarly, PKA activity was enhanced in all cases.

Knockdown of PKA RI α enhances the growth-inhibitory effect of AR downregulation in androgen-sensitive and androgen-ablation-resistant prostate cancer cells

We next assessed whether the depletion of PKA RI α could improve the growth-inhibitory effects of AR knockdown. Androgen-sensitive LNCaP and VCaP and androgen ablation-resistant LNCaPabl were treated with different siRNA combinations for 3 days, followed by measuring [3 H] thymidine incorporation and cell cytotoxicity. Final concentration in double targeting was 20 nM, whereas concentration of each siRNA for single treatment was 10 nM.

In androgen-sensitive LNCaP cells, simultaneous knockdown of AR and PKA RI α resulted in significantly reduced cell proliferation by more than 70% when compared with mock control (siAR-1 + siPKA 75%; $p < 0.05$, siAR-2 + siPKA 72%; $p < 0.05$, siAR-3 + siPKA 72%; $p < 0.05$) (Fig. 5a). By comparison, downregulation of the AR alone with siAR-1, siAR-2 or siAR-3 inhibited cell proliferation only by 43%, 40% and 48% versus mock control ($p < 0.05$), respectively. Silencing of PKA RI α with siPKA also resulted in decreased proliferation (25% decrease over mock control), although the effect was weaker than with AR knockdown. Similar results were obtained with VCaP, although the growth-inhibitory effects were slightly weaker than in LNCaP cells (Fig. 5b). These results showed that combined inhibition of

AR and PKA RI α increases significantly the growth inhibitory effect of AR targeting alone. To further confirm these results, we determined cell viability by MTT assay (Supporting information Fig. 1). Again, combined knockdown of AR and PKA RI α resulted in enhanced growth inhibition (siAR-1 + siPKA 63%; $p < 0.05$, siAR-2 + siPKA 51%; $p < 0.05$ and siAR-3 + siPKA 80%; $p < 0.001$) versus AR depletion alone (siAR-1 40%, siAR-2 30% and siAR-3 32%). Moreover, when cells were treated over a longer period of 6 days, the growth-inhibitory effect was even more pronounced resulting in inhibition of LNCaP cells by 97% (siAR-1+siPKA) and 92% (siAR-2+siPKA), respectively (data not shown).

Because dual targeting was performed with a total siRNA concentration of 20 nM, one could argue that the enhanced effect on cell proliferation was just by doubling the amount of siRNA. However, the combination of each siRNA together with the unspecific negative control siLUC (siAR-1 + siLUC, siAR-2 + siLUC, siAR-3 + siLUC and siPKA + siLUC) did not further decrease cell proliferation when compared with single treatment. In addition, treatment of VCaP cells with 20 nM siLUC did not significantly influence cell proliferation. We therefore concluded from these data that combined inhibition of AR and PKA RI α significantly enhances the growth inhibitory effect of AR knockdown in androgen-sensitive prostate cancer cells.

We next investigated whether this combined treatment strategy would also be effective in androgen ablation-resistant LNCaPabl cells (Fig. 5c). Similarly to androgen-sensitive LNCaP cells, simultaneous downregulation of AR and PKA RI α inhibited LNCaPabl cells to 62% (siAR-1 + siPKA), 65% (siAR-2 + siPKA) and 82% (siAR-3 + siPKA) when compared with AR knockdown alone or in combination with siLUC (siAR-1 + siLUC, siAR-2 + siLUC and siAR-3 + siLUC). Again, these results were further confirmed through measuring cell viability by MTT assay (Supporting information Fig. 1), indicating that combined targeting of AR and PKA RI α is also effective in androgen ablation-resistant prostate cancer cells.

To further exclude off target effects of the siRNAs on cell proliferation, we performed another control experiment in AR negative DU-145 cells. When PKA was arrested by the siPKA, cell

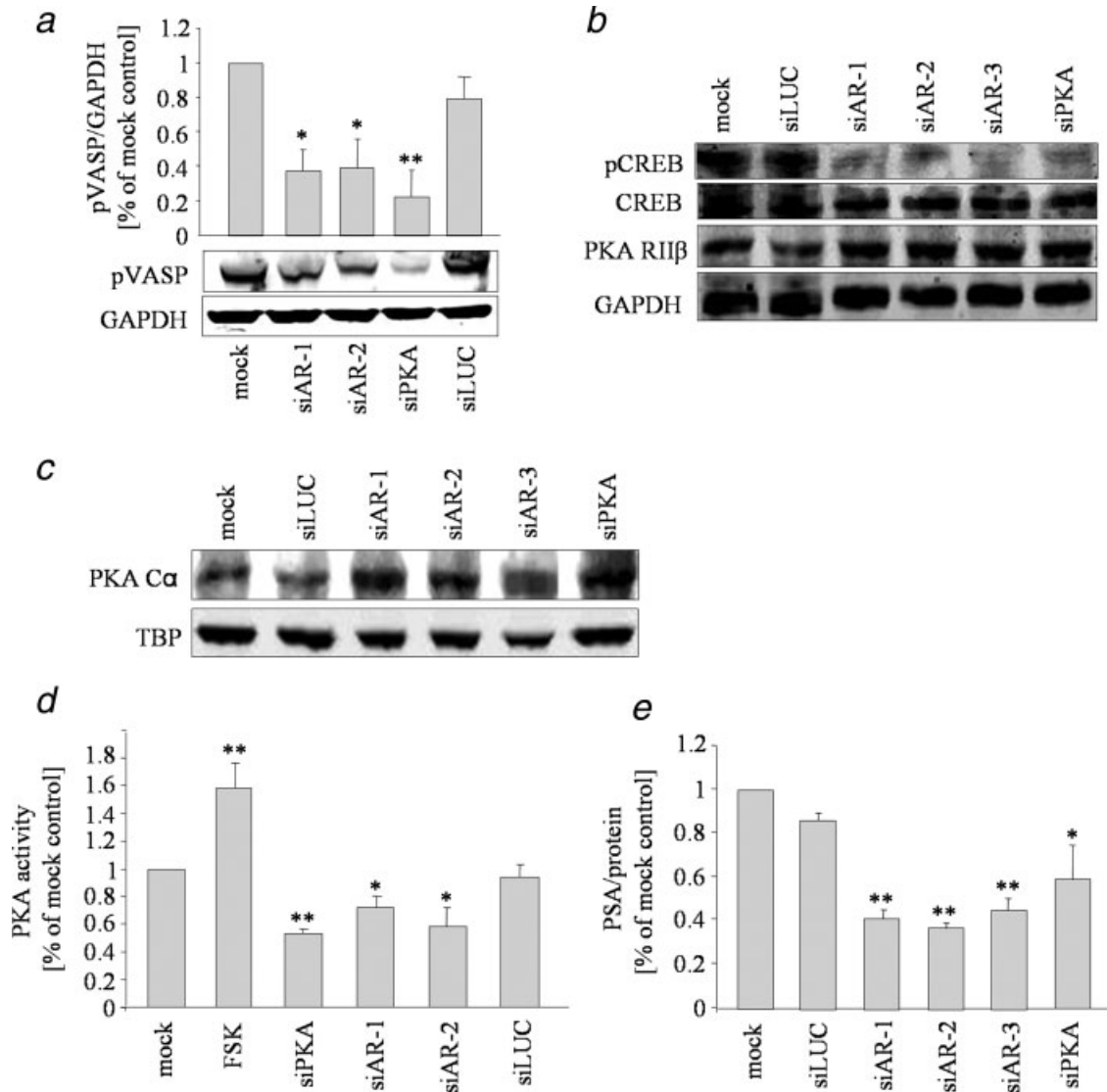


FIGURE 3 – Reduced PKA signaling following AR downregulation in LNCaP cells. (a) pVASP levels are reduced by treatment with 20 nM of siRNAs against AR (siAR-1 and siAR-2) or PKA RI α (siPKA) as determined by Western blotting. We show here 1 representative Western blot out of 3 independent experiments. (b) Phosphorylation status of CREB (pCREB) and expression levels of PKA RII β determined by Western blotting after treatment of cells with 20 nM of siRNAs against AR (siAR-1, siAR-2 and siAR-3) or PKA RI α (siPKA). (c) Nuclear expression of PKA catalytic α (PKA C α) in LNCaP cells after 72-hr treatment. After harvesting the cells, nuclear fractions were extracted by using Ne-Per Nuclear and Cytoplasmic Extraction kit (Thermo scientific, USA) according to the manufacturer's protocol. Levels of PKA C α in the nuclear fraction of LNCaP were determined by Western Blot with TATA binding protein (TBP) as loading control. (d) PKA activity rate measured by ELISA after stimulation of LNCaP with 5 μ M forskolin (FSK) or treatment with siRNAs against AR (siAR-1 and siAR-2) or PKA RI α (siPKA) (20 nM each) for 48 hr. (e) Levels of prostate-specific antigen (PSA) were measured in the cell-culture supernatant of LNCaP cells by immunoassay after siRNA-mediated inhibition of AR (siAR-1, siAR-2 and siAR-3) or PKA RI α (siPKA) for 72 hr in the presence of 1 nM R1881. PSA levels (ng/ml) were normalized to total cell mass (μ g total protein). siLUC was used as a negative control. p values below 0.05 were defined as statistically significant (* p < 0.05; ** p < 0.001 for comparison between single-targeting treatment and mock control).

growth was inhibited by 30% compared to the mock control ($p = 0.039$) (Fig. 5d). In addition, when DU145 cells were treated with a combination of siPKA and either siRNA against AR, cell viability could not be further decreased compared to siPKA single treatment. siAR-2 and siLUC did not affect cell viability, whereas siAR-1 had only a minor effect on DU145 (12% decrease in cell viability, Supporting information Fig. 1).

Increased apoptosis by combined knockdown of AR and PKA RI α

We have previously shown that siRNA-mediated silencing of the AR induces apoptosis in prostate cancer cells.³⁰ This result could be reproduced in the present study, where individual treatment of

LNCaP (Fig. 6a) with either siAR-1, siAR-2 for 72 hr resulted in a 9-fold (siAR-1) and 11-fold (siAR-2) induction of caspase-3 activity rates compared to the mock control, as assessed by ELISA ($p < 0.001$). Downregulating PKA RI α also induced caspase activity to 7.5-fold over the mock control ($p < 0.001$), although the effect was weaker than that obtained by AR knockdown.

Intriguingly, these effects could be strongly enhanced to 17-fold (AR-1 + siPKA) and 18-fold (AR-2 + siPKA) when LNCaP were simultaneously treated with siRNAs against both targets (Fig. 6a). This increase in caspase activity was significantly higher than the value obtained by treatment with each siRNA alone and also over the combination of each siRNA together with the unspecific control siLUC.

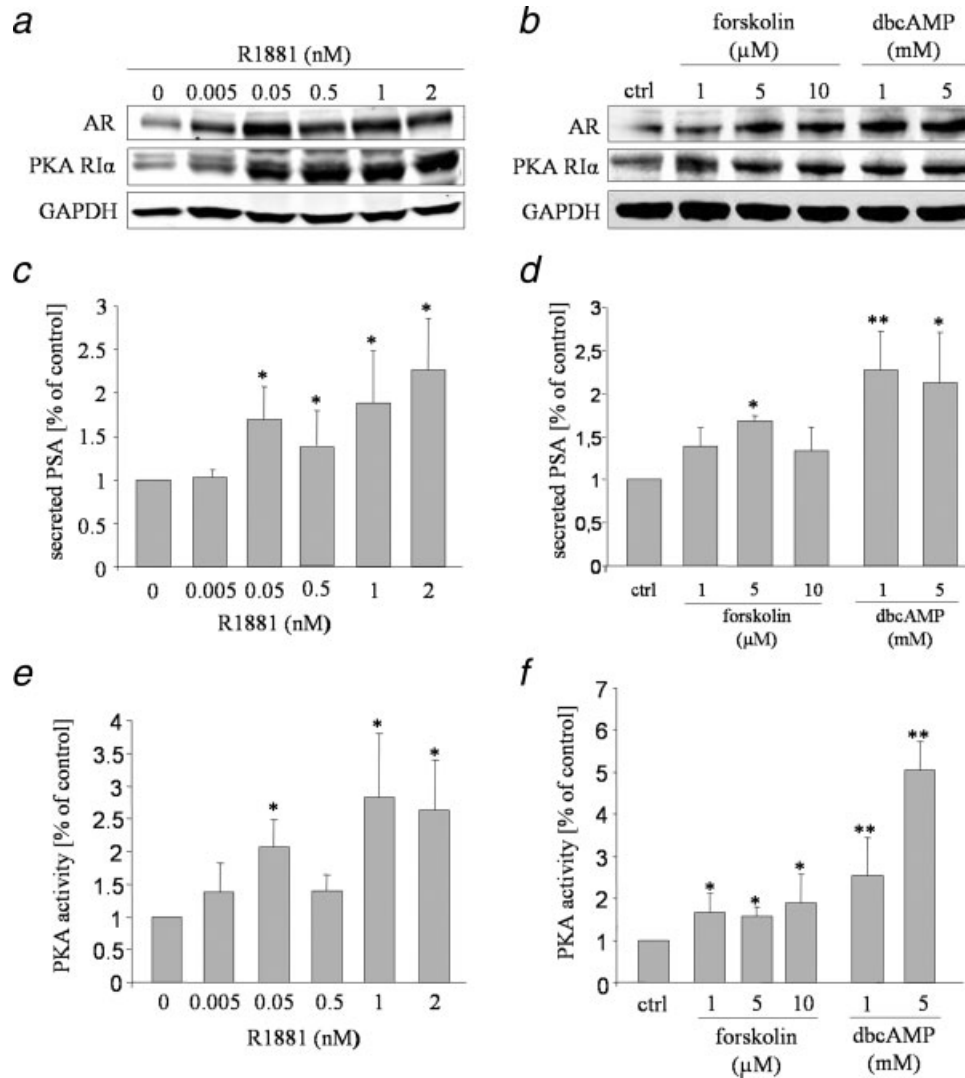


FIGURE 4 – Increased expression and activity of AR and PKA_{RIα} following stimulation of LNCaP cells with androgen, forskolin or dbcAMP over 72 hr compared to mock control (ctrl). (a,b) AR and PKA_{RIα} protein levels determined by Western blotting after treatment of LNCaP in the presence of different concentrations of androgen (R1881), forskolin or dbcAMP for 72 hr. (c,d) AR activity as determined by measuring secreted PSA levels after treatment of LNCaP cells with various concentrations of androgen (R1881), forskolin or dbcAMP for 72 hr. (e,f) PKA activity assay after treatment of LNCaP cells with different concentrations of androgen (R1881), forskolin or dbcAMP for 72 hr. *p* values below 0.05 were defined as statistically significant (**p* < 0.05; ***p* < 0.001 for comparison between single-targeting treatment and mock control).

In VCaP cells that express dramatically higher AR and PKA_{RIα} levels than LNCaP, the effect of the combinatorial treatment in inducing apoptosis was relatively low. Single treatment of VCaP (Fig. 6b) with either siAR-1, siAR-2 or siAR-3 for 72 hr resulted in 43%, 64% and 65% induction of caspase-3 activity rates respectively in comparison with the mock control, while siPKA increased caspase-3 activity by 47%. Nevertheless, the combination of siPKA with each of the 3 siRNAs against AR resulted in 2–2.5-fold induction of apoptosis, an effect that was also significantly higher than combination of each siRNA with negative control siLUC.

A similar result, as in LNCaP, was obtained in androgen ablation-resistant LNCaPabl cells (Fig. 6c). Combined targeting of AR-1 and PKA_{RIα} resulted in 11-fold (siAR-1 + siPKA) and 8-fold (siAR-2 + siPKA) induction of caspase activity, respectively, while treatment with each siRNA alone did not increase the levels of caspase activity for more than 4.5-fold. Thus, combined inhibi-

tion of AR and PKA_{RIα} significantly enhanced the proapoptotic effect of single AR knockdown (*p* < 0.05).

Depletion of PKA_{RIα} potentiates the growth inhibitory effects of the antiandrogen bicalutamide in androgen-sensitive LNCaP cells

We finally tested if enhanced growth inhibitory effects were also achieved by inhibiting the 2 pathways with siRNA knockdown in combination with the chemical inhibitors bicalutamide and H89, respectively. In prostate cancer patients, standard treatment to inhibit AR signaling is by androgen ablation or/and the use of antiandrogens such as bicalutamide, also called Casodex[®]. When androgen-sensitive LNCaP cells were treated with bicalutamide for 3 days, cell proliferation was decreased by 25% compared to mock control (*p* = 0.01; Fig. 7a). This growth-inhibitory effect of bicalutamide was strongly enhanced when it was combined with the siPKA resulting in a 60% inhibition *versus* mock control. Hence, we concluded that the siRNA-mediated inhibition of PKA-I signal-

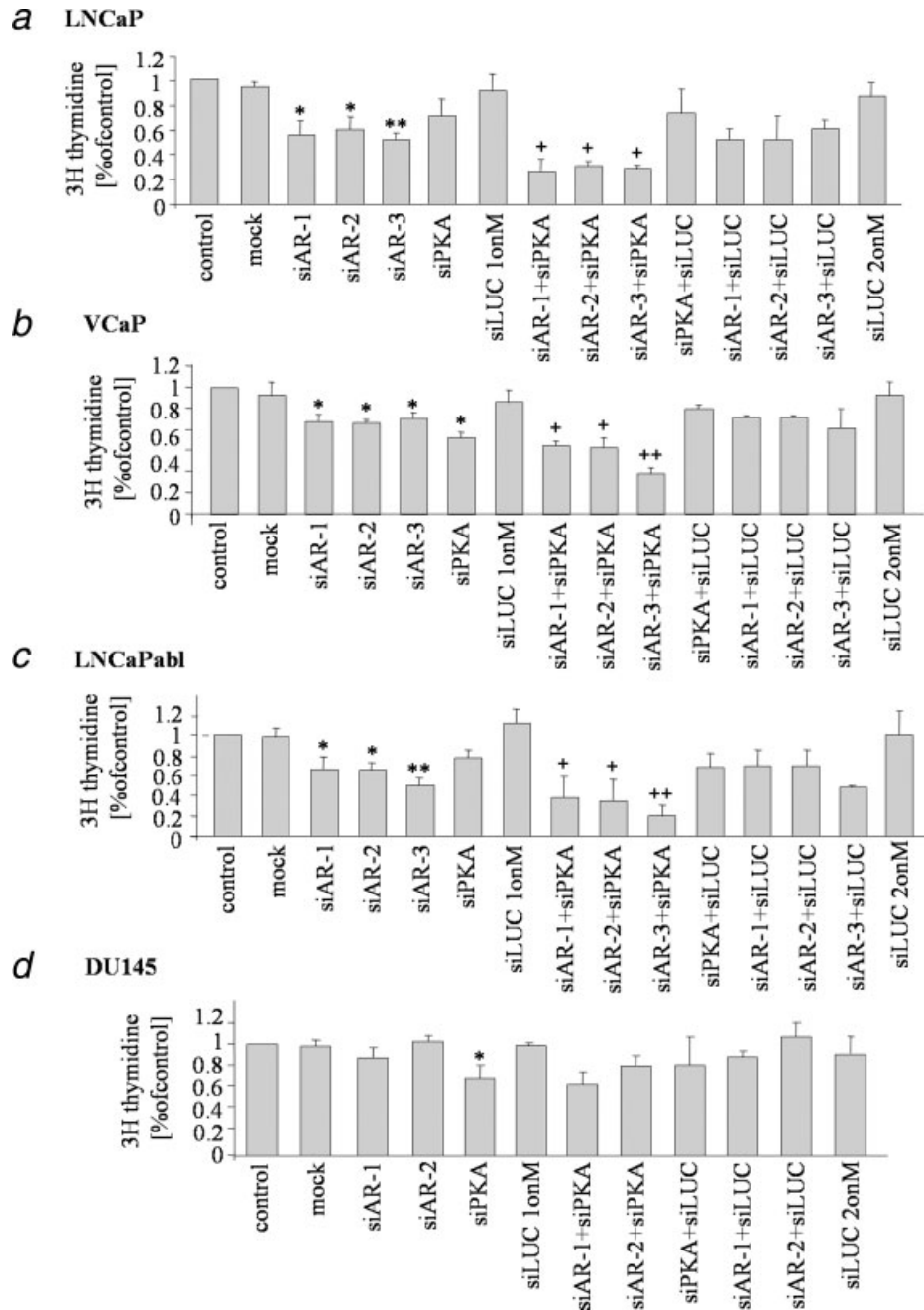


FIGURE 5 – Cell proliferation was recorded by [³H]thymidine incorporation assays in (a) LNCaP, (b) VCaP, (c) LNCaPabl and (d) DU145 in response to treatment. Growth inhibition is enhanced in androgen-sensitive LNCaP and VCaP and androgen-ablation-resistant LNCaPabl cells by simultaneous knockdown of AR and PKA RI α after 72 hr of treatment. *p* values below 0.05 were defined as statistically significant. Statistics were calculated by comparing the effect of single treatment with 10 nM of each siRNA with mock control (**p* < 0.05; ***p* < 0.001), whereas effects of combined treatments with a final siRNA concentration of 20 nM were compared *versus* negative control siLUC combined with each siRNA (+*p* < 0.05; ++*p* < 0.001).

ing significantly potentiates the antiproliferative effect of the anti-androgen bicalutamide in androgen-sensitive LNCaP cells.

In androgen ablation-resistant LNCaPabl cells, the combination of bicalutamide and siPKA by contrast only resulted in a minor enhancement of growth arrest (28% growth inhibition *versus* mock control, *p* = 0.004) compared with PKA-I knockdown alone (23% growth inhibition *versus* mock control, *p* = 0.008) (Fig. 7b). This was probably due to the fact that bicalutamide failed to inhibit cell proliferation in this cell line. By contrary, we even observed a moderate increase (23% *versus* mock control) in prolif-

eration following bicalutamide treatment. This result corresponds well with the previous findings where bicalutamide was shown to loose its growth inhibitory activity and to acquire agonistic properties in androgen ablation-resistant LNCaPabl.²⁶

We next investigated the effect of H89, an assigned inhibitor for PKA, in combination with siRNAs for AR or bicalutamide (Supporting information Fig. 2). LNCaP and LNCaPabl cells were treated with 20 μ M H89 for 72 hr in the presence of 5 μ M bicalutamide. It was revealed that H89 could inhibit proliferation of LNCaP and LNCaPabl by 20 and 33%, respectively, whereas combination of H89

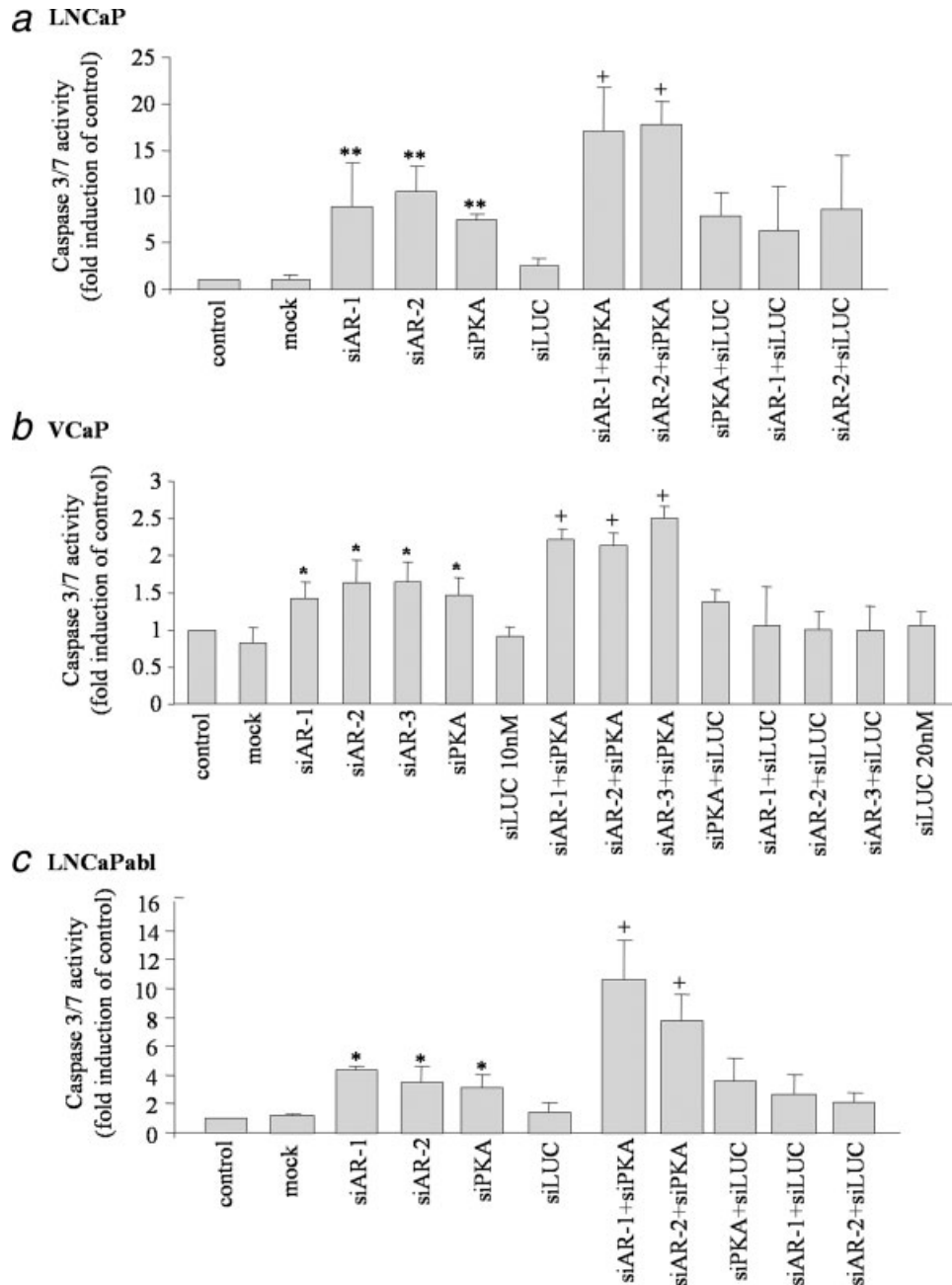


FIGURE 6 – Increased caspase activity in response to concurrent silencing of AR and PKA RI α . (a) Caspase 3 and 7 activity was measured in LNCaP, (b) VCaP and (c) LNCaPabl by ELISA 72 hr after treatment. *p* values below 0.05 were defined as statistically significant. Statistics were calculated by comparing the effect of single treatments with 10 nM of each siRNA with mock control (**p* < 0.05; ***p* < 0.001), whereas effects of combined treatments with a final siRNA concentration of 20 nM were compared *versus* negative control siLUC combined with each siRNA (+*p* < 0.05). In VCaP cells, we included an additional control of siLUC at a concentration of 20 nM.

with bicalutamide further decreased LNCaP proliferation by 58%. In LNCaPabl, the agonistic effect of bicalutamide was abolished in the presence of H89 as in the case of siPKA. When cells were treated with H89 together with siAR-1 or siAR-2, further reduction of proliferation was recorded in both LNCaP and LNCaPabl, whereas H89 treatment combined with siLUC was not more effective in decreasing proliferation than H89 single treatment in either cell line.

Discussion

The AR-signaling pathway is the main mediator of prostate cancer development and progression. It has been indicated that the

AR is expressed significantly in androgen ablation-resistant tumors and is also able to control the transcription of critical genes contributing to tumor progression.⁵⁻⁷ Activation of AR may occur through residual hormone levels within the tumors that can still be detected following androgen ablation.¹⁵ It has also been strongly supported that AR activation, in the absence of androgens, occurs due to genetic alterations within the AR gene, allowing the receptor to accept a broad spectrum of nonandrogenic ligands.¹⁰⁻¹⁴ Another possible mechanism of inappropriate AR activation is through ligand-independent interaction with other signaling pathways including cAMP-dependent PKA.^{2,4,16,19} Two different isoforms of PKA are known, PKA-I and PKA-II, which not only dif-

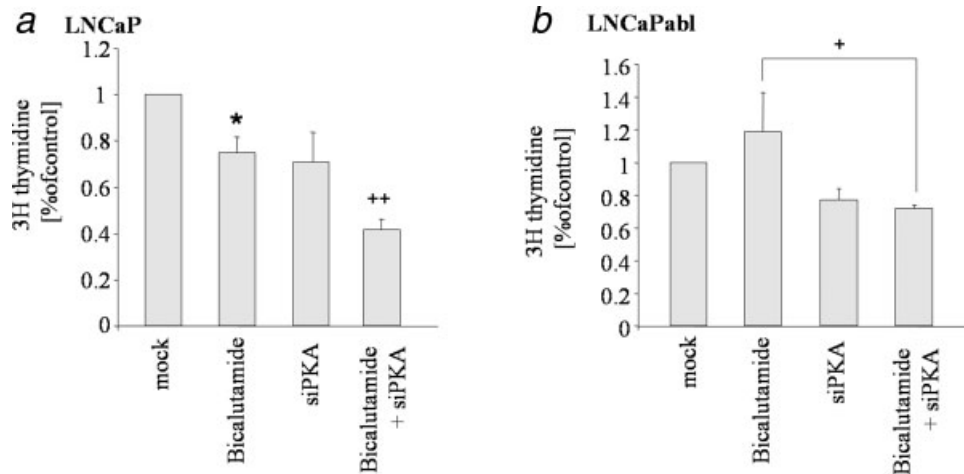


FIGURE 7 – Knockdown of PKA RI α cannot potentiate the antiproliferative effect of the antiandrogen bicalutamide in androgen ablation-resistant LNCaPabl cells. (a) LNCaP and (b) LNCaPabl were subjected to treatment with 5 μ M bicalutamide in the presence or absence of 10 nM siPKA. Cell proliferation was recorded 72 hr afterward by [3 H]thymidine incorporation assays. p values below 0.05 were defined as statistically significant as calculated by comparing the effect of single treatment with mock control (* p < 0.05), whereas effects of combined treatments were compared to single regimens (^+p < 0.05; ^{++}p < 0.001).

fer in terms of their regulatory subunits but also have a strikingly different impact on tumor growth and progression.²⁰ PKA-II is found preferentially in normal nonproliferating tissues and in growth-arrested cells, whereas PKA-I with its regulatory subunit PKA RI α is associated with cell proliferation and survival.²⁰ Overexpression of PKA RI α was revealed in different tumor types and therefore strongly associated with manifestation and progression of cancer cells.^{20,21} PKA RI α has been selected in numerous studies as a target for treatment intervention, and an antisense oligonucleotide targeting PKA RI α has already reached clinical evaluation.^{28,29}

In a previous study, we proposed that inhibiting AR expression with short antisense oligonucleotides^{23,24} or siRNAs³⁰ is an efficient strategy to inhibit androgen-sensitive as well as androgen ablation-resistant prostate cancer cells. The key finding of this study was that simultaneous blockade of PKA RI α significantly enhances the effect of AR knockdown in terms of cell-growth arrest and induction of apoptosis. Importantly, this enhanced effect of combined inhibition of the 2 pathways, AR and PKA-I, was efficient in androgen sensitive (LNCaP, VCaP) and androgen ablation-resistant (LNCaPabl) prostate cancer cells. We believe that such a combined targeting strategy includes several major advantages over single-treatment regimens. First, an enhanced growth-inhibitory effect is achieved without increasing drug doses thereby preventing higher costs of treatment and toxic side effects. A second advantage is that by targeting more than 1 molecule a broader spectrum of tumor cells can be reached, a fact that is especially important in tumor types such as prostate cancer which in general exhibits strong heterogeneity. Targeted therapies are strongly dependent on the strong and broad expression of the target molecules. Immunohistochemical staining has revealed that both target molecules, AR and PKA RI α , are strongly expressed in human prostate cancer. Moreover, we considered both pathways to be active, because we also revealed the downstream effector molecules of PKA and AR, pVASP and PSA, which showed a closely similar expression pattern as PKA RI α and AR. These data on *in vivo* expression pattern suggested that targeting these 2 pathways in prostate cancer represents a reliable approach. AR and PKA RI α are also found to be expressed in benign tissue, but we believe that targeted treatment against them will affect most extensively the prostate cancer sites where the 2 target proteins are overexpressed.

To inhibit AR expression, we used 3 different siRNAs, 1 targeting the N-terminal polyglutamine region (siAR-1) that has previously been shown to efficiently inhibit prostate tumor growth, a

second 1 targeting a specific region nearby the start codon of the AR gene (siAR-2) and a third 1 against the ligand-binding domain of AR (siAR-3). All siRNAs efficiently inhibited AR expression and exhibited a strong antiproliferative and proapoptotic effect in androgen-sensitive LNCaP and VCaP as well as in androgen ablation-resistant LNCaPabl cells. In VCaP cells, which express higher levels of AR and PKA RI α than LNCaP cells, siRNA-mediated target knockdown was less efficient. Correspondingly, the effects on proliferation and apoptosis were slightly less prominent in VCaP than in LNCaP cells, pointing out that the therapeutic effect is strongly dependent on the level of target expression. Nevertheless, combined treatment also had an additive antiproliferative effect in this cell line.

An important finding of our study was that siRNA-mediated AR knockdown is much more efficient in terms of tumor growth arrest than the antiandrogen bicalutamide, which acts *via* inhibition of AR activation. This may be of advantage especially in tumors that escaped from androgen-ablation therapy. To investigate the efficacy of our combination therapy in advanced prostate cancer cells, we used a subline of LNCaP, LNCaPabl, which has acquired specific features of advanced androgen ablation-resistant prostate cancer, including increased AR levels, hypersensitivity to androgen stimulation and the ability to grow in castrated mice.²⁶ Moreover, these cells are less susceptible to growth inhibition and induction of apoptosis than LNCaP,³¹ a phenomenon that could also be confirmed in this study. When LNCaPabl cells were treated with siRNAs to inhibit AR expression, they were inhibited by 35% *versus* mock control. By contrast, blocking AR activation with the antiandrogen bicalutamide failed to inhibit the cells, but even resulted in an increase of cell proliferation by 23%. This loss of antagonistic activity of bicalutamide, which reflects the clinical situation of therapy resistance, has previously been shown by our group.²⁶ Hence, we concluded that especially in advanced therapy-resistant prostate cancer, the target-specific prevention of AR expression could have higher tumor inhibition efficacy than blocking AR activation through antiandrogens.

For knocking down PKA RI α , we used a siRNA targeting, a sequence that corresponds to the target sequence of a previously described efficient antisense oligonucleotide.^{28,29} The regulatory subunit of PKA-I, PKA RI α , is essential for the formation and subsequent activation of the tetrameric core protein. It undergoes dramatic conformational changes upon complex formation with the catalytic subunits, thus resulting in stabilization of the whole complex.³² It has also been shown that overexpression of PKA

RI α in PC3 prostate cancer cells results in elevated PKA catalytic alpha subunit expression but with no increase in mRNA, indicating the posttranscriptional stabilization of the catalytic subunit *via* formation of the tetramer with PKA RI α .³³ In the same context, overexpression of PKA catalytic alpha subunit resulted in concurrent PKA RI α mRNA induction as PKA RI α is the only PKA subunit whose promoter is known to contain cAMP response element (CRE).^{33,34} Thus, PKA RI α is thought to be essential as a mediator of PKA signaling and for the stabilization of PKA catalytic subunits *via* the holoenzyme formation, and in addition, it is assumed that PKA signaling might be autoregulated through the regulation of PKA RI α levels. Here, we have shown that downregulating PKA RI α was sufficient to significantly reduce PKA activity, resulting in cell-growth arrest and induction of apoptosis.

When using siRNA technology, the use of controls is extremely important to exclude unspecific off target effects. Therefore, we used an unspecific siLUC control to demonstrate specificity of our results. The question regarding off target effects arose from the finding that knocking down the AR considerably reduced PKA RI α protein levels. This was associated with the reduction of PKA activity and reduced phosphorylation of the PKA downstream substrates VASP^{35,36} and CREB³⁷ in LNCaP cells. On the other hand, siPKA was found to reduce AR expression. A possible interaction between the 2 pathways has been postulated previously, suggesting an activation of the AR *via* cAMP-dependent PKA activation.^{2,4,16} It has also been shown that androgens cause rapid cAMP-dependent activation of PKA, thereby indirectly stimulating activation of AR signaling in prostate cancer cells.¹⁷ Our study provides evidence that, in AR positive prostate cancer cells, protein expression levels of AR and PKA RI α are possibly indirectly dependent on each other. This was assumed from the results illustrating consistently that AR knockdown by 3 different siRNAs significantly reduced PKA RI α expression and PKA activity, whereas PKA RI α downregulation similarly decreased AR protein levels and signaling in LNCaP. In contrast, knockdown of AR or PKA RI α did not decrease but even slightly increase PKA RII β levels. PKA RII β is a regulatory subtype of PKA-II, which has been associated with cell differentiation and growth-arrest phenotype. This inverse correlation of RI α and RII β expression was shown in this study, therefore agreeing with previously published reports.^{20–22,33} In the same context, PKA catalytic α subunit has been reported to translocate to the nucleus after inhibition of PKA RI α expression.³⁸ This event also occurred in our study after treatment of LNCaP with either siRNA against AR or PKA RI α .

To further assess if this mutual interaction of the 2 molecules is due to siRNA-mediated off target effects, we further tested our siRNAs in AR negative DU145 prostate cancer cells. In this cell line, PKA RI α was significantly inhibited by siPKA, however, none of the 3 used siRNAs against the AR affected PKA RI α levels, again suggesting that expression of PKA RI α is dependent on AR signaling. Moreover, cell-viability assays in DU145 showed that silencing PKA RI α induced growth arrest, whereas AR

knockdown with siAR-2 did not affect viability. Even siAR-1, the siRNA targeting the polyglutamine region of the AR, which is thought to target various CAG repeat containing genes, only had a minor effect on DU145.

To follow the opposite direction and investigate this possible mutual influence between the 2 proteins, we induced AR and PKA signaling by stimulating LNCaP cells with androgen, forskolin and dbcAMP. In earlier studies, it has been proposed that androgen regulation of AR expression occurs in a tissue- and cell-type-specific fashion.³⁹ In particular, we showed here that androgen upregulates AR protein in LNCaP cells. These results are in agreement with the previous studies indicating that androgen stimulation results in increase of AR stability in LNCaP.⁴⁰ Within the same setting, we showed that androgen increased expression of PKA RI α in LNCaP, a consistent and relevant result in comparison with our knockdown studies. The increase of AR and PKA RI α in the presence of androgen was further associated with increased PSA levels and PKA activity in LNCaP. We then assessed AR and PKA RI α levels in LNCaP after stimulation with forskolin and dbcAMP in parallel. We recorded upregulation of AR expression and activity (PSA induction) in the presence of either forskolin or dbcAMP along with expected induction of PKA activity and higher PKA RI α protein levels.^{33,34} This outcome also confirmed an earlier study, in which the authors demonstrated AR induction in LNCaP by cAMP analogues and also identified a potential CRE in the promoter of AR possibly being responsible for this induction.⁴¹ Moreover, our result is consistent with the postulation that the activation of AR can occur through PKA signaling,^{2,4,16,19} and confirms PSA as a common transcriptional target in response to stimulation of both pathways as previously reported.^{5,19,42,43} We additionally identified candidate androgen-response elements that might be responsible for the regulation of PKA RI α by the AR pathway; however, further experiments are required to elucidate the mechanism(s) by which AR signaling might regulate PKA RI α expression in prostate cancer.

In summary, this is the first study showing that simultaneous inhibition of PKA RI α and AR significantly enhances the anti-proliferative and proapoptotic effects of AR knockdown alone in androgen-sensitive and androgen ablation-resistant prostate cancer cells. We therefore conclude that combined targeting of AR and PKA RI α may be a promising new therapeutic option for prostate cancer patients with malignancies of early small tumors, especially when applied as a focal molecular therapy, as well as for advanced tumor stages and might be superior to solely preventing AR expression.

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