

Mechanisms Controlling Cell Cycle Arrest and Induction of Apoptosis after 12-Lipoxygenase Inhibition in Prostate Cancer Cells¹

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ABSTRACT

Extensive studies have implicated the role of dietary fatty acids in prostate cancer progression. Platelet-type 12-Lipoxygenase (12-LOX) has been shown to regulate growth, metastasis, and angiogenesis of prostate cancer. The effect of two 12-LOX inhibitors, Baicalein and *N*-benzyl-*N*-hydroxy-5-phenylpentamide (BHPP), on the mechanisms controlling cell cycle progression and apoptosis were examined in two prostate cancer cell lines, PC3 and DU-145. Treatment with Baicalein or BHPP resulted in a dose-dependent decrease in cell proliferation, as measured by BrdUrd incorporation. This growth arrest was shown to be because of cell cycle inhibition at G₀/G₁, and was associated with suppression of cyclin D1 and D3 protein levels. PC3 cells also showed a strong decrease in phosphorylated retinoblastoma (pRB) protein, whereas the other retinoblastoma-associated proteins, p107 and p130, were inhibited in DU-145 cells. Treatment with 12-hydroxyeicosatetraenoic acid in the presence of Baicalein blocked loss of pRB, whereas 12(S)-HETE alone induced pRB expression. Treatment with either Baicalein or BHPP resulted in significant apoptosis in both cell lines as measured by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling. DU-145 cells underwent apoptosis more rapidly than PC-3 cells. The mechanisms involved were decreased phosphorylation of Akt, loss of survivin and subsequent activation of caspase-3 and caspase-7 in each cell line, decreased Bcl-2 and Bcl-X_L expression in DU-145, and a shift in Bcl-2/Bax levels favoring apoptosis in PC-3 cells. Addition of 12(S)-HETE protected both cell lines from Baicalein-induced apoptosis, whereas other LOX metabolites, 5(S)-HETE, or 15(S)-HETE did not. These results show that the 12-LOX pathway is a critical regulator of prostate cancer progression and apoptosis, by affecting various proteins regulating these processes. Therefore, inhibition of 12-LOX is a potential therapeutic agent in the treatment of prostate cancer.

INTRODUCTION

Arachidonic acid metabolism can be catalyzed by one of two distinct enzyme pathways, cyclooxygenase or LOX.⁴ Its metabolism leads to the generation of biologically active metabolites that may be potentially involved in carcinogenesis by modulating mitogenic signaling and regulating cellular proliferation (1–3). Furthermore, it has been shown that the LOXs in particular are key regulators of cell survival and apoptosis in cells (4). Mammalian LOXs constitute a heterogeneous family of lipid peroxidizing enzymes that are categorized with respect to their regional specificity of arachidonic acid oxygenation. Therefore, the LOXs have been designated as 5-, 8-, 12-, and 15-LOX isoforms, which transiently produce the end products

5(S)-, 8(S)-, 12(S)-, and 15(S)-HETEs, respectively (5, 6). 12-LOX is expressed as two main isoforms, a platelet-type cloned from human platelets (7) and a leukocyte-type from porcine leukocytes, that shares 65% homology to the platelet-type cDNA (8).

Several lines of evidence implicate 12-LOX as a regulator of human cancer development. It is overexpressed in a variety of tumors including breast, colorectal, and prostate cancer (9–11), and has been shown to be present in a number of cancer cell lines (12–14). We have extensively investigated the role of platelet-type 12-LOX and 12(S)-HETE in prostate cancer. We reported previously that 12-LOX is expressed in several prostate cancer cell lines (15). In addition, we have described its involvement in tumor metastasis, as pretreatment of DU-145 prostate cancer cells *in vitro* with the 12-LOX specific inhibitors BHPP or Baicalein significantly inhibited their ability to form lung colonies after tail-vein injection (15). Other reports from our laboratory have demonstrated that 12-LOX levels are correlated with the grade and stage of human prostate tumors (16), and that *s.c.* injection of PC3 cells overexpressing 12-LOX increased the amount of angiogenesis and growth of tumors in mice (11).

Deregulation in the fine balances controlling cellular proliferation and cell death is the hallmark of cancer. Many proteins are involved in the process by which cells choose between growth arrest, apoptosis, or survival. The 12-LOX inhibitor, Baicalein, has been shown previously to induce apoptosis in human gastric, colon, hepatoma, and pancreatic cancer cells (13, 17–20). In addition, an analogue of Baicalein, called baicalin, has been shown to induce apoptosis in prostate cancer cells (20). The underlying mechanism whereby 12-LOX inhibition induces and executes apoptosis is not clearly defined. In the present study we examine the effect of 12-LOX inhibition by Baicalein or BHPP on the mechanisms controlling the cell cycle and apoptosis in prostate cancer cells by examining the cell cycle-regulatory proteins that are involved in the control of the G₁-S transition.

MATERIALS AND METHODS

Cells Lines. Two prostate carcinoma cell lines, DU-145 and PC3, were obtained from the American Type Culture Collection (Rockville, MD) and maintained in a humidified atmosphere of 5% CO₂ in air at 37°C. The cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 2 mM L-glutamine, and 100 μg/ml penicillin-streptomycin. Experiments were performed when cells were ~80% confluent.

Cell Proliferation. PC3 and DU145 prostate cancer cells were seeded into 96-well plates and incubated at 37°C. After 12 h cells were cultured in serum-free medium with or without various concentrations of the selective 12-LOX inhibitors Baicalein (Biomol, Plymouth, PA) or BHPP (Biomide Corp., Grosse Pointe Farms, MI), or a specific 5-LOX inhibitor α-pentyl-4-2-quinolinylmethoxy-benzenemethanol (Rev-5901; Cayman Chemicals, Ann Arbor, MI) for 48 h. Thereafter, cell proliferation was assessed by a specific nonradioactive cell proliferation ELISA based on the measurement of BrdUrd incorporation during DNA synthesis according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Statistical comparison between treatments was carried out using ANOVA with Scheffe post-hoc correction. Data are taken as significant where *P* < 0.05. Separately, cells were examined morphologically by light microscopy.

Flow Cytometry for Cell Cycle Analysis. Prostate cancer cells grown in 25-cm² flasks were treated with various concentrations of Baicalein for 48 h.

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⁴ The abbreviations used are: LOX, lipoxygenase; BHPP, *N*-benzyl-*N*-hydroxy-5-phenylpentamide; BrdU, bromodeoxyuridine; pRB, phosphorylated retinoblastoma; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; PARP, poly-(ADP-ribose) polymerase; CDK, cyclin-dependent kinase; Rb, retinoblastoma; 12(S)-HETE, 12-hydroxyeicosatetraenoic acid; FLAP, 5-LOX-activating protein.

The cells were then digested by trypsin-EDTA, washed, and resuspended in serum-free medium, counted, and then fixed overnight in 75% ethanol at 4°C. The cells were then washed and resuspended in PBS (pH 7.4) containing 0.1% Triton X-100, 0.05 mg/ml DNase-free RNase A, and 50 µg/ml propidium iodide at a concentration of 0.5 ml/10⁶ cells. The cells were incubated in the dark for 30 min at room temperature. The red fluorescence of the single events was recorded using an argon ion laser at 488 nm excitation wavelength and 610 nm as emission wavelength to measure DNA index.

TUNEL Assay. Apoptosis was detected using the terminal incorporation of fluorescein-12-dUTP by terminal deoxynucleotidyl transferase into fragmented DNA in prostate cancer cells treated with Baicalein according to the manufacturer's instructions (Roche Diagnostics GmbH). Briefly, cells were grown in 75-cm² flasks, incubated overnight in serum-free medium, and then treated with various concentrations of Baicalein for 24, 48, or 72 h. The cells were then digested by trypsin-EDTA, washed, and fixed for 1 h in 75% ethanol at 4°C. Thereafter ice-cold permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) was added for 3 min. After this samples were washed twice in PBS, and 50 µl of the working TUNEL reaction mix was added. Samples were incubated at 37°C for 1 h, washed, and resuspended in PBS. Laser flow cytometry was used to quantify the percentage of apoptotic cells indicated by green fluorescence of fluorescein-12-dUTP incorporated by the cell.

Western Blot Analysis. Total cell lysates were prepared following various treatments. Protein (30 µg) was fractionated by precast SDS-PAGE and then transferred to nitrocellulose membranes. After incubating for 1 h in blocking buffer containing 5% skimmed milk dissolved in 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20, blots were probed overnight with primary antibodies against cyclin D1, D2, D3, and cyclin E; CDKs 2, 4, and 6; CDK inhibitors p21, p27, p57, p16, p18, and p19; Rb protein; and various phospho-Rb. In addition apoptosis-related proteins BCL-2, PARP, caspases, and survivin were examined. All of the antibodies, excluding Rb and phosphorylated Rb (Ser 807/811; Cell Signaling Technology, Beverly, MA); p15, p16 and p18 (Upstate Biotechnology, Lake Placid, NY); cyclin D1 (Oncogene Research Products, Boston, MA); cyclins D2, D3, and E, p21, p27, and PARP (BD PharMingen, San Diego, CA) were obtained from Santa Cruz Biotechnology (Berkeley, CA)

RESULTS

Effect of LOX Inhibitors on Prostate Cancer Cell Proliferation.

The 12-LOX inhibitors Baicalein and BHPP both induced a significant growth inhibition in prostate cancer cells in a dose-dependent manner as measured by BrdU incorporation in both PC3 and DU145 cells at 48 h relative to control cells (Fig. 1, A and B). Treatment with 25 µM Baicalein significantly decreased proliferation of PC-3 and DU-145 cells to 51% and 52%, respectively, compared with untreated controls ($P < 0.05$). BHPP also significantly inhibited proliferation of both cell lines at similar concentrations ($P < 0.05$). After 24 h both 12-LOX inhibitors had only minor effects on cell numbers in both prostate cancer cell lines (data not shown). The specific 5-LOX inhibitor Rev-5901 had no effect on cell proliferation in either PC3 or DU145 prostate cancer cells ($P =$ not significant; Fig. 1, A and B).

LOX Inhibition-induced Growth Arrest in Prostate Cancer Cells. To examine where in the cell cycle the growth phase arrest observed in the proliferation experiments occurred, PC3 cells were treated with the LOX inhibitors Baicalein, BHPP, or Rev-5901 for 24 h in serum-free medium. The percentage of cells in G₀/G₁ versus S versus G₂/M phases was then examined by flow cytometry after propidium iodide staining of the cellular DNA (representative DNA histograms are shown in Fig. 2A). Treatment of cells with 25 µM Baicalein resulted in an accumulation of cells in the G₀/G₁ phase (from 45% to 61% and from 51% to 68% in PC-3 and DU-145 cells, respectively) with a comparative drop in the S phase fraction from 36% to 23% and 32% to 24%, respectively. The G₂-M phase decreased slightly in PC-3 cells (from 19% to 16%), whereas a more dramatic decrease was observed in the DU-145 cells (from 17% to

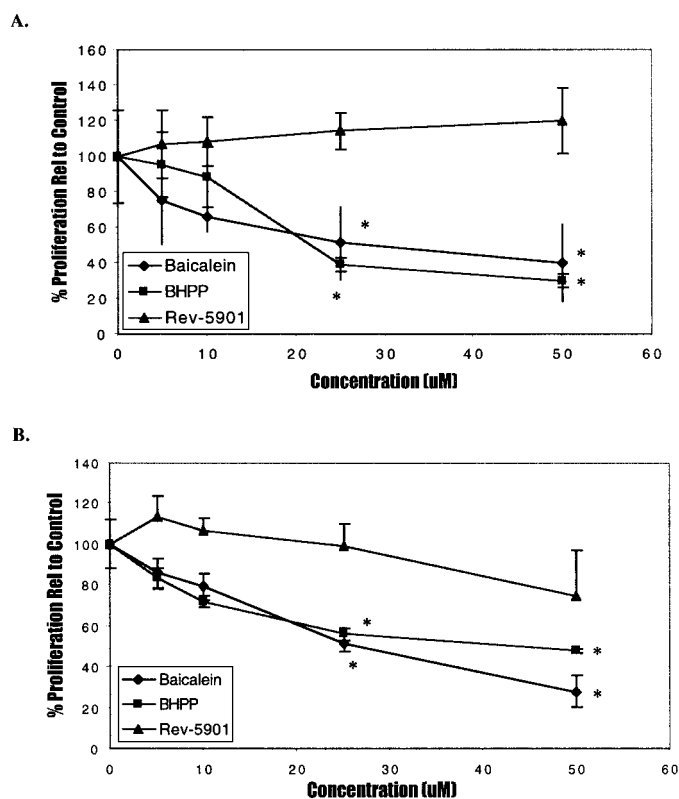


Fig. 1. Effect of LOX inhibition on the proliferation of PC-3 (A) and DU-145 (B) prostate cancer cells. The cells were treated with increasing concentrations of the 12-LOX inhibitors, Baicalein or BHPP, or the 5-LOX inhibitor Rev-5901 (5–50 µM) for 48 h. Viable cells were measured by BrdU incorporation and expressed as a percentage of the controls ($n = 4$); bars, \pm SE. * indicate that levels are significantly different from the untreated controls ($P < 0.05$).

9%; Fig. 2B). A similar effect was observed when both cell lines were treated with BHPP. An accumulation of cells at the G₁ phase was observed, with a proportional reduction in the S and G₂-M phase fractions (Fig. 2B). Therefore, it is obvious that 12-LOX inhibition induced a G₁ phase arrest in both cell lines. Conversely, treatment with the 5-LOX inhibitor Rev-5901 did not alter the growth phase fractions relative to controls in either cell line.

Effect of 12-LOX Inhibition on Proteins Regulating the G₁ to S Transition in the Cell Cycle. To determine the mechanisms involved in the cell cycle arrest caused by 12-LOX inhibition, the expression of a number of molecules that regulate passage of cells from the G₁ to the S phase of the cell cycle were examined. These include cyclins and their catalytic partners, the CDKs, inhibitors of CDKs, and the Rb family of proteins that govern exit from the G₁ phase (21).

Baicalein treatment (25 µM) resulted in a strong reduction in the expression of the D-type cyclins, D1 and D3 in both PC3 and DU-145 cells, whereas no effect was observed on cyclin E expression (Figs. 3A and 4A). Cyclin D1 levels were reduced within 3 h of Baicalein treatment and were undetectable by 14 h. Similar results were observed when the cells were treated with 25 µM BHPP (results not shown). Baicalein also resulted in a moderate reduction in CDK-2 and CDK-4 levels after 14 h, with little or no effect on CDK-6. Unexpectedly, Baicalein also resulted in a strong reduction in the CDK inhibitors p21 and p27 over time, resulting in undetectable levels by 24 h. No effect was observed on p16 (Fig. 3B and Fig. 4B), which inhibits phosphorylation of the Rb protein. The other INK4 family members, p15, p18, and p19, were also unaltered (data not shown). p21 and p27 have a dual function in the cell cycle: inhibition of CDK-cyclin formation, particularly cyclin E-cdk2, as well as facilitating the assembly of cyclinD-cdk4/6 complexes (22).

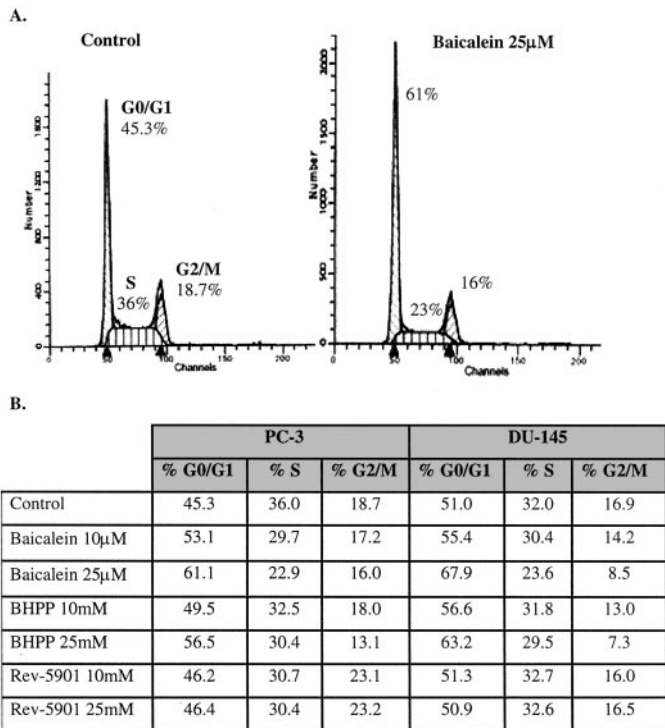


Fig. 2. Representative DNA histogram (A) and tabulated percentages (B) of cells after LOX inhibition and cell cycle analysis by propidium iodide staining. The 12-LOX inhibitors Baicalein and BHPP induced a specific G₀/G₁ growth phase arrest, indicated by accumulation of cells at this phase, with a corresponding decline in cells in the S (mitotic) phase. The tabulated percentages are an average calculated on the results of two separate experiments.

Effect of 12-LOX Inhibition on RB Protein Family Members.

Because cyclin D and CDK-4 govern phosphorylation (inactivation) of the Rb protein during progression of the cell cycle past G₁, the above results would suggest that Rb dephosphorylation may occur in response to 12-LOX inhibition. Rb has been reported recently to be an important factor in directing the choice between permanent arrest and apoptosis in cells (23). Therefore, the phosphorylation state of the Rb protein was examined in PC3 cells. A significant reduction in the phosphorylation of the Rb protein occurred over time after treatment with 25 µM Baicalein (Fig. 3A), resulting in undetectable levels within 24 h of treatment. This observation is consistent with a positive contribution toward growth arrest of PC3 cells. Similar results were obtained when cells were treated with the other 12-LOX inhibitor BHPP (data not shown).

DU-145 cells that contain a mutant form of the Rb protein (24) responded in a similar manner to the PC3 cells. This would indicate that functional Rb is not required to mediate the growth arrest induced by 12-LOX inhibition. To examine if other members of the Rb family are associated with this effect, levels of p107 and p130 were examined in the cells after Baicalein treatment. Baicalein induced a decrease in the expression of both p107 and p130 in DU145 cells (Fig. 4A). Notably, levels of p130 were undetectable by 24 h. These results suggest that these proteins may compensate for the absence of functional Rb in DU-145 cells.

Effect of 12-LOX Inhibition on Prostate Cancer Cell Apoptosis.

To investigate the fate of cells treated with 12-LOX inhibitors, PC3 and DU-145 cells were examined for levels of apoptosis using the TUNEL assay. Baicalein resulted in a dose-dependent increase in apoptosis in both cell lines, determined by FITC-dUTP staining in cells (Fig. 5, A and B). The induction of apoptosis was time dependent, with little effect occurring in either cell line after 24 h. DU145

cells were more sensitive to apoptosis than PC3 cells, with 63% of cells staining positive after 25 µM Baicalein treatment at 48 h (Fig. 5B) compared with 39% in PC3 cells (Fig. 5A). After treatment (72 h), apoptosis was significantly higher in PC3 cells (66%) relative to controls, indicating that apoptosis induction was related to length of drug exposure in PC-3 cells.

Treatment with Baicalein (25 µM) over time resulted in dramatic morphological changes in both of the cell lines examined. This was characterized by decreased cell density, elongation, and filamentous protrusions bridging many cells. By 48 h some cells visibly lost anchorage and were floating. This was more evident in the DU145 cells, which underwent apoptosis more quickly (Fig. 5C).

Effect of 12-LOX Inhibition on Proteins Regulating Apoptosis.

To examine the mechanisms involved in propagating and executing apoptosis in prostate cancer cells, time course experiments were carried out to determine the levels of protein expressed by apoptosis-related genes. Cells were treated with 25 µM Baicalein or BHPP from 0 to 24 h.

In PC-3 cells, Baicalein treatment resulted in a decrease in the antiapoptotic protein Bcl-2 by 14 h (Fig. 6A). The proapoptotic protein Bax, which is known to heterodimer with Bcl-2, was increased after treatment with Baicalein as early as 6 h. DU-145 cells did not express detectable levels of Bax. However, another antiapoptotic member of the Bcl-family, Bcl-X_L, was reduced after treatment with Baicalein (Fig. 6B). Expression of the proapoptotic proteins Bcl-X_S or Bad, which heterodimer with Bcl-X_L, were unaltered by

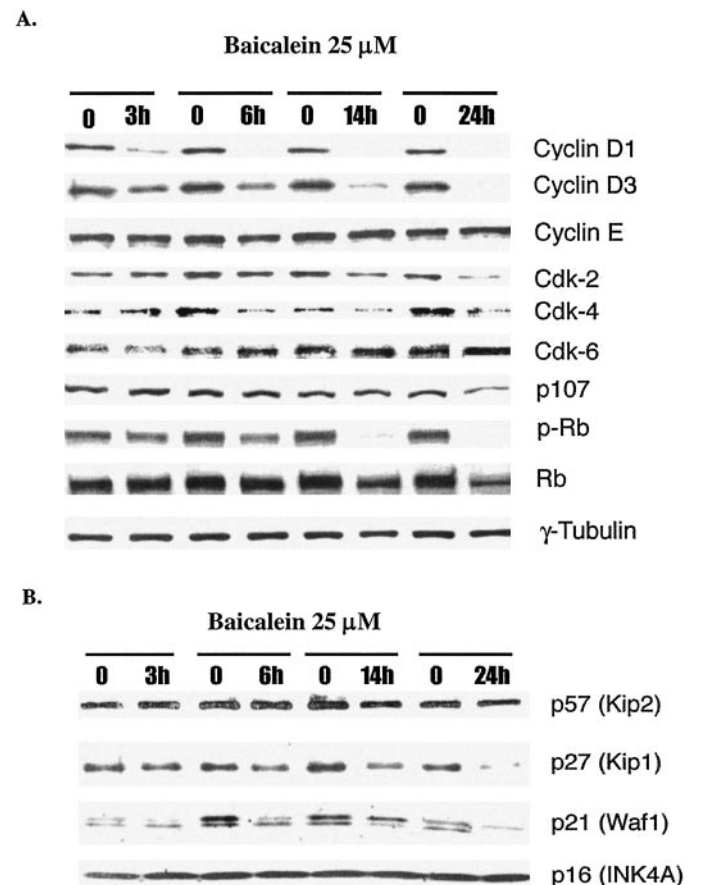


Fig. 3. A, protein levels of cyclin-D1, -D3, and -E; CDK-2, -4, and -6; and Rb family proteins, p107, Phos-RB, and RB in Baicalein-treated PC-3 cells. B, protein levels of the CDK-inhibitors p16, p21, p27, and p57 in Baicalein-treated cells. Cells were treated with 25 µM Baicalein for different time intervals (0, 3, 6, 14, and 24 h). Protein levels were detected by Western blot. Baicalein treatment resulted in decreased expression of cyclin D1 and D3, and also decreased phosphorylation of the Rb protein.

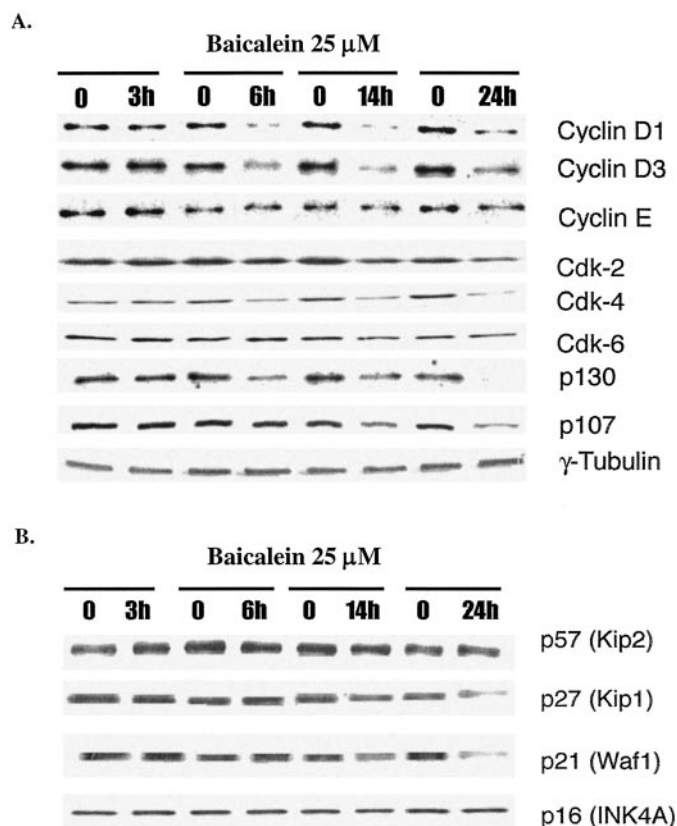


Fig. 4. A, protein levels of cyclin-D1, -D3, and -E; CDK-2, -4, and -6; and Rb family proteins, p130, and p107 in Baicalein-treated DU-145 cells. B, protein levels of the CDK inhibitors p16, p21, p27, and p57 in Baicalein-treated cells. Cells were treated with 25 μ M Baicalein for different time intervals (0, 3, 6, 14, and 24 h). Protein levels were detected by Western blot. The 12-LOX inhibitor Baicalein resulted in a strong decrease in cyclin-D1 and -D3 protein levels, coupled to a decrease in the levels of pRb.

Baicalein treatment. These effects on the Bcl-family proteins would result in a dramatic shift from survival to apoptosis in both cell lines.

The inhibitor of apoptosis family member, survivin, is overexpressed in a variety of human tumors (25). Inhibition of 12-LOX by Baicalein treatment resulted in a strong decrease in survivin protein expression in both PC3 and DU-145 cell lines (Fig. 6, A and B). Survivin has been shown to inhibit apoptosis by binding to active caspase-3 and caspase-7 (26). During apoptosis active caspase-3 and -7 are formed by cleavage of the zymogens. Caspase-7 has been associated previously with Baicalein-induced apoptosis in gastric cancer cells (17). Treatment with 25 μ M Baicalein in both cell lines resulted in active caspase-3 and caspase-7 fragments (Fig. 6B). Active caspase was induced by Baicalein at 24 h in PC-3 cells, at which time PARP expression was also reduced, which occurs downstream of caspase-3/7 cleavage. Therefore, it appears that the caspase cascade is involved in Baicalein-induced apoptosis in PC3 cells. PC3 cells did express high levels of phosphorylated-Akt that reduced over time after serum depletion (Fig. 6A). DU-145 cell expressed lower levels of Akt, and Baicalein treatment resulted in additional loss of phosphorylated Akt-1 in both cell lines, resulting in undetectable levels by 24 h.

Effect of 12(S)-HETE Add-Back on Apoptosis and Protein Expression Induced by 12-LOX Inhibition. To demonstrate that the effects observed in both cell lines after treatment with the 12-LOX inhibitor Baicalein are a consequence of 12-LOX inhibition, an add-back experiment was performed in which cells were treated with 25 μ M Baicalein in the presence of the only product of arachidonic acid metabolism by 12-LOX, *i.e.*, 12(S)-HETE. Treatment with 12(S)-HETE (100 ng/ml) blocked Baicalein-induced apoptosis in both cell

lines, reducing apoptosis to 6.6% in PC-3 and 18.6% in DU-145 cells (Fig. 7, A and B). This protective effect was maintained over time by continued treatment with 12(S)-HETE (at 12-h intervals). Treatment with either 5(S)-HETE or 15(S)-HETE did not block Baicalein-induced apoptosis, with levels of apoptosis similar to Baicalein treated cells (Fig. 7, A and B).

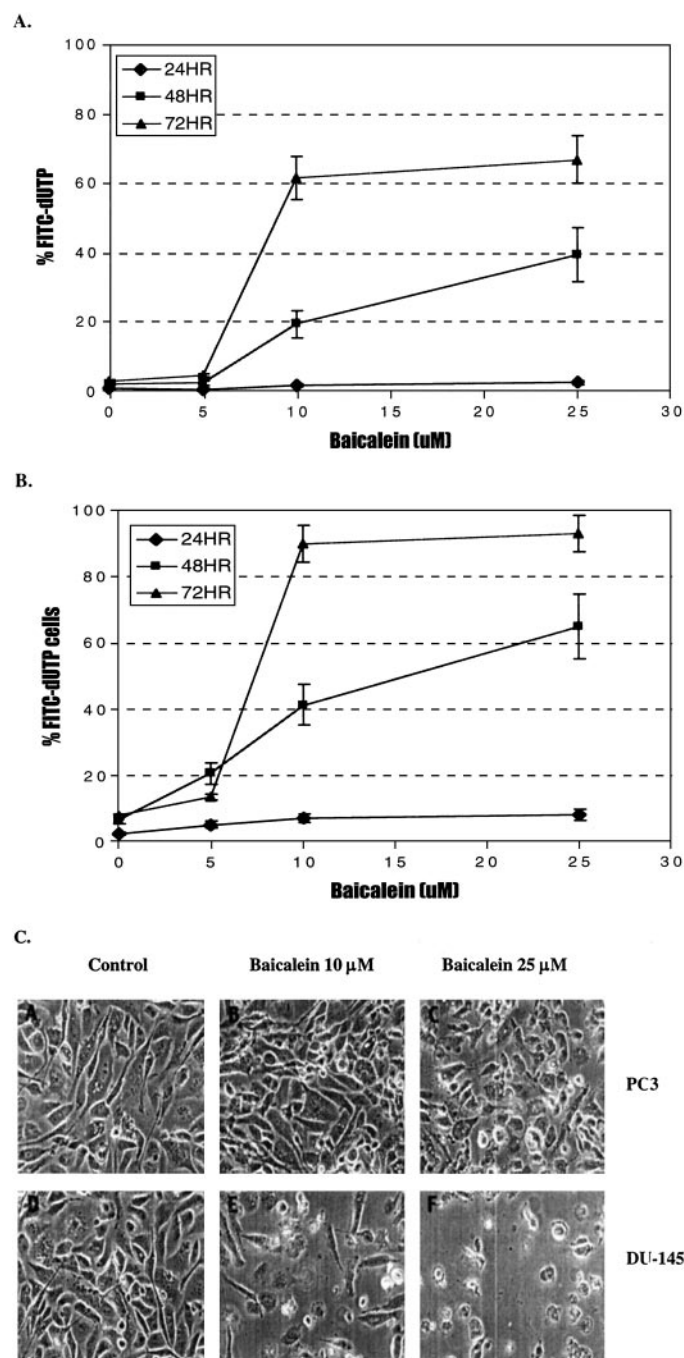


Fig. 5. Effect of Baicalein on apoptosis of PC-3 (A) and DU-145 (B) prostate cancer cells. The cells were treated with increasing concentrations of the Baicalein (5–25 μ M) for 24, 48, or 72 h. DNA fragmentation was measured by FITC-dUTP staining (TUNEL) and detected by flow cytometry. Results were expressed as a percentage of the total cells. Treatment with Baicalein resulted in a dose-dependent increase in apoptosis in both cell lines. DU-145 cells were more sensitive to Baicalein than PC-3 cells, with almost all cells containing DNA fragmentation after 25 μ M treatment for 48 h. The percentages are an average calculated on the results of two separate experiments; bars, \pm SE. C, representative PC-3 and DU-145 cells after Baicalein treatment. DU-145 cells are more sensitive to Baicalein, indicated by cell shrinkage and loss of anchorage, characteristics of apoptosis.

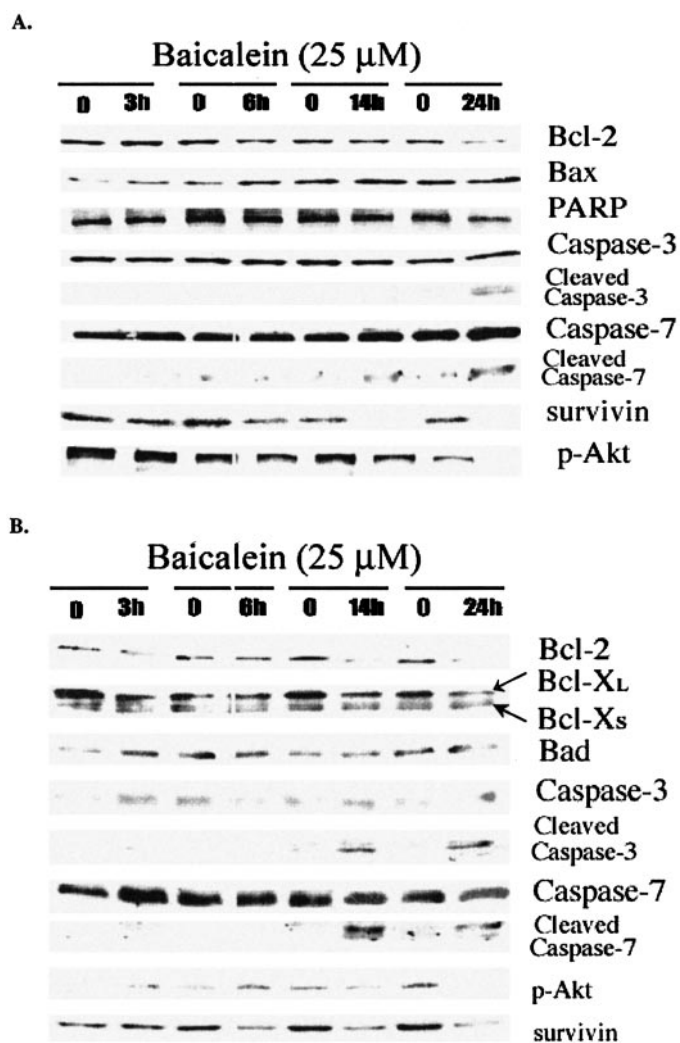


Fig. 6. A, protein levels of PARP, caspase-3, -7, Bcl-2, Bax, survivin, and phosphorylated-Akt in Baicalein-treated PC-3 cells. B, protein levels of caspase-3, -7, Bcl-X_L, Bcl-X_S, Bad, survivin, and p-Akt in Baicalein-treated DU-145 cells. Cells were treated with 25 μ M Baicalein for different time intervals (0, 3, 6, 14, and 24 h). Total protein (30 μ g) was probed by Western blot with different antibodies. Baicalein treatment resulted in a strong decrease over time in the survival proteins survivin and phosphorylated Akt in both cell lines. In addition a shift in the Bcl family proteins favoring apoptosis was observed, with reduced Bcl-2 and increased Bax in PC-3 cells, and decreased Bcl-X_L in DU-145 cells.

To additionally demonstrate that the effects observed after Baicalein or BHPP treatment were through inhibition of 12-LOX, PC-3 cells were treated with 12(S)-HETE for 14 h and phosphorylated RB expression examined by Western blot. The Rb protein was selected for this experiment because its levels were reduced most dramatically in response to 12-LOX inhibition, and Rb has been implicated in both cell cycle progression and apoptosis. 12(S)-HETE treatment (100 ng/ml) induced the expression of phosphorylated Rb protein in PC3 cells, whereas neither 5(S)-HETE nor 15(S)-HETE had any effect (Fig. 7C). In addition, continuous treatment with 12(S)-HETE blocked Baicalein-induced reductions in phosphorylated Rb at 14 h, whereas 5(S)-HETE and 15(S)-HETE had no such effect (Fig. 7C). Treatment with 12(S)-HETE but not 5 or 15(S)-HETE also induced the expression of phosphorylated AKT-1 in both cell lines and blocked Baicalein-induced reductions in this protein (results not shown).

DISCUSSION

The arachidonic acid-metabolizing enzyme 12-LOX and its only metabolite 12(S)-HETE are key regulators of tumor growth. This

pathway has been implicated in tumor cell proliferation and motility, regulation of apoptosis, and in tumor angiogenesis (11–13). We have previously shown 12-LOX to be expressed in the prostate cancer cell lines DU-145 and PC3 (15); however, its exact role in these cells is not fully defined. The present study was aimed at investigating the effect of 12-LOX inhibition on prostate cancer cells and more importantly, examining the mechanisms governing these effects. The results from this study indicate that the 12-LOX pathway is essential for prostate cancer growth and survival. Inhibition of 12-LOX by the specific inhibitors Baicalein or BHPP resulted in a dose-dependent decrease in proliferation of both DU-145 and PC3 cells. Inhibition of 5-LOX with Rev-5901 at similar concentrations had no effect on proliferation. These results are similar to those observed in pancreatic cancer cells after 5- or 12-LOX inhibition (13). However, 5-LOX does not appear to be as critical in the two prostate cancer lines studied

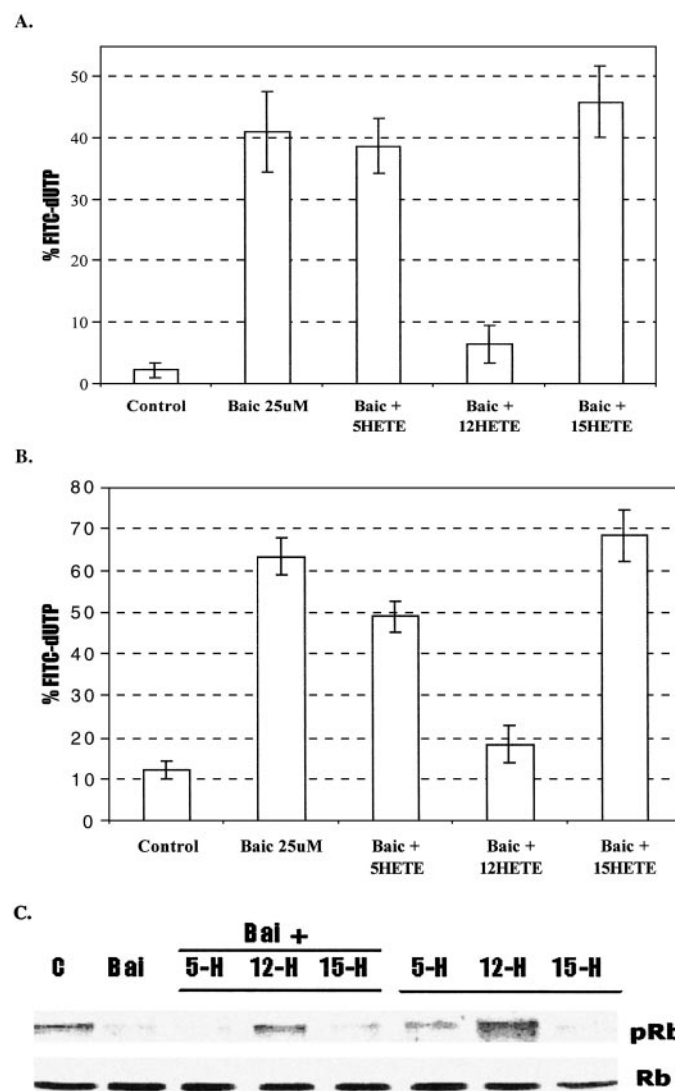


Fig. 7. Effect of 12-HETE on Baicalein-induced apoptosis in PC-3 (A) and DU-145 cells (B). PC-3 or DU-145 cells were incubated with 100 ng/ml 5(S)-HETE, 12(S)-HETE, or 15(S)-HETE and treated with 25 μ M Baicalein for 48 h. HETE incubation was repeated at 12-h intervals. The percentage of apoptotic cells was calculated by FITC-dUTP labeling and flow cytometric determination. Incubation with 12(S)-HETE blocked Baicalein-induced apoptosis, whereas incubation with either 5(S)-HETE or 15(S)-HETE had no protective effect. The percentages are an average calculated on the results of two separate experiments; bars, \pm SE. C, protein levels of pRb in PC-3 cells incubated with 100 ng/ml 5-, 12-, or 15(S)-HETE and treated with 25 μ M 12-LOX inhibitor Baicalein for 14 h. Treatment with 12(S)-HETE alone induced the phosphorylation of RB and blocked Baicalein-induced reductions in its expression.

here. It is likely that the family of LOXs are involved in cell survival and that different isoforms are more important than others depending on the cells being studied. For example, 5-LOX has been shown recently to regulate survival of mesothelial cells (27).

To better understand the anticancer effects of 12-LOX inhibition in prostate cancer cells, we investigated which point in the cell cycle is affected by Baicalein or BHPP treatment. Treatment with either 12-LOX inhibitor resulted in a significant growth arrest in the G₀/G₁ phase of both cell lines. The G₁ arrest observed in this report is similar to that observed in response to Baicalein treatment in Hepatoma cells (19), resulting in an associated decrease in the S phase (or mitotic) and G₂-M fractions of Baicalein-treated cells. In contrast, Ding *et al.* (13) reported that Baicalein treatment in pancreatic cancer cells did not induce a specific cell phase arrest and that the inhibition of pancreatic cancer cell proliferation occurred in all phases of the cell cycle. There is substantial evidence that critical regulatory steps occur during the G₁ phase of the cell cycle, which determine whether or not the cell will synthesize DNA and divide. To examine the molecular mechanisms underlying the G₁ phase arrest observed in prostate cancer cells, we examined the levels of the regulatory proteins required for transition past the G₁ restriction point of the cell cycle. Treatment with Baicalein or BHPP resulted in a time-dependent decrease in cyclin D1 and cyclin D3 levels. Within 6 h of treatment with 12-LOX inhibitors, levels of the D-type cyclins were almost undetectable. Inhibition of 12-LOX also led to reductions in the levels of CDK-4 (the catalytic partner of cyclin D1) in both DU-145 and PC3 cells over time.

Cyclin D1 is a proto-oncogenic regulator of the G₁-S phase checkpoint that has been implicated in the pathogenesis of several cancers (28–30). Cyclin D1 functions upstream of the RB protein by binding to CDK-4 or -6 leading to RB phosphorylation. The phosphorylation of RB in mid-to-late G₁ releases the transcription factors bound by RB, resulting in their subsequent binding to the promoter regions of various genes leading to DNA synthesis (31). Within 14 h of treatment with either 12-LOX inhibitor, levels of phosphorylated RB were undetectable in PC3 cells. These results indicate the mechanism of G₁ growth arrest induced by 12-LOX inhibition in PC3 cells: reduction in D-type cyclins, which then result in decreased phosphorylation of RB, resulting in the RB protein remaining bound to the transcription factors required for DNA synthesis. Because DU-145 cells that contain a mutant form of the RB protein (24) responded in a similar manner to the PC3 cells, we examined whether other members of the RB family compensated for the loss of functional RB. We observed a strong reduction in the levels of both p107 and p130 after treatment with Baicalein in DU-145 cells. These results indicate that 12-LOX inhibition does not only result in blocking phosphorylation of functional RB protein but that it may regulate other cell pathways that compensate for mutations in certain cells. Therefore, it suggests a broader potential for Baicalein and BHPP as anticancer agents in a variety of cancers by blocking cell cycle progression. Interestingly, Baicalein treatment also reduced levels of cdk-2, which complexes with cyclin-E later in G₁, to also phosphorylate the RB protein. However, cyclin-E expression remained unchanged after treatment in either cell line. The effects of Baicalein treatment on cell cycle proteins were shown to be through 12-LOX inhibition, as addition of 100 ng/ml of the end product 12(S)-HETE partially restored phosphorylated RB levels. In contrast, addition of 5(S)-HETE or 15(S)-HETE did not restore phosphorylated RB levels after Baicalein treatment. Furthermore, treatment of PC3 cells with 12(S)-HETE alone resulted in increased expression of phosphorylated RB, an effect that was not observed when cells were treated with similar amounts of 5(S)- or 15(S)-HETE.

Because 12-LOX inhibition decreased cell proliferation and perturbed the cell cycle, we were interested in determining the effects of

12-LOX inhibition on prostate cancer cell apoptosis and the underlying mechanisms responsible for these effects. Induction of apoptosis in prostate cancer cells after treatment with an analogue of Baicalein; *i.e.*, baicalin, was reported previously (20). Our results show that Baicalein treatment in PC3 and DU-145 cells directly induces apoptosis, with DU-145 cells being more sensitive to Baicalein-induced apoptosis compared with the PC3 cells.

As illustrated in Fig. 7, we found that simultaneous addition of 12(S)-HETE to Baicalein-treated cells prevented the induction of apoptosis, lowering the apoptotic fraction to 6.6% and 18.6% in PC-3 and DU-145 cells, respectively. However, addition of other LOX products, 5(S)-HETE or 15(S)-HETE, did not protect the cells from Baicalein-induced apoptosis. These results indicate the specific requirement for the 12-LOX pathway for the survival of these cells. There are several reports suggesting that 5-LOX is the critical LOX enzyme mediating survival in prostate cancer cells (32, 33). In these reports the FLAP inhibitor MK886 was shown to induce apoptosis associated with increased oxidative stress. The differences observed in our study could be because a different 5-LOX inhibitor, Rev-5901, was used. This is supported by the fact that the FLAP inhibitor MK886 has been shown to induce apoptosis independent of FLAP inhibition, suggesting an alternate mechanism unrelated to 5-LOX inhibition (34). In addition, unlike the results observed using Baicalein and BHPP, two inhibitors of 5-LOX induced apoptosis by two morphologically distinct pathways in the same cell line, suggesting that 5-LOX inhibition is not the convergent point for apoptosis induction by these inhibitors (35).

In this study, we examined the mechanisms underlying the induction of apoptosis after 12-LOX inhibition. Results from this laboratory have reported previously that the Bcl family of proteins are involved in apoptosis induced by LOX inhibition in rat Walker-256 carcinoma cells (4). In that study LOX inhibition resulted in a down-regulation of the antiapoptotic Bcl-2 protein and a dramatic decrease in the Bcl-2:Bax ratio, which could be blocked by Bcl-2 overexpression. In this study, we found a similar decrease in Bcl-2 levels in both cell lines after 12-LOX inhibition, and this reduction was coupled to an increased expression of the proapoptotic protein Bax in the PC3 cells. DU-145 cells, on the other hand, did not express Bax; however, treatment with Baicalein resulted in decreased expression of another antiapoptotic protein of the same family, Bcl-X_L, whereas levels of its proapoptotic partner, Bcl-X_S, were unaltered. Therefore, in each cell line, Baicalein altered the expression of different Bcl-family protein members, resulting in a shift in their ratios favoring apoptosis, once again suggesting the broad applicability of these inhibitors to cancer treatment.

Survivin, a member of the inhibitor of apoptosis family, is overexpressed in the majority of human cancers, including cancer of the prostate (25, 36). Treatment with Baicalein resulted in a strong decrease in survivin expression over time, resulting in undetectable levels in both PC3 and DU-145 cells by 14 h. Survivin has been shown to inhibit apoptosis by binding to active caspase-3 and caspase-7 (26). In our study, we observed that treatment of both cells with Baicalein resulted in increased expression of active caspase-3 and caspase-7. These time points correlated with decreased levels of the survivin protein. In addition, we observed decreased PARP levels in PC3 cells after 24-h treatment with Baicalein, which occurs downstream of caspase-3/7 cleavage. These results indicate that caspase-mediated apoptosis, in response to decreases in survivin expression, is responsible for apoptosis observed after 12-LOX inhibition. A recent report has observed a strong association of survivin expression with Bcl-2 expression in cervical carcinoma tissues (37), indicating that survivin may be related to the Bcl family protein expression. Our results support this association, as we observed decreased survivin and

reduced Bcl-2 protein expression in response to 12-LOX inhibition in both cell lines. We also observed decreased levels of phosphorylated Akt-1 in both PC3 and DU-145 cells after treatment with Baicalein or BHPP. Akt activation has been shown previously to induce survival and suppress apoptosis through increased phosphorylation of Bad and subsequent liberation of antiapoptotic proteins of the Bcl family (38). Therefore, decreased expression of Bcl-2 and Bcl-X_L in the cells may also be a result of decreased levels of phosphorylated Akt after 12-LOX inhibition. Interestingly, Akt has also been shown to enhance the translation of cyclin D1 (39), and previous reports have shown that treatment with antisense cyclin D1 resulted in a strong induction of apoptosis in human squamous carcinoma (40). Our results implicate a similar mechanism may be involved in prostate cancer cells, and that Baicalein-induced decreases in phosphorylated-Akt may affect both progression through the cycle and apoptosis in prostate cancer cells. In either case, the fact that two major apoptotic pathways are stimulated simultaneously in response to 12-LOX inhibition highlight its critical role in cell survival.

In summary, we have shown that the 12-LOX pathway of arachidonic acid metabolism regulates cell growth, survival, and apoptosis of human prostate cancer cells. Inhibition of 12-LOX led to growth inhibition associated with a specific G₁ arrest, followed by induction of apoptosis through caspase and Bcl-mediated mechanisms. Therefore, the inhibition of 12-LOX is a potential therapeutic approach in the treatment of prostate cancer.

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