Cytosolic Phospholipase A$_2$-α: A Potential Therapeutic Target for Prostate Cancer
Manish I. Patel,$^{1,4}$ Jaskirat Singh,$^{2,5}$ Marzieh Niknami,$^{2,6}$ Caroline Kurek,$^{2,5}$ Mu Yao,$^{2,5}$ Sasa Lu,$^{2,5}$ Fiona Maclean,$^7$ Nicholas J.C. King,$^3$ Michael H. Gelb,$^{10}$ Kieran F. Scott,$^8$ Pamela J. Russell,$^9$ John Boulas,$^6$ and Qihan Dong$^{2,5,6}$

Abstract

**Purpose:** Cytosolic phospholipase A$_2$-α (cPLA$_2$-α) provides intracellular arachidonic acid to supply both cyclooxygenase and lipoxygenase pathways. We aim to determine the expression and activation of cPLA$_2$-α in prostate cancer cell lines and tissue and the effect of targeting cPLA$_2$-α in vitro and in vivo.

**Experimental Design:** The expression of cPLA$_2$-α was determined in prostate cancer cells by reverse transcription-PCR, Western blot, and immunocytochemistry. Growth inhibition, apoptosis, and cPLA$_2$-α activity were determined after inhibition with cPLA$_2$-α small interfering RNA or inhibitor (Wyeth-1). Cytosolic PLA$_2$-α inhibitor or vehicle was also administered to prostate cancer xenograft mouse models. Finally, the expression of phosphorylated cPLA$_2$-α was determined by immunohistochemistry in human normal, androgen-sensitive and androgen-insensitive prostate cancer specimens.

**Results:** cPLA$_2$-α is present in all prostate cancer cells lines, but increased in androgen-insensitive cells. Inhibition with small interfering RNA or Wyeth-1 results in significant reductions in prostate cancer cell numbers, as a result of reduced proliferation as well as increased apoptosis, and this was also associated with a reduction in cPLA$_2$-α activity. Expression of cyclin D1 and phosphorylation of Akt were also observed to decrease. Wyeth-1 inhibited PC3 xenograft growth by ~33% and again, also reduced cyclin D1. Immunohistochemistry of human prostate tissue revealed that phosphorylated cPLA$_2$-α is increased when hormone refractory is reached.

**Conclusions:** Expression and activation of cPLA$_2$-α are increased in the androgen-insensitive cancer cell line and tissue. Inhibition of cPLA$_2$-α results in cells and xenograft tumor growth inhibition and serves as a potentially effective therapy for hormone refractory prostate cancer.

Previous studies have shown 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 pathways, contribute to cancer progression by promoting cell proliferation, motility, invasion, and angiogenesis (8–10). Eicosanoids are synthesized from intracellular arachidonic acid, which is released from membrane phospholipids by the action of phospholipase A$_2$ (PLA$_2$; refs. 11, 12). Of the known mammalian PLA$_2$ enzymes, cytosolic PLA$_2$-α (cPLA$_2$-α), an 85-kDa protein, is the predominant source of intracellular arachidonic acid for eicosanoid production.

We have previously shown that expression of Annexin II, a calcium-dependent phospholipid-binding protein and inhibitor regulator of cPLA$_2$-α, is lost in human prostate cancer (13). We have also reported that secretory PLA$_2$-IIA (sPLA$_2$-IIA), one of the secretory PLA$_2$s, is overexpressed in prostate cancer cells including those remaining after androgen ablation therapy (14). The growth-promoting effect of sPLA$_2$-IIA seems to be via cPLA$_2$-α (14). These data have prompted us to investigate the role of cPLA$_2$-α in prostate cancer growth, and to determine if it is a feasible target for treatment of the hormone refractory stage of prostate cancer.

In this study, we show that cPLA$_2$-α is present in prostate cancer cell lines and increases in androgen-insensitive cells. We also show that small interfering RNA (siRNA) knockdown or specific inhibition of cPLA$_2$-α results in decreased prostate cancer 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...
Cytosolic Phospholipase A2α and Prostate Cancer

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 cytosolic phospholipase A2α (cPLA2-α) is present in prostate cancer cell lines and showing that inhibition of cPLA2-α will suppress cancer cell growth in vitro and in vivo raise cPLA2-α as a potential therapeutic target. The identification of cPLA2-α and its increase in hormone refractory disease confirms its presence and relevance to human prostate cancer. Our results give evidence that cPLA2-α is a potentially effective therapeutic target and support the need for further studies in the context of clinical trials validating cPLA2-α–targeted therapy for men with prostate cancer.

Akt phosphorylation and a decrease in cyclin D1 expression. We also show that, as seen in vitro, in vivo, inhibition of cPLA2-α results in a significant decrease in xenograft tumor growth. Finally, we show that the phosphorylated cPLA2-α (p-cPLA2-α) is present in human prostate tissues and its levels increase in hormone refractory prostate cancer.

Materials and Methods

Reagents and cell lines. The LNCaP-FGC (LNCaP), DU1145, and PC3 human prostate cancer cell lines were purchased from the American Type Culture Collection. Cells were maintained in RPMI 1640 (Sigma-Aldrich), supplemented with 10% fetal bovine serum (ICN Biomedicals) with all cell cultures at 37°C in a humidified environment of 5% CO2. The passage numbers of cells described in this article were between 30 and 45 for LNCaP, 65 and 80 for DU1145, and 25 and 40 for PC3. The cPLA2-α inhibitor Wyeth-1 was prepared as previously described (ref. 15; U.S. Patent 6,797,708, Sanmar Chemical Company). Pyrrolidine-2 (also known as pyrophenone) was prepared as previously described (16). Both inhibitors were reconstituted in DMSO. All antibodies [p (Ser457)-Akt, total Akt, GAPDH, and α-tubulin] were obtained from Cell Signaling except cyclin D1 (Sigma).

Reverse transcription-PCR. Levels of cPLA2-α mRNA were measured by end-point and real-time reverse transcription-PCR (RT-PCR). After cell treatments, total RNA was isolated using Trizol reagent (Sigma-Aldrich) according to the manufacturer’s instructions. The first-strand complementary DNA was synthesized from 2 μg of total RNA using a combination of random hexamers and oligo-dT as described previously (13). Endpoint primers were designed based on the human cPLA2-α mRNA (NM_024420), forward 5’-ACAGTGGGCCCTACATTTAACCT, reverse 5’-CTTCCCGATCAAACATAAGG. GAPDH was normalized to protein concentration. Protein concentration was quantified using Bio-Rad Protein Assay (Bio-Rad). Cell lysates (60 μg) were separated on 8% SDS-PAGE and then transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat 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). Blocking peptide (total and p-cPLA2-α; Cell Signaling) was added at a concentration of 2 μg/mL to the primary antibody, before being added to the blot. NIH-3T3 and HeLa cells were used as positive controls for densitometric scanning. Blocking peptide was added to the primary antibody, before being added to the blot. NIH-3T3 and HeLa cells were used as positive controls for densitometric scanning.

Western blotting. Cell lysates were prepared using lysis buffer (20 mMol/L Tris, 150 mMol/L NaCl, 1 mMol/L EDTA, 1 mMol/L EGTA, 1% Triton X-100, 2.5 mMol/L sodium PPI, 1 mMol/L β-glycerophosphate), supplemented with 1:50 dilution of protease inhibitor cocktail (Sigma-Aldrich). Protein concentration was quantified using Bio-Rad Protein Assay (Bio-Rad). Cell lysates (60 μg) were separated on 8% SDS-PAGE and then transferred onto a nitrocellulose membrane. The membranes were blocked with 5% nonfat 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). Blocking peptide (total and p-cPLA2-α; Cell Signaling) was added at a concentration of 2 μg/mL to the primary antibody, before being added to the blot. NIH-3T3 and HeLa cells were used as positive controls for densitometric scanning. Densitometric scanning was done to quantify band intensities.

Immunohistochemistry. Prostate cancer 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 prostate cancer specimens from the peripheral zone of the prostate were obtained from men who had radical prostatectomy as treatment for their prostate cancer (n = 12). Paired prostate cancer specimens before and after reaching hormone refractory stage were also obtained (n = 7 pairs). These men initially presented with prostate cancer, and tissue was obtained when they underwent transurethral resection of prostate. After the cancers progressed and androgen ablation therapy failed, these men required repeat transurethral resection of prostate. 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-cPLA2-α was added at a concentration of 2 μg/mL to the primary antibody, before addition to the slide. This was followed by the application of biotinylated secondary antibody and Vectastain ABC kit (Vector) and staining revealed using 3,3′-diaminobenzidine (DakoCytomation). The staining intensity was graded as low or high, and the percentage of cells stained at each intensity was recorded.

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 AQueous One Solution Cell Proliferation Assay; Promega) as described previously (17). Values are expressed relative to untreated controls. Inhibitory concentrations IC25, IC50, and IC75 were calculated by plotting graphs of cell growth measured by MTS versus concentration of drug.

Cytosolic PLA2 activity assay. Arachidonoyl thioc-PC was used as the substrate to measure cPLA2 activity in vitro. The cPLA2 Assay Kit was purchased from Cayman Chemical and procedures were done according to the manufacturer’s instructions. After treatment of cells with Wyeth-1 or siRNA, the cells were homogenized and treated with 7.5 μmol/L bromoelol lactone, a specific independent PLA2 inhibitor. We did not add a cPLA2 inhibitor as it is barely detectable in the PC2 cell line. Twenty microliters of cell lysate were finally subjected to the assay, and the absorbance value was measured at 414 nm and normalized to protein concentration.

www.aacrjournals.org
8071
DNA synthesis assay. Incorporation of $[^3]H$thymidine was used to measure DNA synthesis. Prostate cancer 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) to the equivalence of 1 µCi/100 µL was added for the final 6 h. At the end of treatment, the cells were trypsinized and cellular DNA was harvested onto filter mats using a cell harvester (Perkin-Elmer). Following drying, scintillation fluid was added and radioactivity read in a β 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 fluorometrically activated caspase-3 and caspase-7 with ApoOne caspase-3/7 Homogenous Assay (Promega) and done as per the manufacturer's instructions. Prostate cancer cells were plated onto white 96-well plates (Greiner Bio-One) and after 48 h were treated with siRNA or inhibitors. The caspase substrate and buffer solution were added at the end of treatment and allowed to incubate for 18 h before detection with a Fluoroskan Ascent fluorometer (Thermo Labsystems) at excitation wavelength 485 nm and emission wavelength 515 nm. All relative fluorescence units were normalized to cell numbers.

Terminal deoxynucleotidyl transferase-mediated nick-end labeling. The Promega DeadEnd Colorimetric TUNEL System was used to measure the effects of inhibitor on late prostate cancer cell apoptosis and done according to the manufacturer's instructions. Cell blocks were dewaxed, antigen retrieved, and covered with equilibration slides at 37°C. The reaction was terminated by immersing slides in 2× SSC solution and biotinylated nucleotides detected using 3,3′-diaminobenzidine. Positive staining was counted and expressed as the average of 10 high-power fields (>40).

PC3 xenografts. PC3 cells (0.5 × 10⁷–1 × 10⁸) were implanted s.c. in the flanks of 6- to 7-wk athymic male nude mice. Mice were randomly distributed into two groups once the tumor size was between 150 and 200 mm³ (randomly distributed into two groups once the tumor size was between 150 and 200 mm³). Mice were weighed to 1,500 mm³ (the allowable limit specified by the institution's equation). The IC25, IC50, and IC75 for Wyeth-1 treatment of DU145 and PC3 (P < 0.05) as did the PC3 cell line consistent with the total cPLA2-α expression. We believe antibody binding was specific, as binding was depleted by the addition of excess peptide (Fig. 1C). Further experiments were done only on LNCaP (androgen sensitive) and PC3 (androgen insensitive) prostate cancer cell lines.

To visualize the proportions of cells with active cPLA2-α, cell blocks of LNCaP and PC3 cells were stained by immuonochemistry with anti-p-cPLA2-α antibody (Fig. 1D). Phosphorylated cPLA2-α stained cytoplasm, perinuclear as well as intranuclear regions. Consistent with the immunoblot findings, LNCaP showed some cells with weak p-cPLA2-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.

Knockdown or inhibition of cPLA2-α results in inhibition of prostate cancer cell growth. To determine if cPLA2-α enzymatic activity is involved in prostate cancer growth, we examined prostate cancer cell growth in vitro, after cPLA2-α mRNA was silenced with siRNA. Figure 2A shows that siRNA at 10 nmol/L results in efficient knockdown of cPLA2-α mRNA in LNCaP and PC3 cells at 48 hours, with levels at ~30% to 40% compared with control cells transfected with nonmammalian siRNA. To determine cell growth, cells were treated with siRNA to cPLA2-α for 48 hours and cell number was estimated by the MTS assay. Results show that both LNCaP and PC3 cell numbers were significantly reduced compared with cells transfected with nonmammalian siRNA (Fig. 2B). At doses of 5 nmol/L and 10 nmol/L siRNA 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 knockdown of cPLA2-α mRNA resulted in significant reductions in cell growth in both cell lines, we aimed to determine if a newly available specific inhibitor of cPLA2-α named Wyeth-1 by Ni et al. (15) and also named Gplipladib could also reduce prostate cancer cell growth. This compound has been shown not to inhibit sPLA2.11 LNCaP and PC3 were treated with increasing concentrations of the inhibitor, and growth was measured after 72 hours treatment by MTS assay (Fig. 2C). LNCaP cells showed 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% (IC25), 50% (IC50), and 75% (IC75) reduction in cell number was calculated by constructing a line of best fit and applying the resulting equation. The IC25, IC50, and IC75 for Wyeth-1 treatment of LNCaP cells were 11.1, 15.9, and 21.0 µmol/L, respectively, and 15.1, 22.2, and 27.6 µmol/L for PC3, respectively. These

11M.H. Gelb, unpublished data.
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 cPLA2-α inhibitors, the experiment was repeated with pyrophenone-2, another specific cPLA2-α inhibitor. Pyrophenone also decreased LNCaP and PC3 cell growth in a dose-dependent manner identical to Wyeth-1 except that the inhibitory concentrations were lower (IC50 was 3.3 μmol/L for LNCaP and 8.8 μmol/L for PC3). The ability of two structurally different inhibitors, pyrophenone-2 and Wyeth-1, as well as cPLA2-α silencing, to block cell growth is evidence supporting an important role for cPLA2-α in prostate cancer cell growth.

To further confirm that the inhibitor Wyeth-1 inhibited cell growth by inhibiting cPLA2-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 independent PLA2 inhibitor to ensure all measured hydrolysis was a result of cPLA2 activity. Secretory PLA2 protein is barely detectable in PC3 cells by Western blot (data not shown). Figure 2D confirms that at IC50 Wyeth-1 results in a 41% reduction in cPLA2 activity in PC3 cells (P < 0.001). To verify that the reduction in PLA2 activity was due to cPLA2-α, not any other PLA2, we also showed a significant reduction in cPLA2-α activity (26%; P < 0.01) with knockdown of cPLA2-α mRNA using 10 nmol/L siRNA.

**Prostate cancer cell proliferation and apoptosis are regulated by cPLA2-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 [3H]thymidine incorporation in prostate cancer cell lines after cPLA2-α mRNA silencing with 10 nmol/L 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 hours after treatment with siRNA when compared with cells treated with nonmammalian siRNA. Wyeth-1 treatment at IC25 and IC50 also resulted in reduced LNCaP cell proliferation by 22% (P = 0.006) and 75% (P = 0.001), respectively. PC3 cells showed a 50% (P < 0.001) and 76% (P < 0.001) reduction in proliferation, respectively (Fig. 3A).

Early apoptosis was measured by activated caspase-3/7 assay. Cytosolic PLA2-α mRNA silencing with siRNA results in a 26% increase in apoptosis in LNCaP cells (P = 0.03) and a 19% increase in PC3 cells (P = 0.02; Fig. 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 (Fig. 3B). PC3 cells showed a lesser increase at IC_{25} and IC_{50} concentration (P = 0.02).

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 hours and then measured late apoptosis by TUNEL assay of the fixed cell block. Figure 3C shows that compared with 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 cPLA2-α are a result of proliferation reduction and apoptosis induction in both androgen-sensitive and -insensitive cell lines.

**Inhibition of cPLA2-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 phosphatidylinositol 3-kinase-Akt pathway that regulates cell growth and survival. We have previously shown that the treatment of prostate cancer cells with celecoxib, a selective COX-2 inhibitor, results in decreased expression of cyclin D1 (18) and also reduction of p-Akt.\(^2\) We aimed to determine if p-Akt or cyclin D1 were regulated by cPLA2-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 total Akt was also reduced, but only at concentrations of IC_{75}. Thus, a reduction in total 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 (Fig. 4A). To confirm this finding, we did immunocytochemistry for cyclin D1 on cell blocks of LNCaP cells that 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 (Fig. 4C).

**Inhibition of cPLA2-α results in PC3 xenograft growth retardation.** Based on the in vitro findings described above, it was important to investigate whether inhibition of cPLA2-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 200 mm\(^3\) (Fig. 5A). The control (vehicle-treated mice) showed very rapid xenograft tumor growth, and at 14 days of treatment all mice were sacrificed as they had reached the average size of 1,500 mm\(^3\). Comparatively, the Wyeth-1–treated mice showed 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 with 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 with control tumors (54%; P = 0.005; \(^{12}\) unpublished data.)
Fig. 5B and C). H&E staining also revealed that control xenograft tumors showed very little necrosis on cross section of the tumor (Fig. 5C) in contrast to the smaller Wyeth-1–treated xenograft tumors, in which areas of necrosis were seen (Fig. 5C).

Phosphorylated cPLA2-α is increased in human prostate cancer. To determine if cPLA2-α was a potential target in human prostate cancer, we evaluated the expression of p-cPLA2-α in human prostate cancer specimens by immunohistochemistry. As shown in Fig. 6A, prostatic peripheral zone obtained from organ donors without prostate cancer showed weak p-cPLA2-α staining of the normal epithelial compartment of prostatic acini (n = 7). Prostate cancer tissue from radical prostatectomy (Fig. 6B) and transurethral resection of prostate specimens (Fig. 6C) showed slightly higher intensity levels of p-cPLA2-α immunostaining compared with normal. Analysis of the percentage of cells with low or high intensity staining between normal and cancer specimens was not significantly different (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-cPLA2-α staining (19).

To investigate whether development of hormone refractory is associated with changes in p-cPLA2-α, we compared the immunostaining of p-cPLA2-α in androgen-sensitive prostate cancer obtained by transurethral resection of prostate (Fig. 6C) with the same patient samples once the patient had reached hormone refractory status and required a repeat transurethral resection of prostate (Fig. 6D). In three of seven paired samples there was a clear further increase in p-cPLA2-α staining intensity in the AI disease status. Illustrated in Fig. 6C and D is one such pair. For the remaining four samples, 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 cPLA2-α staining in tissue specimens using antibodies from Cell Signaling and Santa Cruz for total cPLA2-α as both displayed poor staining immunohistochemically. Figure 6E and F show little background staining on the isotype and method control slides.

Discussion

The arachidonic acid pathway is highly active in prostate cancer (2). Prostaglandin E2, one of the major eicosanoid end-products from the COX arm of the arachidonic acid pathway, results in increased proliferation (20) and decreased apoptosis (21) in prostate cancer cell lines. Inhibition of COX-2 results in decreased prostate cancer cell and PC3 xenograft growth (18, 22). Lipoxygenases, the other arm of the arachidonic acid pathway, produce hydroxyeicosatetraenoic acids (HETE). 5-Lipoxygenase, the enzyme responsible for 5-HETE production,
is overexpressed in prostate cancer (4), and inhibition of 5-lipoxygenase results in decreased prostate cancer cell proliferation and increased apoptosis (23). 12-Lipoxygenase is also overexpressed in prostate cancer (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 lipoxygenase arms of the arachidonic acid pathway, as well as loss of the natural inhibitor to cPLA2-\( \alpha \), (13), led us to hypothesize that cPLA2-\( \alpha \) would be a potential target of the treatment of prostate cancer, as inhibition would block a number of very active downstream pathways.

All the human prostate cancer cell lines tested expressed cPLA2-\( \alpha \) (Fig. 1). The androgen-sensitive cell line LNCaP expressed considerably less cPLA2-\( \alpha \) mRNA and protein compared with the androgen-insensitive DU145 and PC3. A proxy for cPLA2-\( \alpha \) activity, \( \beta \)-cPLA2-\( \alpha \), was correlated with the total cPLA2-\( \alpha \) levels.

Increased expression of cPLA2-\( \alpha \) in prostate cancer cell lines is an interesting phenomenon with a currently unknown mechanism. Cytosolic PLA2-\( \alpha \) is part of the inflammatory cascade and can be induced by tumor necrosis factor-\( \alpha \) (26), interleukin-1 (27), and nuclear factor-\( \kappa \)B (28, 29). The nuclear factor-\( \kappa \)B pathway has been shown to be more active in androgen-insensitive prostate cancer cells compared with androgen-sensitive prostate cancer cell lines, and may be a potential mechanism of cPLA2-\( \alpha \) up-regulation (30). In addition, Hughes-Fulford et al. have shown that arachidonic acid added exogenously to PC3 cells can up-regulate the expression of cPLA2-\( \alpha \) via the production of prostaglandin E2 (28). This is of great importance as dietary intake of fat has been correlated with increased incidence and mortality (31) as well as prostate cancer 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 cPLA2-\( \alpha \) or COX (28).

There are several potential mechanisms by which cPLA2-\( \alpha \) activity may be aberrantly regulated in prostate cancer cells. First, loss of inhibitory regulators Annexin I and Annexin II occurs in prostate cancer (13, 33). Second, increased mitogen-activated protein kinase activity observed in prostate cancer (34) could be responsible for the phosphorylation-activation of cPLA2-\( \alpha \) (35). Third, sPLA2-IIA, which is overexpressed in human prostate cancer (14), could contribute to the phosphorylation of cPLA2-\( \alpha \). In human kidney mesangial cells, cPLA2-\( \alpha \) has been shown to be activated by sPLA2-IIA (36) and in a human astrocytoma cell line, sPLA2-IIA has been shown to bind to sPLA2-IIA cell surface receptors, which then stimulate the mitogen-activated protein kinase pathway (37). The activated mitogen-activated protein kinase pathway in turn phosphorylates cPLA2-\( \alpha \), leading to its activation and arachidonic acid production (37, 38).

To determine if cPLA2-\( \alpha \) represented a potential target for the treatment of prostate cancer, we initially silenced cPLA2-\( \alpha \) mRNA and found a significant reduction in prostate cancer growth (Fig. 2). We then treated the prostate cancer cells with a specific inhibitor of cPLA2-\( \alpha \) (Wyeth-1) and showed a dose-dependent inhibition of prostate cancer cell growth. PC3 exhibited more resistance to the effects of Wyeth-1, despite expressing much more cPLA2-\( \alpha \). This may reflect the more aggressive nature of this cell line, which may harbor more active survival pathways. To confirm that the effects of Wyeth-1 were not the result of cPLA2-\( \alpha \)–independent effects, we tested the prostate cancer cells with another specific cPLA2-\( \alpha \) inhibitor, pyrophenone (39), and observed similar results. We also showed that Wyeth-1 and cPLA2-\( \alpha \) gene silencing effectively inhibit the cPLA2-\( \alpha \) activity in prostate cancer cells (Fig. 2D).

To understand the mechanism of proliferation inhibition (Fig. 3A), we elected to study pathways known to be involved in prostate cancer. 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 prostate cancer cells, and that Wyeth-1 treatment results in reduced p-Akt (Fig. 4). This mechanism may be mediated by cPLA2-\( \alpha \)–facilitated eicosanoid production, as studies of vascular smooth muscle cells show that either arachidonic acid, 5(S)-, 12(S)-, 15(S)-, or 20-HETE addition will increase Akt phosphorylation. Prostaglandin E2 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 prostate cancer cells (41). A decrease in p-Akt may be a
mechanism by which proliferation is decreased and apoptosis increased after cPLA2-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 G0-G1 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 G0-G1 and H&E. Tumors from Wyeth-1–treated mice stained significantly less for cyclin D1 than control. H&E staining showed very little necrosis in control tumors but striking necrosis in the Wyeth-1–treated tumors.

As the ultimate aim is to find new treatments for men with high-volume hormone refractory prostate cancer, we chose to use the PC3 xenograft model, and started treatment at a high tumor volume (200 mm³) to mimic the clinical disease. Despite the large initial sizes of the tumors, Wyeth-1 significantly reduced the rate of growth of tumors by ~33% compared with vehicle-treated mice (Fig. 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 that 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 cPLA2-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-1 caused a reduction in cells staining for cyclin D1 in PC3 xenografts, a finding that has been observed in PC3 xenografts treated with celecoxib (18).

It was important to determine whether the target cPLA2-α was present and active (phosphorylated) in human prostate cancer as this is ultimately where the therapy will be used. In addition, we
study was small in numbers, the expression of p-cPLA2-
tissue. We have shown that phosphorylation of cPLA2-
increased significantly in three of seven men who developed
higher in cancer compared with normal tissue; but the levels
magnification. The inserts were magnified (×40).

Fig. 6. Activity of cPLA2-α in prostate tissues by immunohistochemical staining of p-cPLA2-α. A, normal peripheral zone prostate samples exhibited low levels of phosphorylation of cPLA2-α in epithelial elements, and no staining of stromal tissue. B, localized prostate cancer samples from radical prostatectomy exhibited slightly stronger cPLA2-α phosphorylation. C, patients who initially underwent transurethral resection of prostate 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 p-cPLA2-α staining compared with before androgen deprivation (3 of 7 samples). E, isotype control and (F) method control show no background staining. All photographs at ×40 magnification. The inserts were magnified (×200) to view nuclear staining.

wanted to confirm whether men with hormone refractory prostate cancer gained increased expression of p-cPLA2-α compared with the androgen-sensitive disease. We are unable to report on expression levels of total cPLA2-α as the immunohistochemical staining of total cPLA2-α, with all available commercial antibodies, was of poor quality in human prostate tissue. We have shown that phosphorylation of cPLA2-α is present in prostate cancer tissue (Fig. 6) and levels may not be much higher in cancer compared with normal tissue; but the levels increased significantly in three of the seven men who developed hormone refractory prostate cancer (P = 0.03). Although this study was small in numbers, the expression of p-cPLA2-α was determined in prostate cancer tissue before 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 cPLA2-α inhibition will decrease cPLA2-α activity and prostate cancer cell growth, we have not investigated another effect of cPLA2-α inhibition such as reduction in platelet activating factor. In cancer cells and hematopoetic cells, platelet activating factor can be produced from the remaining lysophospholipid after arachidonic acid cleavage by cPLA2-α. Platelet activating factor has been shown to stimulate nuclear factor-κB as well as angiogenesis (42), and has been shown to be oncogenic in a number of cancers (42–44). Its role in prostate cancer has not been well studied.

A number of other cancers have also been noted to show elevated levels of cPLA2 with the potential for therapeutic targeting (12). In colon cancer, however, the reported actions of cPLA2-α have been controversial. Studies have found that cPLA2-α expression is increased in colorectal cancer (45, 46), but in the Apcmin mouse model of colon tumorigenesis, the deletion of cPLA2-α did not result in a change in the number of colon tumors observed (47). Similarly, in azoxymethane-induced mouse colon cancer, cPLA2-α was observed to decrease (48), and when cPLA2-α was deleted in this mouse model, an increase in colon tumors was observed (49). It has been suggested that a loss of arachidonic acid–derived ceramide, a proapoptotic mediator, may result in increased tumor formation in this disease.

Before human phase I studies, further research needs to be done in the area of cPLA2-α inhibition and prostate cancer. Initially, as the class of specific cPLA2-α inhibitors is quite new, pharmacokinetic studies need to be done 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 cPLA2-α are present in prostate cancer cell lines. Cytosolic PLA2-α plays a role in cancer cell proliferation and apoptosis, and its inhibition or knockdown results in decreased cell growth. The drug Wyeth-1 seems to act specifically through the cPLA2-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 remaining cancer cells. Cytosolic PLA2-α is a potentially effective target for therapy of prostate cancer, and therefore warrants further investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


