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Cytosolic Phospholipase A_2-α: A Potential Therapeutic Target for Prostate Cancer

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Abstract

Purpose—Cytosolic Phospholipase A2-α (cPLA2-α) provides intracellular arachidonic acid to supply both cyclooxygenase and lipoxygenase pathways. We aim to determine the expression and activation of cPLA2-α in prostate cancer (PC) cell line and tissue and the effect of targeting cPLA2-α *in-vitro* and *in-vivo*.

Experimental Design—The expression of cPLA2-α was determined in PC cells by RT-PCR, Western blot and immunocytochemistry. Growth inhibition, apoptosis and cPLA2-α activity were determined after inhibition with cPLA2-α siRNA or inhibitor (Wyeth-1). cPLA2-α inhibitor or vehicle was also administered to PC xenograft mouse models. Finally the expression of phospho-cPLA2-α was determined by immunohistochemistry in human normal, androgen sensitive and insensitive PC specimens.

Results—cPLA2-α is present in all PC cells lines, but increased in androgen insensitive cells. Inhibition with siRNA or Wyeth-1 results in significant reductions in PC cell numbers, as a result of reduced proliferation as well as increased apoptosis and this was also associated with a reduction in cPLA2-α activity. Expression of cyclin D1 and phosphorylation of Akt were also observed to decrease. Wyeth-1 inhibited PC3 xenograft growth by approximately 33% and again, also reduced cyclin D1. Immunohistochemistry of human prostate tissue revealed that phospho-cPLA2-α is increased when hormone refractory is reached.

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Statement of translational relevance. The poor response to current therapies by men with hormone refractory prostate cancer highlights the importance of identifying the biological drivers of prostate cancer growth and survival, which may be used as therapeutic targets. Such specific targets have the potential for greater cancer control and significantly less toxicity than current chemotherapy regimens. Identifying that $cPLA_2$-α is present in prostate cancer cell lines and demonstrating that inhibition of $cPLA_2$-α will suppress cancer cell growth *in-vitro* and *in-vivo* raise $cPLA_2$-α as a potential therapeutic target. The identification of $cPLA_2$-α, and its increase in hormone refractory disease confirms its presence and relevance to human prostate cancer. Our results give evidence that $cPLA_2$-α is a potentially effective therapeutic target and support the need for further studies in the context of clinical trials validating $cPLA_2$-α targeted therapy for men with prostate cancer.

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Conclusions—cPLA2-α expression and activation is increased in the androgen insensitive cancer cell line and tissue. Inhibition of cPLA2-α results in cells and xenograft tumor growth inhibition and serves as a potentially effective therapy for hormone refractory PC.

Keywords

prostate cancer; $cPLA_2$-α; eicosanoids; angiogenesis

Introduction

Previous studies have demonstrated that the eicosanoid pathway is activated in many types of cancers (1) including prostate (2–9). Eicosanoids, which are the products of the cyclooxygenase (COX) and lipoxygenase (LOX) pathways, contribute to cancer progression by promoting cell proliferation, motility, invasion and angiogenesis (8–10). Eicosanoids are synthesized from intracellular arachidonic acid (AA), which is released from membrane phospholipids by the action of phospholipase A_2 (PLA_2) (11,12). Of the known mammalian PLA_2 enzymes, cytosolic PLA_2-α ($cPLA_2$-α), an 85kDa protein, is the predominant source of intracellular AA for eicosanoid production.

We have previously shown that expression of Annexin II, a calcium dependent phospholipid-binding protein and inhibitory regulator of $cPLA_2$-α, is lost in human prostate cancer (PC) (13). We have also reported that $sPLA_2$-IIA, one of the secretory PLA_2s, is overexpressed in PC cells including those remaining after androgen ablation therapy (14). The growth-promoting effect of $sPLA_2$-IIA appears to be via $cPLA_2$-α (14). These data have prompted us to investigate the role of $cPLA_2$-α in PC growth, and determine if it is a feasible target for treatment of the hormone refractory stage of PC.

In this study we show that $cPLA_2$-α is present in PC cell lines and increases in androgen insensitive cells. We have also shown that siRNA knockdown or specific inhibition of $cPLA_2$-α results in decreased PC cell growth, through a mechanism of decreased proliferation and, to a lesser extent, increased apoptosis. These effects are at least partially mediated through an inhibition of Akt phosphorylation and decrease in cyclin D1 expression. We also show that, as seen *in-vitro*, *in-vivo* inhibition of $cPLA_2$-α results in a significant decrease in xenograft tumor growth. Finally we also demonstrate that the phosphor-$cPLA_2$-α (p-$cPLA_2$-α) is present in human prostate tissues and its levels increases in hormone refractory PC.

Materials and Methods

Reagents and cell lines

The LNCaP-FGC (LNCaP), DU145 and PC3 human PC cell lines were purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 (Sigma-Aldrich, Sydney, Australia), supplemented with 10% FBS (ICN Biomedical, Sydney, Australia) with all cell cultures at 37°C in a humidified environment of 5% CO_2. The passage numbers of cells described in this article were between 30 and 45 for LNCaP, 65 and 80 for DU145, 25 and 40 for PC3. The $cPLA_2$-α inhibitor, Wyeth-1 was prepared as previously described (15) (US Patent 6,797,708, Sanmar Chemical Company, India). Pyrrolidine-2 (also known as pyrrophenone) was prepared as previously described (16). Both inhibitors were reconstituted in dimethylsulfoxide (DMSO). All antibodies (phosphor (Ser^{505})-$cPLA_2$-α, total $cPLA_2$-α, phosphor (Ser^{457})- Akt, total Akt, GAPDH and alpha-tubulin) were obtained from Cell Signaling except Cyclin D1 (Sigma, St. Louis, Missouri).

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RT-PCR

Levels of $cPLA_2$-α mRNA were measured by end-point and real time RT-PCR. After cell treatments, total RNA was isolated using Trizol reagent (Sigma-Aldrich, Sydney, Australia) as per the manufacturers' instructions. The first-strand cDNA was synthesized from 2 μg of total RNA using a combination of random hexamers and oligo-dT as described previously (13). End-point primers were designed based on the human $cPLA_2$-α mRNA (NM_024420). Forward: 5′-ACAGTGGGCTCACATTTAACCT, Reverse: 5′-CTTCCCGATCAAACACATAAGG. GAPDH was used as the house-keeping gene and its primer sequences are: Forward 5′-TGGACCTGACCTGCCGTCTA, Reverse- 5′-CCTGTTGCTGTAGCCAAATTC. Conditions for PCR were: one cycle of 2 minutes at 94°C; 40 cycles of 20 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C; then one cycle of 5 minutes at 72°C. For real time PCR, the $cPLA_2$-α primer sequence: Forward: 5′-ATCCTGATGAATTTGAGCGA, Reverse: 5′-CAAGTAGAAGTTCCTTGAACG. TATA box Binding Protein (TBP) was chosen as the house-keeping gene: Forward: 5′-GAACCACGGCACTGATTTTC, Reverse: 5′-CCCCACCATGTTCTGAATCT. Quantitative PCR measurements were performed with SYBR-Green and ROX as a passive reference using the Rotor-Gene system. Conditions for PCR were: one cycle of 2 minutes at 50°C and 2 minutes at 95°C; 50 cycles of 30 seconds at 95°C, 30 seconds at 65°C and 30 seconds at 55°C followed by one cycle for 10 seconds at 25°C. The Δ–Δ method was used to calculate relative changes in $cPLA_2$-α compared to housekeeping gene (TBP).

Western Blotting

Cell lysates were prepared using lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β-Glycerolphosphate), supplemented with 1:50 dilution of protease inhibitor cocktail (Sigma-Aldrich, Sydney, Australia). Protein concentration was quantified using Bio-Rad Protein Assay (Bio-Rad, Sydney, Australia). Cell lysates (60 μg) were separated on an 8% SDS-PAGE and then transferred onto a nitrocellulose membrane. The membranes were blocked with 5% non-fat milk containing 0.1% Tween for 1 h. Primary antibodies were incubated overnight at 4°C, washed and probed with secondary antibodies coupled with peroxide and detected by enhanced chemiluminesence (Amersham Biosciences, Buckinghamshire, UK). Blocking peptide (total and phosphor-$cPLA_2$-α, Cell Signaling) was added at a concentration of 2μg/ml to the primary antibody, prior to being added to the blot. NIH-3T3 and HeLa cells were used as a positive control for detection. Densitometric scanning was performed to quantify band intensities.

Immunohistochemistry

PC cells were scraped with a rubber policeman and fixed in 10% formalin and embedded in paraffin wax. Human prostate tissue was collected from consenting patients, under Central Sydney Area Health Service (X04–0138) and Western Area Health Service Ethics Committee (HREC 2000/9/4.18(1089)) approval. Localized PC specimens from the peripheral zone of the prostate were obtained from men who had radical prostatectomy as treatment for their PC (n=12). Paired PC specimens before and after reaching hormone refractory stage were also obtained (n=7 pairs). These men initially presented with PC, and tissue was obtained when they underwent transurethral resection of prostate (TURP). After the cancers progressed and androgen ablation therapy failed, these men required repeat TURP. Normal prostate tissue from the peripheral zone was also obtained from organ donors as the control.

Cut sections were subject to dewaxing, antigen retrieval and incubation with primary antibody. Blocking peptide for p-$cPLA_2$-α was added at a concentration of 2 μg/ml to the primary antibody, prior to addition to the slide. This was followed by application of biotinylated secondary antibody and Vectastain ABC kit (Vector, Sydney, Australia) and staining revealed

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using DAB (DakoCytomation, Glostrup, Denmark). The staining intensity was graded as low or high and the percentage of cells stained at each intensity was recorded.

$cPLA_2$-α gene silencing with siRNAs

The $cPLA_2$-α specific sequence: TTG AAT TTA GTC CAT ACG AAA (Qiagen, Doncaster, Australia) and negative control against the non-mammalian gene, fluorescein: AAT TCT CCG AAC GTG TCA CGT was used. PC cells were transfected with 5nM or 10 nM of siRNA duplexes using HiPerfect Transfection Reagent (Qiagen, Doncaster, Australia). Transfection efficiency was confirmed by fluorescence microscopy and counting.

Cell Growth

The cells were plated in triplicate in 96 well plates. After 48 h, cells adherent on 96 well plates were exposed to the indicated treatments for the stated time intervals. Cell growth was assessed using MTS (CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay, Promega, Madison, WI) as described previously (17). Values are expressed relative to untreated controls. Inhibitory concentrations IC_{25}, IC_{50} and IC_{75} were calculated by plotting graphs of cell growth measured by MTS verses concentration of drug.

cPLA2 Activity Assay

Arachidonoyl Thio-PC was used as the substrate to measure $cPLA_2$ activity *in vitro*. The $cPLA_2$ Assay Kit was purchased from Cayman Chemical (Cat NO 765021) and procedures were performed according to the manufacturer's instructions. After treatment of cells with Wyeth-1 or siRNA, the cells were homogenized and treated with 7.5μM bromoenol lactone, a specific $iPLA_2$ inhibitor. We did not add a sPLA2 inhibitor as it is barely detectable in the PC3 cell line. Twenty μl of cell lysate was finally subjected to the assay, and the OD value was measured at 414 nm and normalized to protein concentration.

DNA Synthesis Assay

Incorporation of [^{3}H]thymidine was used to measure DNA synthesis. PC cells were plated in 96 well plates and allowed to seed for 48 h. The cells were then treated with siRNA or inhibitors. [^{3}H]thymidine (TRK686, Amersham Biosciences, Buckinghamshire, UK) to the equivalence of 1 μCi/100μL was added for the final 6 h. At the end of treatment, the cells were tryspinised and cellular DNA was harvested onto filtermats using a cell harvester (PerkinElmer, Wellesley, MA). Following drying, scintillation fluid was added and radioactivity read in a beta plate counter. Counts were normalized to cell number in replicate plates treated the same way.

Apoptosis Assay -Caspase 3/7

This was quantitated by measuring fluorimetrically activated Caspase 3 and 7 with ApoOne caspase 3/7 Homogenous Assay (Promega, Madison) and performed as per the manufacturers instructions. PC cells were plated onto white 96 well plates (Greiner Bio-One, Frickenhausen, Germany) and after 48 h were treated with siRNA or inhibitors. The caspase substrate and buffer solution was added at the end of treatment and allowed to incubate for 18 h before detection with a Fluroskan Ascent fluorometer (Thermo Labsystems, Beverley, MA) at excitation wavelength 485 nm and emission wavelength 515 nm. All Relative Fluorescence Units were normalized to cell numbers.

TUNEL

The Promega DeadEnd™ Colorimetric TUNEL System was used to measure the effects of inhibitor on late PC cell apoptosis and performed as per the manufacturer's instructions. Cell blocks were dewaxed, antigen retrieved and covered with equilibration buffer at room

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temperature for 10 min followed by 1.5 h incubation in rTdT Reaction Mix at 37°C. The reaction was terminated by immersing slides in 2×SSC solution and biotinylated nucleotides detected using DAB. Positive staining cells were counted and expressed as the average of 10 high power fields (40X).

PC3 Xenografts

PC3 cells ($0.5–1 \times 10^6$) were implanted subcutaneously in the flanks of 6–7 week athymic male nude mice. Mice were randomly distributed to two groups once the tumor size was between 150–200mm^3(n=8 mice/group): (i) control treated with vehicle (DMSO) daily ip; or (ii) treated with Wyeth-1, 10 mg/kg daily ip. Tumor growth was assessed twice a week by caliper measurement of tumor diameter in the longest dimension (L) and at right angles to that axis (W). Tumor volume was estimated by the formula, $L \times W \times W \times \pi/6$. Mice were sacrificed when the tumors grew to 1500mm^3 (the allowable limit specified by the institution animal ethics committee) and tumors excised for immunohistochemical studies. The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Sydney (K14/7-2006/3/4429).

Statistical Analysis

All in-vitro experiments were performed in triplicate and the experiments were repeated at least three times. The statistical software SPSS version 12.0 (Chicago, IL) was used for analysis. Data were analyzed by student's t-test, χ^2 or analysis of variance (ANOVA) as appropriate. A two-tailed *P* value <0.05 was considered significant. Xenograft tumor growth was compared between groups by fitting a repeated measures covariate model (ANCOVA), where the actual time measurements are viewed as a covariate. Results are expressed descriptively as the mean ± SE.

Results

cPLA$_2$-α is present in PC cell lines

To determine if cPLA$_2$-α was a potential therapeutic target for the treatment of PC, we initially determined the expression levels of this enzyme in the three commonly used PC cell lines. Expression of cPLA$_2$-α mRNA transcripts by end-point RT-PCR (data not shown) revealed that the androgen sensitive PC cell line, LNCaP, expressed far less cPLA$_2$-α mRNA than DU145 and PC3, the two androgen insensitive cell lines. This was confirmed by quantitative RT-PCR (Fig. 1A, $p<0.05$). To verify this, protein expression of total cPLA$_2$-α was determined by Western blot analysis of PC cell lysates and quantitated with densitometric analysis of the protein bands from 3 separate experiments (Fig 1B). Immunoblots of total- cPLA$_2$-α revealed that LNCaP expressed very small amounts of the protein compared to DU145 and PC3 ($p<0.05$). As cPLA$_2$-α needs to be activated by phosphorylation, we also determined the level of p-cPLA$_2$-α by Western blot analysis (Fig 1B). LNCaP again demonstrated very low p-cPLA$_2$-α expression, however the intensity significantly increased for DU145 and even further for PC3 ($p<0.05$), consistent with the total cPLA$_2$-α expression. We believe antibody binding was specific, as binding was depleted by the addition of excess peptide (Fig 1C). Further experiments were performed only on LNCaP (androgen sensitive) and PC3 (androgen insensitive) PC cell lines.

To visualize the proportions of cells with active cPLA$_2$-α, cell blocks of LNCaP and PC3 cells were stained by immunocytochemistry with anti p-cPLA$_2$-α antibody (Fig 1D). Phosphor-cPLA$_2$-α stained cytoplasm, peri-nuclear as well as intra-nuclear regions. Consistent with the immunoblot findings, LNCaP demonstrated some cells with weak p-cPLA$_2$-α and PC3 cells had much more intense staining of a larger proportion of cells. Specificity was again confirmed by the addition of a blocking peptide.

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Knock-down or inhibition of $cPLA_2$-α results in inhibition of PC cell growth

To determine if $cPLA_2$-α enzymatic activity is involved in PC growth, we examined PC cell growth *in vitro*, after $cPLA_2$-α mRNA was silenced with siRNA. Figure 2A shows that siRNA at 10nM results in efficient knock-down of $cPLA_2$-α mRNA in LNCaP and PC3 cells at 48 h, with levels at approximately 30–40% compared to control cells transfected with non-mammalian siRNA. To determine cell growth, cells were treated with siRNA to $cPLA_2$-α for 48 h and cell number was then estimated by the MTS assay. Results show that both LNCaP and PC3 cell numbers were significantly reduced compared with cells transfected with non-mammalian siRNA (Figure 2B). siRNA at doses of 5nM and 10nM significantly reduced LNCaP cell numbers to 81% ($p=0.008$) and 70% ($p<0.001$) respectively, and PC3 cell numbers to 79% ($p=0.007$) and 65% ($p<0.001$) respectively.

As specific knock-down of $cPLA_2$-α mRNA resulted in significant reductions in cell growth in both cell lines, we aimed to determine if a newly available specific inhibitor of $cPLA_2$-α named Wyeth-1 by Ni et al (15) and also named Gfipladib could also reduce PC cell growth. This compound has been shown not to inhibit sPLA2 (unpublished data from M.H. Gelb). LNCaP and PC3 were treated with increasing concentrations of the inhibitor, and growth was measured after 72 h treatment by MTS assay (Fig. 2C). LNCaP cells demonstrated dose-dependent reductions in cell number ($p<0.001$) as did the PC3 cell line ($p<0.001$), but at higher concentrations. The concentration of Wyeth-1 required to achieve a 25% (IC_{25}), 50% (IC_{50}) and 75% (IC_{75}) reduction in cell number was calculated by constructing a line of best fit and applying the resulting equation. The IC_{25}, IC_{50} and IC_{75} for Wyeth-1 treatment of LNCaP cells were 11.1μM, 15.9 μM and 21.0 μM respectively and 15.1 μM, 22.2 μM and 27.6 μM for PC3 respectively. These specific Wyeth-1 concentrations were used for further experiments. To determine if the growth inhibitory effects of Wyeth-1 could be repeated using other specific $cPLA_2$-α inhibitors, the experiment was repeated with pyrrophenone-2, another specific $cPLA_2$-α inhibitor. Pyrrophenone also decreased LNCaP and PC3 cell growth in a dose dependent manner identical to Wyeth-1 except that the inhibitory concentrations were lower (IC_{50} for LNCaP was 3.3 μM and for PC3 8.8 μM). The ability of two structurally different inhibitors, pyrrolidine-2 and Wyeth-1 as well as $cPLA_2$-α silencing, to block cell growth is evidence supporting an important role for cPLA2-α in PC cell growth.

In order to further confirm that the inhibitor, Wyeth-1, inhibited cell growth by inhibiting $cPLA_2$-α activity, we determined the effect of Wyeth-1 on inhibition of cPLA2-α activity *in-vitro*. Activity was quantitated by measuring conversion of a PLA2 substrate, arachidonoyl thio-PC to free thiol by cPLA2. Cells were pretreated with an iPLA2 inhibitor to ensure all measured hydrolysis was a result of cPLA2 activity. sPLA2 protein is barely detectable in PC3 cells by Western (Data now shown). Figure 2D confirms that at IC_{50} Wyeth-1 results in a 41% reduction in cPLA2 activity in PC3 cells ($p<0.001$). To verify the reduction in PLA2 activity was due to $cPLA_2$-α, not any other PLA2, we also showed a significant reduction in $cPLA_2$-α activity (26%, $p<0.01$) with knockdown of $cPLA_2$-α mRNA using 10nM siRNA.

PC Cell Proliferation and Apoptosis are regulated by $cPLA_2$-α function

The decreases in cell growth observed with Wyeth-1 treatment could be a result of inhibition of proliferation and/or induction of apoptosis. To determine which processes were involved, we first assessed proliferation by measuring ^{3}H-thymidine incorporation in prostate cancer cell lines after $cPLA_2$-α mRNA silencing with 10nM siRNA. Figure 3A shows that LNCaP cells and PC3 cells undergo a 28% ($p=0.04$) and 43% ($p<0.001$) reduction in proliferation, respectively, 24 h after treatment with siRNA when compared to cells treated with non-mammalian siRNA. Wyeth-1 treatment at IC_{25} and IC_{50} also resulted in reduced LNCaP cell proliferation by 22% ($p= 0.006$) and 75% ($p=0.001$), respectively. PC3 cells showed 50% ($p<0.001$) and 76% ($p<0.001$) reduction in proliferation, respectively (Figure 3A).

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Early apoptosis was measured by activated caspase 3/7 assay. $cPLA_2$- α mRNA silencing with siRNA results in a 26% increase in apoptosis in LNCaP cells (p=0.03) and 19% increase in PC3 cells (p=0.02, Figure 3B). Wyeth-1 treatment of LNCaP cells showed 82% (p=0.03) and 96% (p= 0.05) increases in apoptosis at IC_{25} and IC_{50} respectively (Figure 3B). PC3 cells showed a lesser increase at IC_{25} and IC_{50} concentration (p=0.02).

In order to confirm that the measure of apoptosis by caspase 3/7 activation was also representative of late apoptotic events, we treated LNCaP cells with Wyeth-1 (IC_{50}) for 72 h and then measured late apoptosis by TUNEL assay of the fixed cell block. Figure 3C shows that compared to control, Wyeth-1 treated cells underwent a 2 fold increase in the percentage of TUNEL positive cells under high power field.

We conclude that the growth inhibitory effects of $cPLA_2$- α are a result of proliferation reduction and apoptosis induction in both androgen sensitive and insensitive cell lines.

Inhibition of $cPLA_2$- α results in a reduction in phosphorylated Akt and expression of cyclin D1

Cyclin D1 is critical for the cell cycle progression and phosphorylated Akt (p-Akt) is an important part of the PI3K-Akt pathway which regulates cell growth and survival. We have previously demonstrated that treatment of PC cells with celecoxib, a selective COX-2 inhibitor results in decreased expression of cyclin D1 (18) and also reduction of p-Akt (unpublished data). We aimed to determine if p-Akt or Cyclin D1 were regulated by $cPLA_2$- α. Figure 4A shows that increasing concentrations of Wyeth-1 treatment of LNCaP cells resulted in reductions in p-Akt, particularly at IC_{50} and above. The expression of t-Akt, was also reduced, but only at concentrations of IC_{75}, thus a reduction in t-Akt only partially explains the reduction in p-Akt; the remainder must be due to a decrease in proportion phosphorylated.

Wyeth-1 treatment of LNCaP cells also decreased the expression of cyclin D1 at concentrations of IC_{50} or greater (Figure 4A). To confirm this finding, we performed immunocytochemistry for cyclin D1 on cell blocks of LNCaP cells which had been treated with IC_{25}, IC_{50} and IC_{75} concentrations of Wyeth-1 compound for 72 hours. Figure 4B shows the decrease in nuclear cyclin D1 expression with increasing concentrations of Wyeth-1. Cell counting of cyclin D1 positive cells showed that positive staining cells decreased from 19% in control to 17% after IC_{25} (p=0.31), 14% after IC_{50} (p=0.02) and 10% after IC_{75} (p= 0.005) treatment of cells (Figure 4C).

Inhibition of $cPLA_2$-α results in PC3 Xenograft Growth Retardation

Based on the *in vitro* findings described above, it was important to investigate whether inhibition of $cPLA_2$- α, had comparable growth inhibitory effects *in vivo*. The PC3 xenograft model was used to assess whether Wyeth-1 inhibited tumor growth. Treatment with vehicle or Wyeth-1 (10 mg/kg i.p. daily) was initiated once tumors had formed and reached $200mm^3$(Fig. 5A). The control (vehicle treated mice) demonstrated very rapid xenograft tumor growth, and at 14 days of treatment all mice were sacrificed as they had reached the average size of $1500mm^3$. Comparatively the Wyeth-1 treated mice, demonstrated significantly slower rates of tumor growth. The slopes of the two regression lines, separate for each treatment group, are significantly different ($p<0.05$). At sacrifice tumor volumes were 33% smaller compared to control. Mouse health, average weights and unplanned deaths did not differ between the two groups, suggesting that no major toxicity was suffered as a result of the treatment. Immunohistochemistry for cyclin D1 on xenograft tumors after sacrifice showed considerable reduction in the number of cells staining positive for cyclin D1 in the treated tumors (36%) compared to control tumors (54%, p=0.005) (Figure 5B and C). H&E staining also demonstrated that control xenograft tumors demonstrated very little necrosis on cross section

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of the tumor (Fig 5C) in contrast to the smaller Wyeth-1 treated xenograft tumors, where areas of necrosis were seen (Fig 5C).

Phosphorylated $cPLA_2$-α is increased in human PC

To determine if $cPLA_2$-α was a potential target in human PC, we evaluated the expression of p-$cPLA_2$-α in human PC specimens by immunohistochemistry. As shown in Fig. 6A, prostatic peripheral zone obtained from organ donors without PC demonstrated weak p-$cPLA_2$-α staining of the normal epithelial compartment of prostatic acini (n=7). PC tissue from radical prostatectomy (Fig. 6B) and TURP specimens (Fig. 6C) demonstrated slightly higher intensity levels of p-$cPLA_2$-α immunostaining compared to normal. Analysis of the percentage of cells with low or high intensity staining between normal and cancer specimens was not significantly different though (p=0.20). The staining was predominantly localized to the nucleus but some slides showed weak cytoplasmic staining, consistent with other reported patterns of p-$cPLA_2$-α staining (19).

To investigate whether development of hormone refractory is associated with changes in p-$cPLA_2$-α, we compared the immunostaining of p-$cPLA_2$-α in androgen sensitive PC obtained by TURP (Fig. 6C) with the same patient samples once the patient had reached hormone refractory status and required a repeat TURP (Fig. 6D). In 3 of 7 paired samples there was a clear further increase in p-$cPLA_2$-α staining intensity in the AI disease status. Illustrated is one such pair (Fig. 6C and D). For the remaining 4, the staining intensity did not change appreciably with progression of disease. The percentage of cancer cells with low or high intensity staining was significantly higher in hormone refractory status (p=0.03). We were unable to determine total $cPLA_2$-α staining in tissue specimens using antibodies from Cell Signaling and Santa Cruz for total $cPLA_2$-α as both displayed poor staining immunohistochemically. Figures 6E and F demonstrate little background staining on the isotype and method control slides.

Discussion

The AA pathway is highly active in PC (2). PGE2, one of the major eicosanoid end-products from the COX arm of the AA pathway, results in increased proliferation (20) and decreased apoptosis (21) in PC cell lines. Inhibition of COX-2 results in decreased PC cell and PC3 xenograft growth (18,22). LOXs, the other arm of the AA pathway, produce HETEs. 5-LOX, the enzyme responsible for 5-HETE production, is over expressed in PC(4), and inhibition of 5-LOX results in decreased PC cell proliferation and increased apoptosis (23). 12-LOX is also over expressed in PC (24) and its product, 12-HETE has been shown to increase cancer cell adhesion, migration and angiogenesis (25). The high activity of both COX and LOX arms of the AA pathway, as well as loss of the natural inhibitor to $cPLA_2$-α (13) led us to hypothesize that $cPLA_2$-α would be a potential target of the treatment of PC, as inhibition would block a number of very active downstream pathways.

All the human PC cell lines tested expressed $cPLA_2$-α (Fig. 1). The androgen sensitive cell line, LNCaP, expressed considerably less $cPLA_2$-α mRNA and protein compared to the androgen insensitive DU145 and PC3. A proxy for $cPLA_2$-α activity, p-$cPLA_2$-α, were correlated with the total $cPLA_2$-α levels.

Increased expression of $cPLA_2$-α in PC cell lines is an interesting phenomenon with a currently unknown mechanism. $cPLA_2$-α is part of the inflammatory cascade and can be induced by TNF-α (26), IL-1 (27) and NF-kB (28,29). The NF-kB pathway has been shown to be more active in androgen insensitive PC cells compared to androgen sensitive PC cell lines, and may be a potential mechanism of $cPLA_2$-α upregulation (30). In addition, Huges-Fulford et al. have shown that AA added exogenously to PC3 cells, can upregulate the expression of $cPLA_2$-α, via the production of PGE_2 (28). This is of great importance as dietary intake of fat has been

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correlated with increased incidence and mortality (31) as well as PC growth (32). Based on this, Hughes-Fulford et.al. have hypothesized that there is a potential for prevention or treatment by dietary reduction of fatty acids or drug inhibition of $cPLA_2$-α or COX (28).

There are several potential mechanisms by which $cPLA_2$-α activity may be aberrantly regulated in PC cells. First, loss of inhibitory regulators, Annexin I and Annexin II occurs in PC (33) (13). Second, increased MAPK activity observed in PC (34) could be responsible for the phosphorylation-activation of $cPLA_2$-α (35). Third, $sPLA_2$-IIA which is overexpressed in human PC (14) could contribute to the phosphorylation of $cPLA_2$-α. In human kidney mesangial cells, $cPLA_2$-α has been shown to be activated by $sPLA_2$-IIA (36) and in a human astrocytoma cell line, $sPLA_2$-IIA has been shown to bind to $sPLA_2$-IIA cell surface receptors, which then stimulate the MAPK pathway (37). The activated MAPK pathway in turn phosphorylates $cPLA_2$-α, leading to its activation and AA production (37,38).

To determine if $cPLA_2$-α represented a potential target for the treatment of PC, we initially silenced $cPLA_2$-α mRNA and found a significant reduction in PC growth (Fig 2). We then treated the PC cells with a specific inhibitor of $cPLA_2$-α (Wyeth-1) and demonstrated a dose dependent inhibition of PC cell growth. PC3 exhibited more resistance to the effects of Wyeth-1, despite expressing much more $cPLA_2$-α. This may reflect the more aggressive nature of this cell line which may harbor more active survival pathways. In order to confirm that the effects of Wyeth-1 were not the result of $cPLA_2$-α independent effects, we tested the PC cells with another specific $cPLA_2$-α inhibitor pyrrophenone (39) and observed similar results. We also demonstrated that Wyeth-1 and $cPLA_2$-α gene silencing effectively inhibits the $cPLA_2$-α activity in PC cells (Fig 2D).

To understand the mechanism of proliferation inhibition (Figure 3A), we elected to study pathways known be involved in PC. Akt, also known as protein kinase B, is activated by phosphorylation on the membrane. Once phosphorylated it travels to the cytosol and phosphorylates a variety of target proteins leading to an increase in cell proliferation and survival. Consistent with other reports (40), we have shown that Akt is strongly phosphorylated in PC cells, and that Wyeth-1 treatment results in reduced p-Akt (Figure 4). This mechanism may be mediated by $cPLA_2$-α facilitated eicosanoid production, as studies of vascular smooth muscle cells show that either AA, 5(S)-, 12(S)-, 15(S)-, or 20-HETE addition will increase Akt phosphorylation. PGE_2 may not be the mediator of Wyeth-1 effect, as inhibition of Akt phosphorylation caused by celecoxib treatment has been shown to be independent of COX-2 in PC cells (41). A decrease in p-Akt may be a mechanism by which proliferation is decreased and apoptosis increased after $cPLA_2$-α inhibition.

We have previously shown that celecoxib will decrease expression of cyclin D1 (one of the downstream effectors of Akt pathway) and result in an accumulation of cells in G_0/G_1 with a corresponding decrease in S phase in LNCaP and PC3 cells (18). Consistent with those findings, we found that cyclin D1 expression decreased with Wyeth-1 treatment in LNCaP cells. This also resulted in an accumulation of cells in G_0/G_1 and a corresponding decrease in S phase on flow cytometry (data not shown). It is not yet certain though that this is a result of decreased PGE_2 production as we have previously shown that celecoxib's effect on cyclin D1 could be independent of COX inhibition (18).

As the ultimate aim is to find new treatments of men with high volume hormone refractory PC, we chose to use the PC3 xenograft model, and started treatment at a high tumor volume ($200m^3$) to mimic the clinical disease. Despite the large initial sizes of the tumors, Wyeth-1 significantly reduced the rate of growth of tumors by approximately 33% compared to vehicle treated mice (Figure 5). In addition, not only were the Wyeth-1 treated tumors smaller, but substantial volumes of the tumors were necrotic, unlike the larger control tumors, suggesting

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actual viable cancer volume would be even smaller. The cause of the necrosis is not clear but may be due to an inhibition of angiogenesis by $cPLA_2$-α inhibition, as has been shown by COX-2 inhibitors (18,22); this will be an area of further study in our laboratory. Based on our *in vitro* findings, levels of cyclin D1 were measured in xenograft tumor samples. Similar to what was observed in cultured LNCaP cells, Wyeth-1caused a reduction in cells staining for cyclin D1 in PC3 xenografts, a finding which has been observed in PC3 xenografts treated with celecoxib (18).

It was important to determine whether the target $cPLA_2$-α was present and active (phosphorylated) in human PC as this is ultimately where the therapy will be used. In addition we wanted to confirm whether men with hormone refractory PC gained increased expression of p-$cPLA_2$-α compared to the androgen sensitive disease. We are unable to report on expression levels of total $cPLA_2$-α as the immunohistochemical staining of total $cPLA_2$-α, with all available commercial antibodies, was of poor quality in human prostate tissue. We have shown that phosphorylation of $cPLA_2$-α is present in PC tissue (Figure 6) and levels may not be much higher in cancer compared to normal tissue; but the levels increased significantly in 3/7 of the men who developed hormone refractory PC ($p=0.03$). Although this study was small in numbers, the expression of p-$cPLA_2$-α was determined in PC tissue prior to androgen ablation, and the tissues from the same men were examined when they reached hormone refractory status, thus each man acted as his own control. Collectively, the immunohistochemical analyses show that the target enzyme is present and could be higher in some men reaching hormone refractory state.

Although we have shown that $cPLA_2$-α inhibition will decrease $cPLA_2$-α activity and PC cell growth, we have not investigated another effect of $cPLA_2$-α inhibition such as reduction in platelet activating factor (PAF). In cancer cells and haematopoetic cells, PAF can be produced from the remaining lysophosphoplipid after AA cleavage by $cPLA_2$-α. PAF has been shown to stimulate NF-kB as well as angiogenesis (42), and has been shown to be oncogenic in a number of cancers (42–44). Its role in PC has not been well studied.

A number of other cancers have also been noted to demonstrate elevated levels of cPLA2 with the potential for therapeutic targeting (12). However, in colon cancer the reported actions of $cPLA_2$-α have been controversial. Studies have found that $cPLA_2$-α expression is increased in colorectal cancer (45,46), however, in the Apc^{min} mouse model of colon tumorigenesis, the deletion of $cPLA_2$-α did not result in a change in the number of colon tumors observed (47). Similarly, in azoxymethane (AOM) induced mouse colon cancer, $cPLA_2$-α was observed to decrease (48) and when $cPLA_2$-α was deleted in this mouse model, an increase in colon tumors was observed (49). It has been suggested that a loss of AA derived ceramide, a pro-apoptotic mediator, may result in increased tumor formation in this disease.

Prior to human phase I studies, further research needs to be performed in the area of $cPLA_2$-α inhibition and PC. Initially, as the class of specific $cPLA_2$-α inhibitors is quite new, pharmaco-kinetic studies need to be performed so proper dosing schedules can be calculated. Further studies in animal models need to look at the effect of varying doses and different stages of disease, such as chemoprevention.

In summary, total and phosphorylated $cPLA_2$-α are present in PC cell lines. These levels are increased further in androgen insensitive PC cell lines. $cPLA_2$-α plays a role in cancer cell proliferation and apoptosis, and its inhibition or knock-down results in decreased cell growth. The drug Wyeth-1, appears to act specifically through the $cPLA_2$-α enzyme and not through an off-target effect. Some of these effects can be explained by Wyeth-1 mediated inactivation of Akt and reductions in cyclin D1. These findings are replicated *in-vivo*, with decreased PC3 xenograft growth with Wyeth-1 treatment and resulting decrease in cyclin D1 staining of

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remaining cancer cells. cPLA$_2$-α is a potentially effective target for therapy of prostate cancer, and therefore warrants further investigation.

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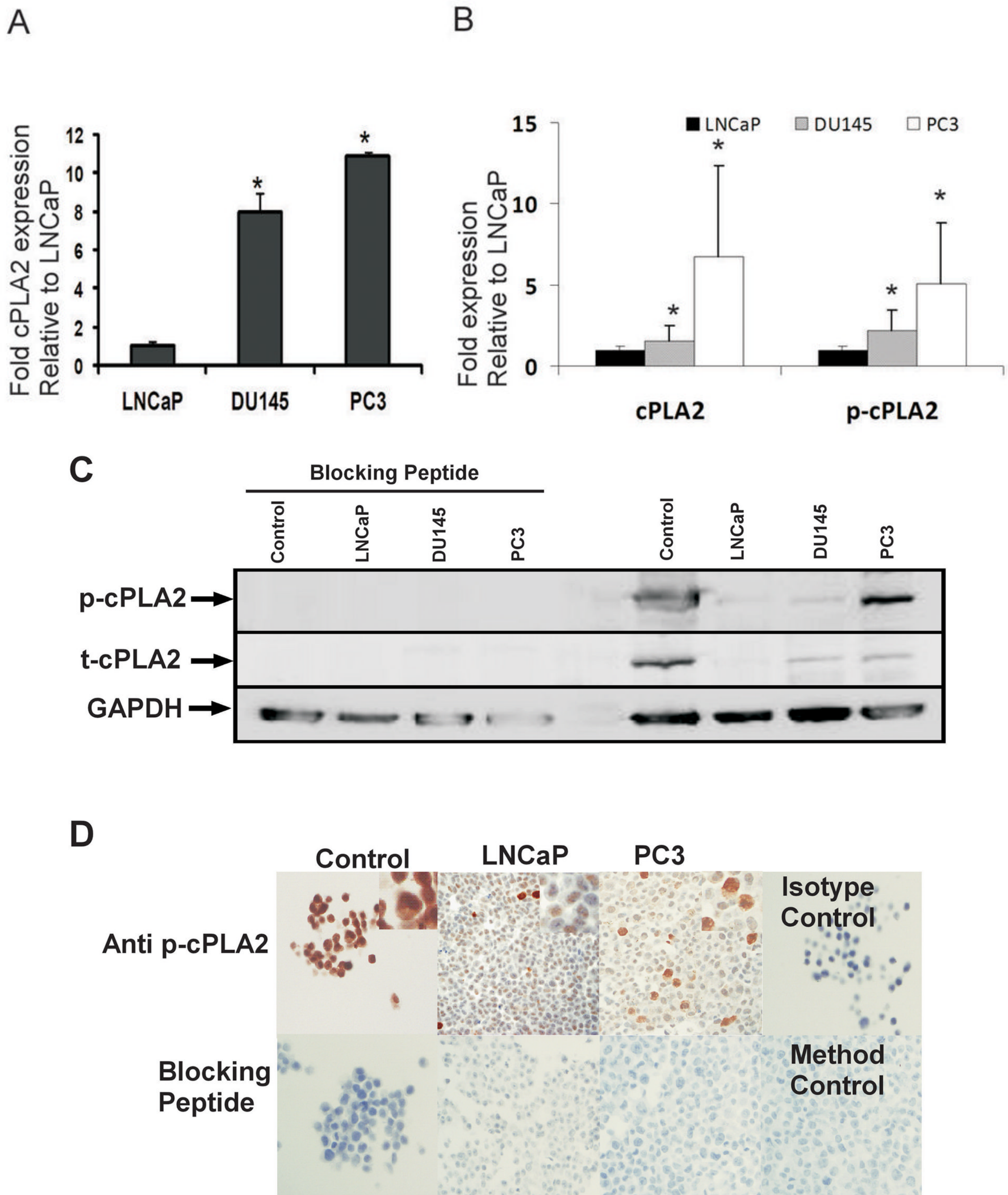

Figure 1. cPLA$_2$-α is present and levels are higher in androgen insensitive prostate cancer cell lines
(A) Quantitative real time RT-PCR from the cell lines (Mean±S.D.) demonstrates cPLA$_2$-α expression in PC3 > DU145 > LNCaP (*$p<0.05$). (B) Staining on immunoblot of cell lines for total and p-cPLA$_2$-α was quantitated by densitometric analysis (Mean±SD) of protein expression on 3 separate immunoblots (*$p<0.05$ compared to LNCaP). (C) Protein levels of total and phosphor-cPLA$_2$-α on immunoblot. Staining on immunoblot was specific for the

target protein as bands were absent when the primary antibody was preincubated with the specific blocking peptide.(D) Immunocytochemistry of control HeLa cells show strong staining for p-cPLA$_2$-α which is abolished with preincubation with blocking peptide. LNCaP and PC3 also show strong staining for p-cPLA$_2$-α. There is little staining with isotype control or method control in either cells line (PC3 shown). All photographs taken at X40, inserts magnified a further X4.

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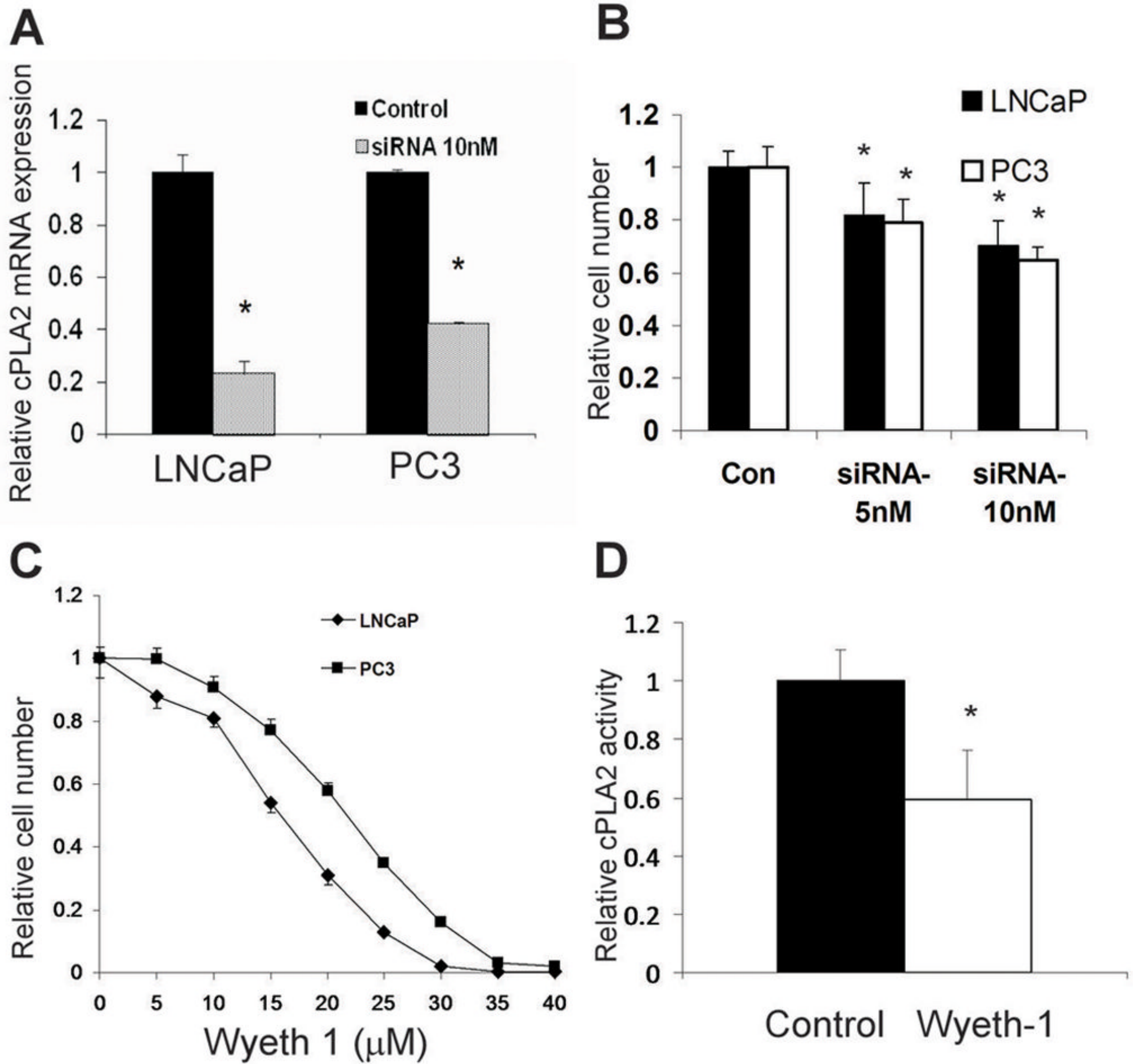

Figure 2. cPLA$_2$-α gene silencing or inhibition results in decreased prostate cancer cell growth
(A) cPLA$_2$-α mRNA silencing was achieved with siRNA treatment for 24 hours. Quantitative RT-PCR for cPLA$_2$-α shown effective knock-down in LNCaP and PC3 cells. (B) cPLA$_2$-α mRNA silencing after 24 hours treatment, results in significant reductions in cell number by MTS assay, with increasing concentrations of siRNA in LNCaP and PC3 cell lines (±S.D.). (C) Inhibition of cell growth by Wyeth-1. Prostate cancer cells were plated onto 96 well plates and treated for 72 hours with increasing concentrations of Wyeth-1. Cell growth was measured by MTS assay, and results expressed relative to control cells (±S.D.). (D) Inhibition of cPLA2-α activity by Wyeth-1. Cell lysates of PC3 cells treated with DMSO (control) or Wyeth-1 IC_{50} were measured for cPLA$_2$-α activity. Mean activity relative to control were expressed (±S.D.). * $p<0.05$ when compared to control.

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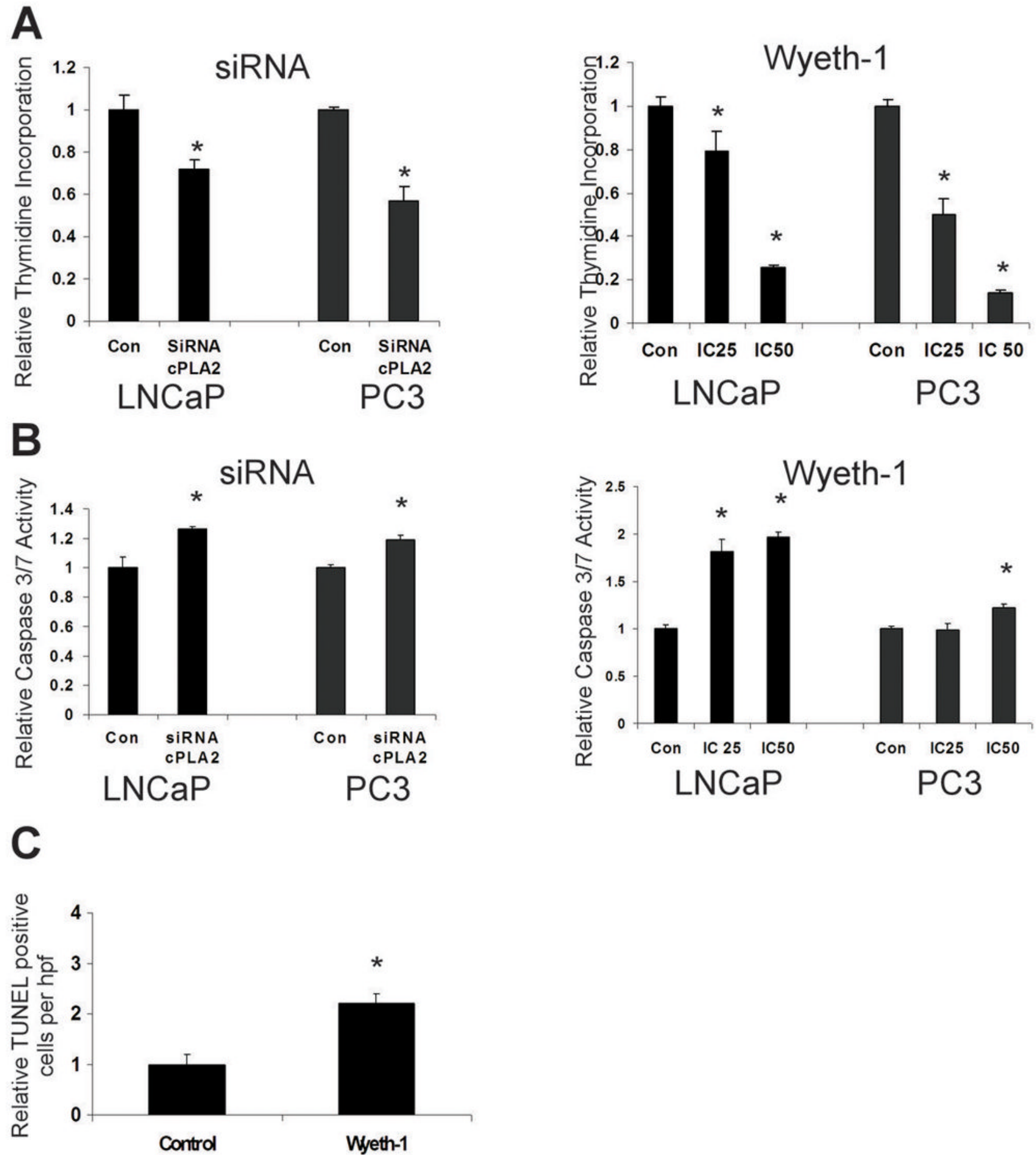

Figure 3. cPLA$_2$-α mRNA silencing or inhibition results in decreased proliferation and increased apoptosis

(A) Prostate cancer cell lines were plated in 96 well plates and treated with siRNA for 24 hours or Wyeth-1 at concentrations of IC_{25} and IC_{50} for 72 hours. ^{3}H-thymidine was added in the final 6 hours. ^{3}H-thymidine incorporation was measured at the end of treatment to quantify DNA synthesis. (B) Prostate cancer cells were treated as in (A) and apoptosis measured by a caspase 3/7 assay. (C) Apoptosis as measured by TUNEL. LNCaP cells were treated with IC_{50} Wyeth-1 for 72 hours and the cells then fixed and assayed by the TUNEL method. Percentage of stained cells were counted and expressed as the average of ten high power fields

(×40). All results are plotted relative to control cells (±S.D.).* $p<0.05$ when compared to vehicle treated control for the individual cell lines.

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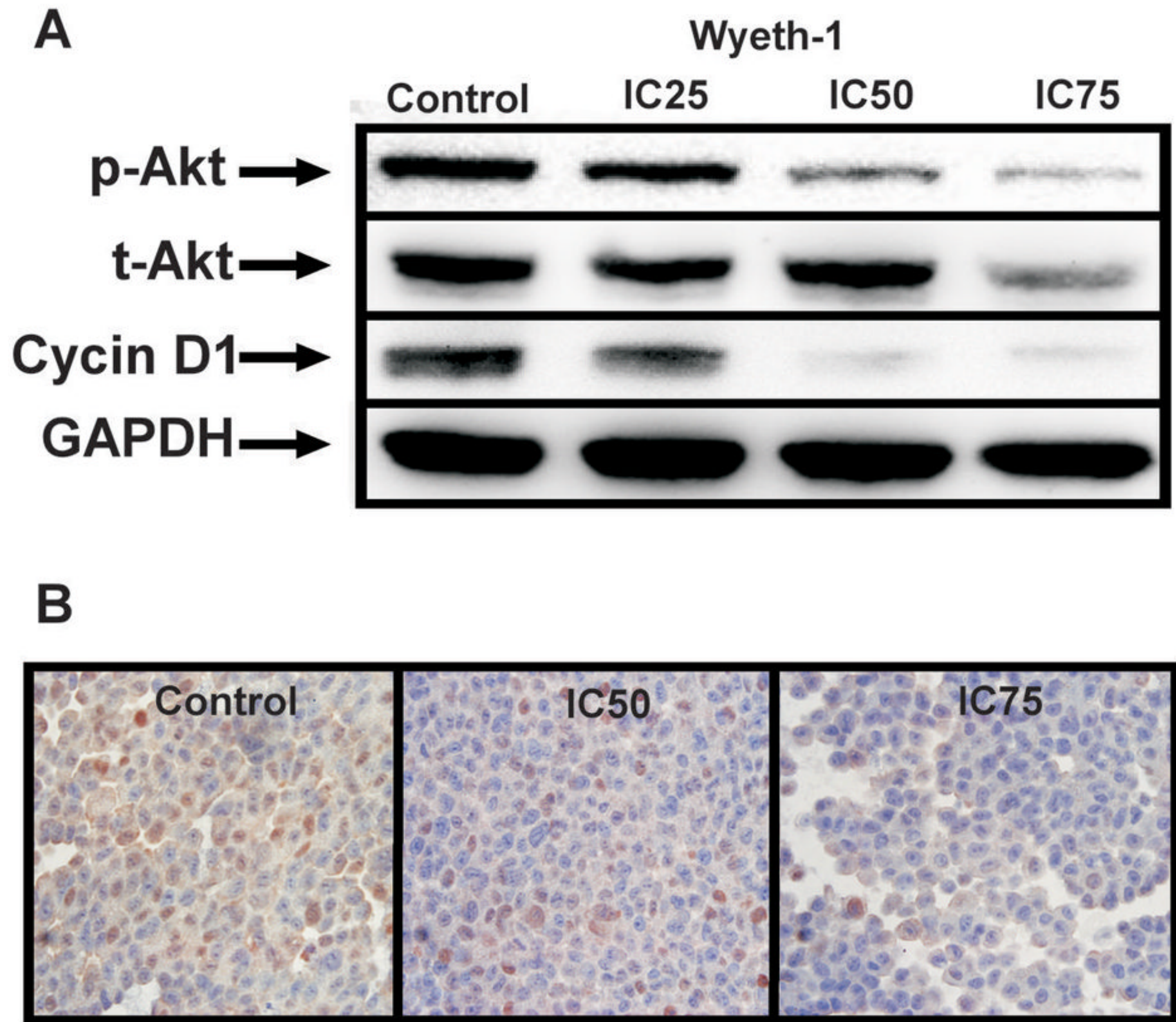

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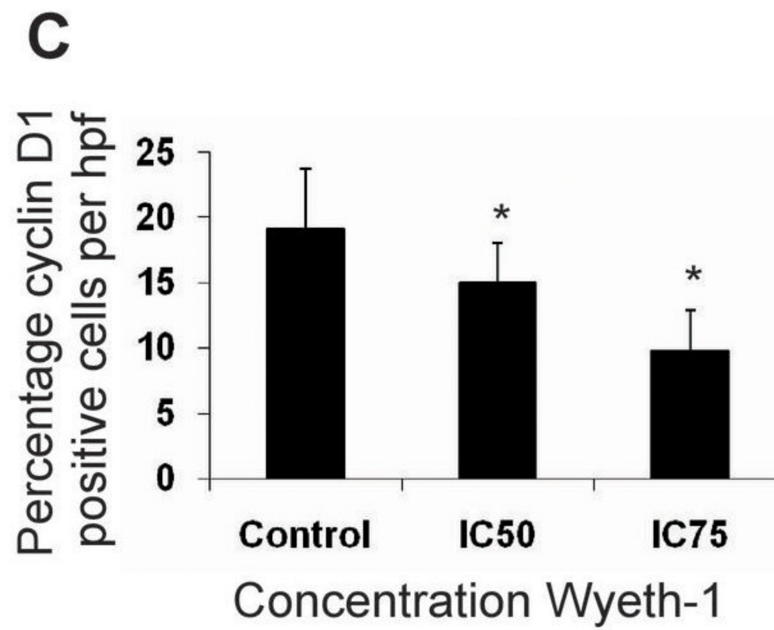

Figure 4. cPLA$_2$-α inhibition reduces cyclin D1 and Akt expression in prostate cancer cells
(A) LNCaP cells were treated with vehicle (Control) or increasing concentrations of Wyeth-1 for 24 hours. Cellular lysate protein (50 μg/lane) was analyzed by Western blot. Reduced levels of p-Akt and cyclin D1 were detected at concentrations of IC_{50}. Reduced levels of t-Akt were detected at IC_{75}. (B) LNCaP cell was treated with increasing concentrations of Wyeth-1. The cell was then paraffin fixed and stained for cyclin D1. LNCaP exhibited reduced numbers of cells positive for Cyclin D1 with increasing Wyeth-1 concentrations. (C) The percentage of Cyclin D1 positive cells with increasing concentration of Wyeth-1. (*$p<0.05$)

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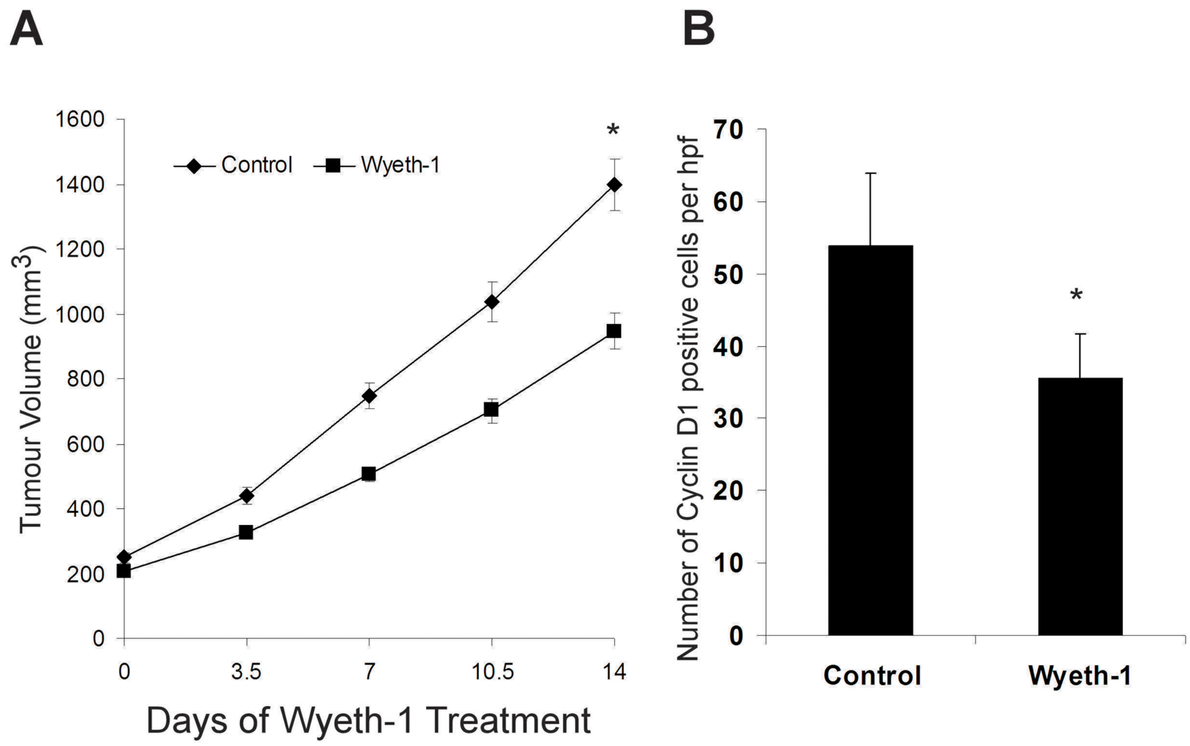

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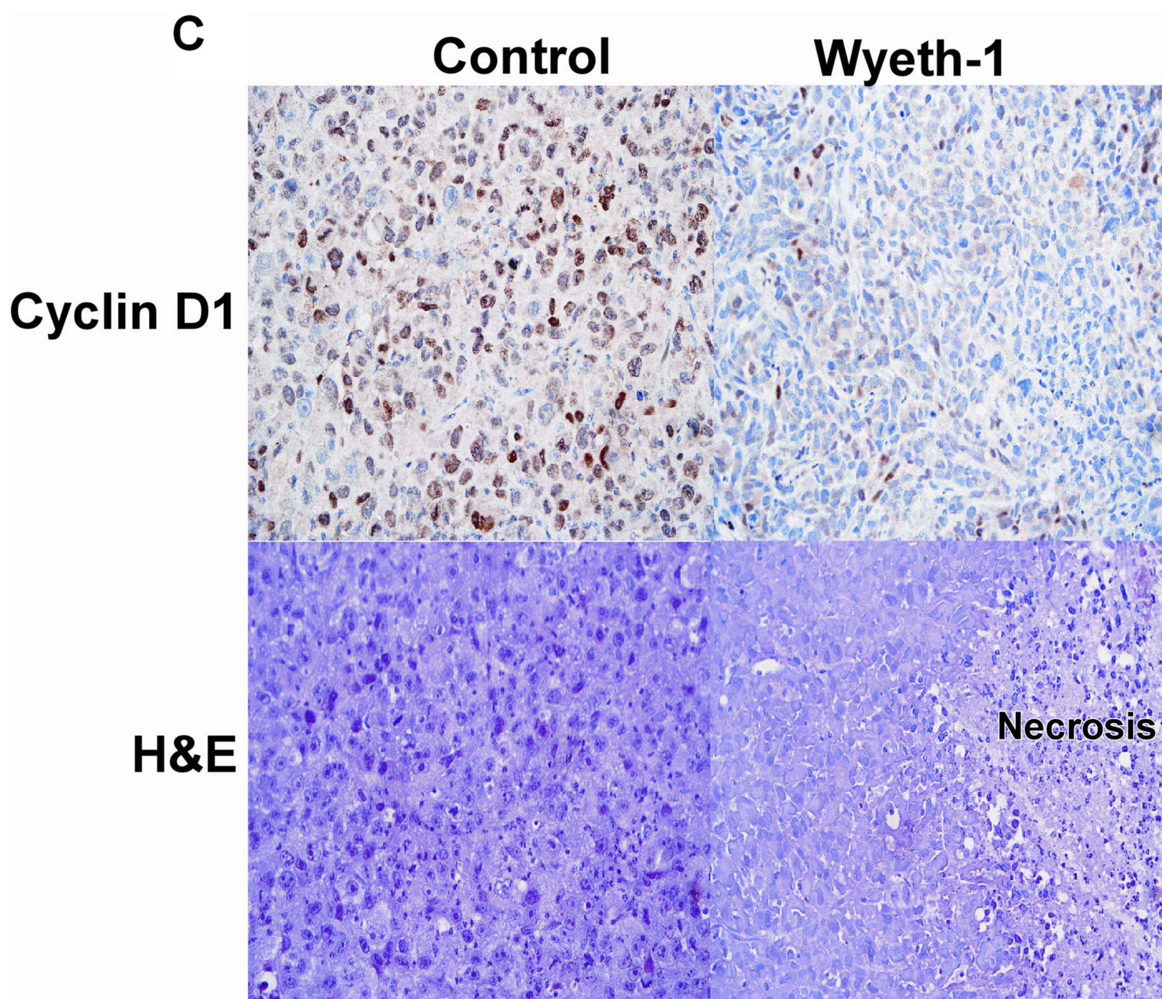

Figure 5. c-PLA$_2$-α inhibition impedes the growth of PC3 xenografts

(A) PC3 cells were inoculated into the flanks of nude mice. When established xenograft tumors had reached 200m^3 in size, the mice were randomized to control (vehicle) or Wyeth-1 treatment, given intraperitoneally daily at a dose of 10 mg/kg (8 mice/group). Statistically significant inhibition of tumor growth was achieved in the Wyeth-1 treated mice compared to the control animals (Mean±SEM, $*p < 0.05$). (B) At the time of sacrifice, tumors were harvested and fixed in formaldehyde and paraffin embedded. Control tumors demonstrated significantly more cyclin D1 positive cells than did Wyeth-1 treated cells. ($*p<0.05$). (C) The tumors in control and Wyeth-1 treated mice stained for cyclin D1 and H&E. Tumors from Wyeth-1 treated mice, stained significantly less for cyclin D1 than control. H&E staining demonstrated very little necrosis in control tumors but striking necrosis in the Wyeth-1 treated tumors.

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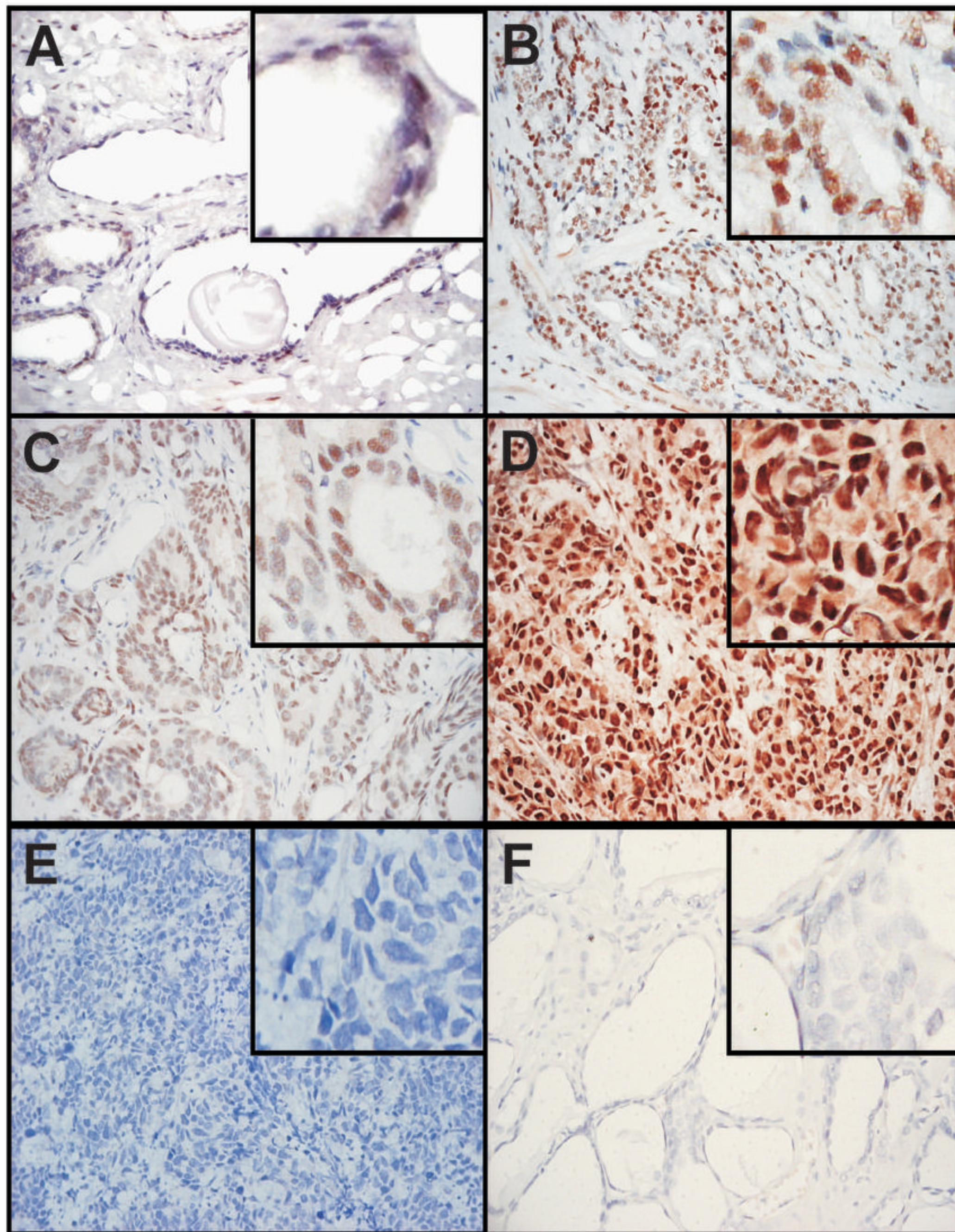

Figure 6.

Activity of cPLA$_2$-α in prostate tissues by immunohistochemical staining of phosphorylated cPLA$_2$-α. (A) Normal peripheral zone prostate samples exhibited low levels of phosphorylation of cPLA$_2$-α in epithelial elements, and no staining of stromal tissue. (B) Localized prostate cancer samples from radical prostatectomy exhibited slightly stronger cPLA$_2$-α phosphorylation. (C) Patients who initially underwent TURP and diagnosed with prostate cancer show the same staining pattern as in (B). (D) After androgen deprivation, some patients who became hormone refractory exhibited increases in phosphorylated cPLA$_2$-f staining compared to before androgen deprivation (3/7 samples). (E) Isotype control and (F) method

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control show no background staining. All photographs at X40 magnification. The inserts were magnified (X200) to view nuclear staining.