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Zyflamend®, a Unique Herbal Preparation With Nonselective COX Inhibitory Activity, Induces Apoptosis of Prostate Cancer Cells That Lack COX-2 Expression

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Abstract: *Cyclooxygenase (COX) inhibitors have suppressive effects on several types of cancer cells including prostate cancer. In this study, we considered the potential COX-inhibitory activity of a unique anti-inflammatory herbal preparation (Zyflamend®; New Chapter, Inc., Brattleboro, VT) and analyzed its effects on the human prostate cancer cell line LNCaP. COX inhibitory activity of Zyflamend was determined by a spectrophotometric-based assay using purified ovine COX-1 and COX-2 enzymes. Effects of Zyflamend on LNCaP cell growth and apoptosis in vitro were assessed by cell counting, Western blot detection of poly ADP-ribose polymerase (PARP) cleavage, and measurement of caspase-3 activity in treated and control cell extracts. Western blotting techniques were conducted to determine the effects of this herbal preparation on the expression of the cell signaling proteins, p21, androgen receptor (AR), phospho-protein kinase C (pPKC)$_{\alpha/\beta}$, and phospho (p)Stat3. The phospohorylation status of several signal transduction phosphoproteins was profiled using a high-throughput phosphoprotein screening assay in treated cells and compared to controls. Zyflamend dramatically decreased COX-1 and COX-2 enzymatic activity. Elevated p21 expression coincided with attenuated cell growth following treatment of LNCaP cells with Zyflamend. PARP cleavage fragments were evident, and caspase-3 activity was upregulated over the control indicating the ability of Zyflamend to induce apoptosis of these cells. Androgen receptor expression levels declined by 40%, and decreases were observed in the active forms of Stat3 and PKC$_{\alpha/\beta}$ in Zyflamend-treated LNCaP cells. Zyflamend inhibited both COX-1 and COX-2 enzymatic activities, suppressed cell growth, and induced apoptosis in LNCaP cells. However, our data suggests that the effects are likely due to COX-independent mechanisms potentially involving enhanced expression of p21 and reduced expression of AR, pStat3, and pPKC$_{\alpha/\beta}$.*

Introduction

Although progress has been made in the early diagnosis and treatment of prostate cancer (1), it remains the most common malignancy and the second leading cause of male cancer-related deaths in the United States (2). One of the more interesting aspects of this disease is the fact that latent prostate cancer occurs at equal rates in both Asian and American men, whereas the incidence of clinically significant prostate cancer is much greater in the United States than in Asia (3–5). There are many reasons to believe that this discrepancy is related to the dietary intake of different populations, and these observations have stimulated extensive research into various dietary factors that might influence progression of prostate cancer. Some epidemiological studies have suggested this may be in part due to the lower fat intake in Asian diets compared to the typical Western diet, as high-fat diets have been linked to elevated risks of prostate cancer (6,7).

Arachidonic acid and its precursor, linoleic acid, are present in significant quantities in animal fats and a variety of vegetable oils, both of which are generally thought to be consumed in greater quantities in the typical Western diet in comparison to Eastern diets. The elevated intake of these fatty acids provides increased substrate availability for cyclooxygenases (COX), enzymes responsible for converting arachidonic acid into potent signaling molecules termed *prostaglandins.* In addition to their vital role as second messengers in many important biological pathways, prostaglandins have recently been implicated in tumor development, progression, and metastasis (8,9). The COX enzymes that mediate this conversion are represented by two species: COX-1, which is constitutively expressed in many tissues, and COX-2, which is typically induced during disease states such as inflammation and cancer (10). Data

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from many molecular and cellular biology studies have also suggested that the COX-2 gene is an early growth response gene affecting pathways that modulate apoptosis, proliferation, adhesion, angiogenesis, and differentiation (8,11,12).

COX and especially COX-2 inhibition has been a main target for anti-inflammatory drug design for many years; however, the link between COX-2 expression and cancer has only been more recently recognized. Observations from several population-based studies have documented a significant decrease in the risk of colorectal cancer in people who regularly take nonsteroidal anti-inflammatory drugs that have potent COX inhibitory activity (13,14). Histological studies that have followed colorectal tumor development have determined that most human and animal colorectal tumors express elevated COX-2 levels, whereas adjacent normal colorectal epithelial cells have low to undetectable COX-2 levels (15–17). Similar to these observations, several laboratories have also reported that COX-2 expression is elevated in prostate tumor cells during both initiation and progression compared to normal epithelial cells (18,19); however, this finding is controversial (20). It is clear though that several pharmacological COX-2 inhibitory drugs have shown the ability to suppress prostate cancer cell growth in vitro, induce apoptosis, and suppress growth of human prostate tumor xenografts in immunodeficient mouse models or transgenic models of prostate cancer such as the TRAMP mouse (12,21–23). Given the controversy as to whether COX-2 is a factor in prostate cancer development or progression, several of the known COX inhibitors are thought to have a variety of COX-independent anticancer effects, and these actions appear to differ amongst inhibitors (24–30).

In this regard, it is very interesting that many plants that are prominent in regional diets contain substances that have COX inhibitory activity. Extracts from these plants, both in the crude form and as isolated components, have been found to have potent anti-inflammatory and anticancer activities. Salicylic acid, for example, is a traditional inflammatory inhibitor agent found in willow tree bark, and the chemical derivative of this agent, aspirin, remains one of the most commonly used COX inhibitory substances in the world. In this study, we considered the potential of a unique commercially available herbal preparation, Zyflamend®, for its ability to affect COX-1 and COX-2 enzyme activities and influence the behavior of a commonly used human prostate cancer cell model system, LNCaP cells. Zyflamend is comprised of 10 standardized herbal extracts (rosemary, turmeric, ginger, holy basil, green tea, hu zhang, Chinese goldthread, barberry, oregano, and *Scutellaria baicalensis*). Each of these herbs has been shown to contain unique chemical constituents that influence COX activity or expression, and each has been studied for either anti-inflammatory or anticancer activity (31–45). This testing has tended to focus on the predominant compound found in any given herb; however, there is reason to believe that there may be additive benefits in the combination of multiple herbal/dietary agents for diseases such as cancer (46–49). The multiple and chemically diverse constituents present in Zyflamend, each of which is an integral component of the typical Asian diet, may be more effective against prostate cancer than any single herbal extract alone.

Here we present the results of in vitro studies that evaluated the effects of Zyflamend on purified COX-1 and COX-2 enzymes and the human prostate cancer cell line LNCaP. We determined that Zyflamend inhibits cell growth and induces apoptosis in LNCaP cells, and these actions appear to occur independently of COX-2 enzyme inhibition. The results of this study are a critical step toward considering this agent as a chemopreventative or adjuvant therapeutic agent against human prostate cancer.

Materials and Methods

Reagents and Chemicals

Zyflamend was provided by the manufacturer (New Chapter, Inc., Brattleboro, VT). The product is an encapsulated olive-oil based suspension, and experiments were conducted using the liquid material that was directly removed from the capsules. For all of our experiments, the liquid was dissolved in dimethyl sulfoxide (DMSO) at a 1/10 dilution and filtered through a 0.2 µm filter. For descriptive purposes, the Zyflamend solution is expressed as total µl/ml Zyflamend. Compositional analysis of the extract reveals that Zyflamend contains approximately 13 µM curcumin. All controls contained DMSO at similar concentrations. NS-398, indomethacin, and prostaglandin E_2 (PGE_2) were obtained from Cayman Chemical Company (Ann Arbor, MI). Curcumin was obtained from J. T. Baker (Phillipsburg, NJ).

COX Inhibitory Analysis

Zyflamend, indomethacin, and NS-398 were prepared as described previously. The assay was performed according to the procedure outlined by the manufacturer, Cayman Chemical Company (Ann Arbor, MI), for the COX (ovine) inhibitor screening assay. The final dilutions of Zyflamend tested in the assays were 0.45 µl/ml and 0.90 µl/ml, which contain approximately 6 nM and 12 nM curcumin, respectively. Absorbance of the samples correlating to the activity of COX-1 or COX-2 was detected at 600 nm using a 96-well spectrophotometric plate reader (SLT Spectra, Tecan, Mannedorf/Zurich, Switzerland). This assay was completed in duplicate.

Cell Culture

The androgen sensitive human prostate cancer cell line LNCaP was purchased from the American Type Culture Collection (Manassas, VA). LNCaP cells were maintained in RPMI-1640 media supplemented with 10% fetal bovine serum, L-glutamine, antibiotics (Gibco® Invitrogen Corporation, Carlsbad, CA), and the synthetic androgen R1881 (0.05 nM; Perkin Elmer Life Sciences, Wellesley, MA). The cells

were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell Proliferation and Viability Assays

LNCaP cells were seeded in 12-well plates at a density of 38,000 cells per well in a final volume of 1 ml. At 24 h after seeding, the medium was removed and replaced with fresh medium containing the same concentration of DMSO (0.1%) as a vehicle control or with medium containing Zyflamend at concentrations of 0.1 µl/ml, 0.05 µl/ml, or 0.001 µl/ml in a final volume of 1 ml. The cultures were maintained in the incubator for a period of 3 days. At the end of the 24-, 48-, and 72-h time periods, cells were harvested, trypsinized, and counted using a hemocytometer. Trypan blue dye exclusion (Sigma-Aldrich, St. Louis, MO) was also performed to ensure that cell viability was 98% or greater on plating. The cell counting experiments were repeated four times.

Poly ADP-Ribose Polymerase (PARP) Immunoblotting

LNCaP cells were treated with 0.1 µl/ml Zyflamend for 24, 48, and 72 h. Cells were washed twice in ice cold phosphate buffered saline (PBS); scraped into a buffer containing 50 mM Tris HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), and 5% glycerol with a 1× protease inhibitor cocktail (Sigma, Inc., St. Louis, MO); and lysed via probe sonication and incubation on ice for 10 min. The lysates were then centrifuged (15,000 *g*) for 10 min at 4°C to remove insoluble debris. Protein quantification was conducted using the BioRad D_C protein assay (BioRad, Inc., Hercules, CA). Equal quantities of protein were electrophoresed through 7.5% polyacrylamide gels, transferred to polyvinylidene diflouride membranes, and immunoblotted according to standard methods. Following a 2-h incubation in a 6% nonfat dry milk blocking buffer prepared in TBST (tris buffered saline plus 0.1% tween 20, pH 7.8), the membranes were probed with a rabbit polyclonal antibody against human PARP (Roche Applied Science, Indianapolis, IN) diluted 1/2000 in blocking buffer. Protein bands were detected by the enhanced chemiluminesence procedure using luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA) as described by the manufacturer.

Active Caspase-3 Activity Assay

Apoptotic activity was determined via a colorimetric assay measuring caspase-3 activity (Clontech, Palo Alto, CA). LNCaP cells were treated with 0.1 µl/ml Zyflamend as described previously for 72 h. As a positive control, LNCaP cells were treated for 24 h with 10 nM 12-O-tetradeconoylphorbol-13-acetate (TPA), a known inducer of apoptosis in this cell line. Cells were then trypsinized, counted, and processed according to the manufacturer.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) of COX-2 in LNCaP Cells

To determine if the COX-2 message is expressed in LNCaP cells, RT-PCR was conducted using two different sets of COX-2 specific primers (50,51). RNA was isolated from LNCaP cells in Tri Reagent (Sigma), and then reverse transcribed using SuperScript™ III Reverse Transciptase (Invitrogen). cDNA from normal human prostate epithelial cells (Cambrex, Walkersville, MD) was also analyzed for comparative purposes. The PCR reaction was conducted using DNA *Taq* polymerase (New England Biolabs, Ipswich, MA) as follows: 1) 95°C for 3 min; 2) 95°C for 20 s, 50°C for 30 s, 72°C for 40 s for 42 cycles; and 3) 72°C for 10 min. Following the reaction, samples were electrophoresed on a 1% agarose gel containing ethidium bromide, and amplicons were visualized under ultraviolet light.

PGE_2 Add-Back Assay

To gain evidence of the potential involvement of COX inhibition in Zyflamend-induced apoptosis, LNCaP cells were treated with the herbal COX inhibitor in conjunction with PGE_2, the main prostaglandin produced following COX activity. LNCaP cells were plated to 60% confluency in 25 cm^2 flasks and exposed to 0.1 µl/ml Zyflamend alone or in combination with freshly diluted PGE_2 (1 nM or 10 nM) for 24 h. The cells were then collected and processed for flow cytometric analysis as follows. Adherent cells were trypsinized and pooled with the cells in suspension, centrifuged, and washed thrice with ice cold PBS. The cell count of each sample was adjusted to 500,000 cells per ml and fixed in a 2:1 ratio (vol/vol) in chilled ethanol overnight before staining with propidium iodide (PI) in the presence of RNAse. Cell cycle distribution was analyzed on a Becton Dickinson Flow Cytometer (Becton Dickinson, San Jose, CA), and at least 10,000 cells were analyzed for each experimental condition. Data analysis was performed using the CellQuest Pro cell cycle analysis software (Becton Dickinson). The percentage of the cell population that partitioned out into the SubG0 fraction, indicating cells undergoing apoptosis, was determined for each treatment. The experiment was performed in duplicate, and a Student's *t*-test was utilized to compare the percentage of cells undergoing apoptosis in the cells treated with both Zyflamend and PGE_2 to cells treated with Zyflamend alone.

Phosphoprotein Cell Signaling Analysis

To determine the possible involvement of Zyflamend on signal transduction phosphoproteins in LNCaP cells, protein phospho-site screening using the Kinetworks™ technology was conducted on 31 phosphorylation sites of 26 different cell signaling proteins (Kinexus, Inc., Vancouver, CA). LNCaP cells were treated with a concentration of 0.1

µl/ml Zyflamend or vehicle for 24 h. Protein lysates were prepared in a 1x lysis buffer plus protease and phosphatase inhibitors [20 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 5 mM ethylenediaminetetraacetic acid (EDTA), 2 mM ethyleneglycol-*bis*(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), 30 mM NaF, 20 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM phenlymethylsulfonylfluoride, 40 mM β-glycerophosphate, 5 µM pepstatin A, 10 µM leupetin, and 0.5% Nonidet P-40]. The harvested lysates were then quantitated as described previously, diluted in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, and submitted to the company for analysis. The screening assay was repeated twice using two different batches of treated cells to verify the results.

Western Immunoblotting for Cell Signaling and Cell Cycle Regulators

Following a 24-h exposure to 0.1 µl/ml Zyflamend, LNCaP cellular lysates were prepared as described earlier, electrophoresed through 10% polyacrylamide gels, transferred to polyvinylidene difluoride membranes, and immunoblotted according to standard methods. The phospho-Stat3 (pStat3; Ser 727) and phospho-protein kinase C (p-PKC)$_{\alpha/\beta}$ (Thr 638/641) antibodies were purchased from Cell Signaling Technology (Beverly, MA), and both were diluted 1/1,000 prior to use. The p21 (clone 187) and androgen receptor (clone 441) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at dilutions of 1/100 and 1/250, respectively. All antibodies were diluted in blocking buffer (6% nonfat dry milk in TBST).

Results

Zyflamend Inhibits COX-1 and COX-2 Enzyme Activities in a Biochemical-Based Assay

The ability of Zyflamend to inhibit COX activity was analyzed using a colorimetric screening assay with purified ovine COX-1 or COX-2 enzymes. Results shown in Table 1 demonstrate that the two different concentrations of Zyflamend tested significantly inhibited COX-2 activity to an extent that was greater than the inhibitory concentration 50% (IC_{50}) of NS-398, a specific COX-2 inhibitor. Zyflamend also inhibited COX-1 activity in a similar manner to the IC_{50} of indomethacin. These results suggest that Zyflamend is a general COX-inhibitory agent.

Zyflamend Decreases the In Vitro Growth of the Prostate Cancer Cell Line, LNCaP, by Affecting Proliferation and Apoptosis

Zyflamend (0.1 µl/ml) was found to significantly inhibit the growth of LNCaP cells over a 72-h time period compared to untreated, control cells ($P = 0.01$; Fig. 1). Lower doses tested did not have a significant growth inhibitory effect on the LNCaP cells. To determine if the observed decrease in cell growth was due, in part, to proapoptotic stimuli elicited by Zyflamend, we performed Western blot analysis to detect the presence of the cleaved form of PARP, an indicator of caspase activation. PARP cleavage was evident in the cells treated with 0.1 µl/ml Zyflamend for 72 h indicating that Zyflamend induced apoptosis in LNCaP cells (Fig. 2). Direct evaluation of caspase-3 activity was performed using a colorimetric substrate assay. Caspase-3 activity in LNCaP cells was increased by 17-fold [standard error of the mean (SEM) ± 0.110] following a 72-h incubation with Zyflamend (0.1 µl/ml). A known inducer of apoptosis in the LNCaP cell line, TPA, was used as a positive control in this experiment and induced caspase-3 activity by 12-fold (SEM ± 0.005) over control levels. The presence of PARP cleavage products and elevated caspase-3 activity indicate that Zyflamend is inducing apoptosis in LNCaP cells.

Table 1. Inhibition of Cyclooxygenase (COX) Activity by Zyflamend[a]

	Percent Inhibition	
	COX-1	COX-2
Zyflamend (0.90 µl/ml)	73.8 ± 1.83	85.7 ± 5.60
Zyflamend (0.45 µl/ml)	36.5 ± 10.46	80.9 ± 12.00
NS-398 (0.15 µM)	ND	52.5 ± 21.26
Indomethacin (6 µM)	45.0 ± 23.32	58.0 ± 13.18

a: Zyflamend inhibits both COX-1 and COX-2 enzyme activity as determined in a colorimetric screening assay using purified ovine COX-1 and COX-2 (Cayman Chemical, Ann Arbor, MI). Two dilutions of Zyflamend (0.90 µl/ml and 0.45 µl/ml) were compared to a specific COX-2 inhibitor, NS-398 [inhibitory concentration of 50% (IC_{50}) = 0.15 µM], and a general COX inhibitor, indomethacin (COX-1 IC_{50} = 6 µM). The findings are reported as means ± standard error of the mean; $n = 3$ for all data points. Abbreviation is as follows: ND, not determined.

COX-2 Expression Is Not Detected in LNCaP Cells

Given the controversy in the literature as to whether the LNCaP cells express COX-2 enzyme, we also felt it was appropriate to determine if the LNCaP cells that were employed in our experiments showed evidence for the expression of this enzyme. We used two different oligonucleotide primer sets specific for human COX-2 in an RT-PCR procedure and applied these primers to cDNA prepared either from LNCaP cell RNA or from cultured normal human prostate epithelial cell (hPEC) RNA. As is shown in Fig. 3A, we were unable to detect amplification of COX-2 specific cDNA fragments from reverse transcribed LNCaP messenger (m)RNA, whereas both of the primer sets were readily able to identify the presence of appropriate-sized COX-2 cDNA fragments from reverse transcribed hPEC mRNA.

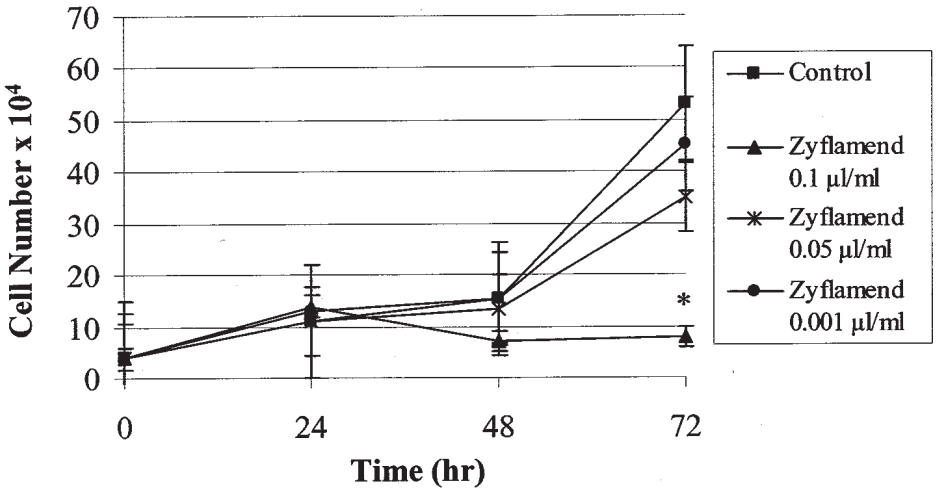

Figure 1. Growth inhibition of LNCaP cells following exposure to Zyflamend. LNCaP cells were seeded at a density of 38,000 cells per well in 12-well plates and treated in duplicate with 0.1 µl/ml Zyflamend, 0.05 µl/ml Zyflamend, 0.001 µl/ml Zyflamend, or vehicle control (0.1% DMSO). Five separate cell counts of each well were obtained for all treatments at 24, 48, and 72 h. Data points are reported as mean and standard error of the mean. $*P \leq 0.0001$ as determined by Student's *t*-test. This experiment was repeated four times with similar results.

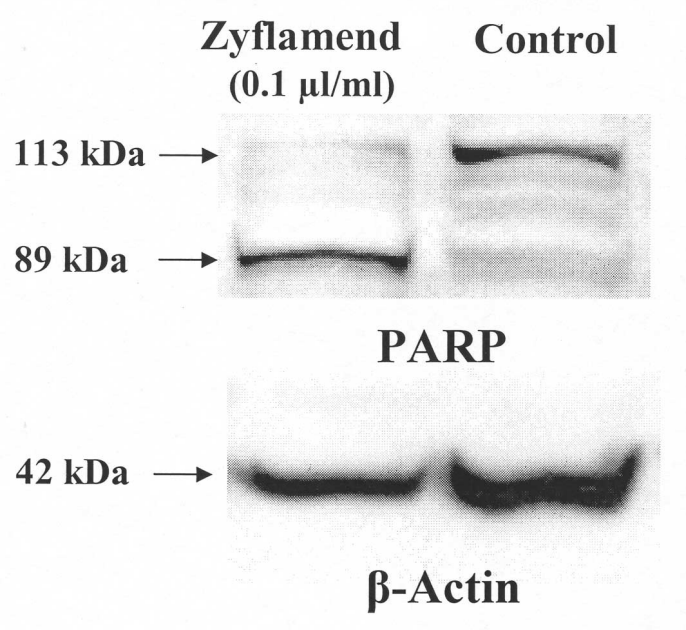

Figure 2. Zyflamend induces apoptosis in LNCaP cells. Cells were treated with 0.1 µl/ml Zyflamend or vehicle alone (0.1% DMSO) for 72 h. Cell lysates were prepared as described previously (see **Materials and Methods**), followed by SDS PAGE and Western analysis. Membranes were probed with poly ADP-ribose polymerase (PARP) antibody, and the presence of the immunoreactive PARP cleavage product at 89 kDa indicated cells were undergoing apoptosis. Membranes were stripped and reprobed with β-actin as a lane loading control. Blots are representative of two individual experiments.

To further determine if Zyflamend-induced apoptosis in LNCaP cells was dependent on COX inhibition, we tested whether the primary product of COX-2 enzyme activity, PGE_2,could suppress this response. As is shown in Fig. 3B, even at a concentration of PGE_2 as high as 10 nM, it was unable to suppress the apoptosis induced by treatment of LNCaP cells with Zyflamend at 0.1 µl/ml for 24 h. These results support the idea that induction of apoptosis by Zyflamend is independent of COX-2 enzyme or the products of either COX-1 or COX-2 in LNCaP cells.

Zyflamend Increases Expression of the Cell Cycle Inhibitory Protein p21 and Decreases Expression of Androgen Receptor, Phosphorylated Stat3, and Phosphorylated PKC$_{\alpha/\beta}$ in LNCaP Cells

To investigate potential molecular effectors involved in the antiproliferative and proapoptotic activities of Zyflamend in LNCaP cells, we considered its influence on the expression and phosphorylation of several important cell cycle regulatory and cell signaling molecules. Western blot analysis

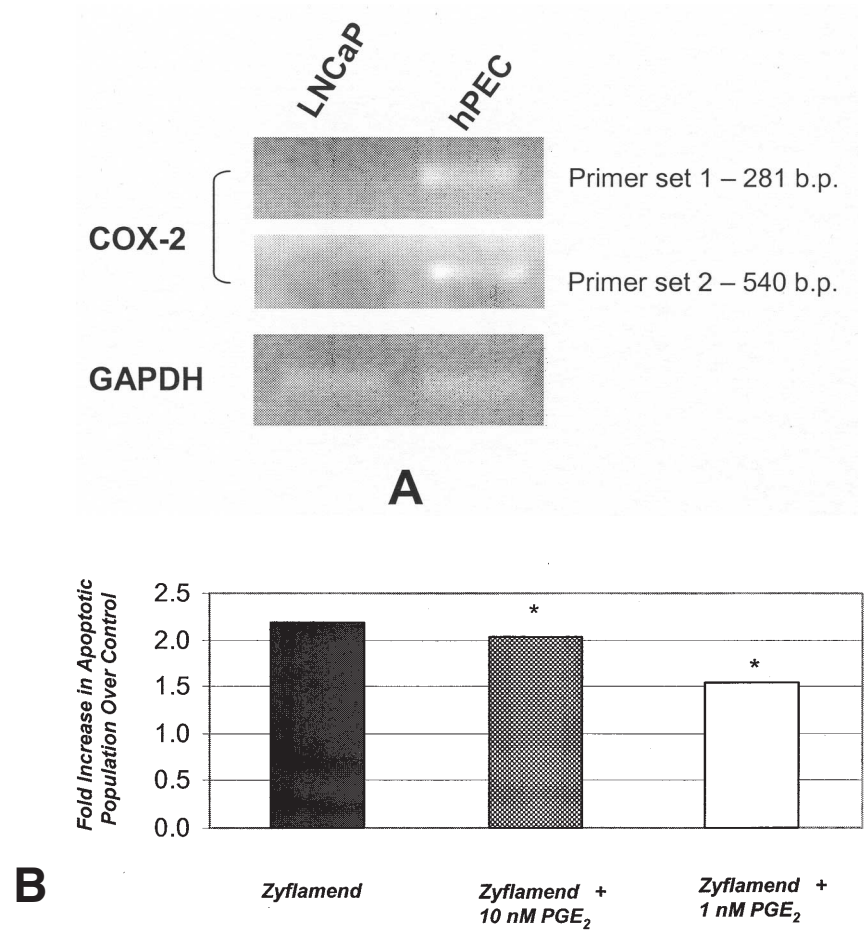

Figure 3. Cyclooxygenase (COX)-2 expression is not detectable in LNCaP cells, and inhibition of COX activity does not appear to be responsible for Zyflamend-induced apoptosis. A: Representative analyses of COX-2 mRNA expression in LNCaP and human prostate epithelial cells (hPECs) using reverse transcription polymerase chain reaction with COX-2 specific primers (46,47). No COX-2 messenger RNA was detectable in any of the LNCaP samples. Detection of GAPDH was utilized for normalization. These results are representative of three individual experiments. B: Flow cytometric analysis of LNCaP cells treated with 0.1 µl/ml Zyflamend alone or in conjunction with prostaglandin E_2 (PGE_2; 1 nM and 10 nM) for 24 h. Cells undergoing apoptosis were identified as the SubG0 population of each sample. Flow cytometric analysis of each sample was conducted in duplicate. *$P > 0.05$, no significant difference from Zyflamend alone.

demonstrated that within 24 h, Zyflamend (0.1 µl/ml) induced the expression of the cyclin dependent kinase inhibitor protein p21, which is known to suppress transition of cell cycle into the S-phase (Fig. 4). Concurrently, expression of androgen receptor (AR) protein was observed to decrease by 39.5% as determined by densitometric analysis of a Western blot probed with an antibody to this protein (Fig. 4). The phosphorylation status of several cell-signaling proteins in Zyflamend-treated LNCaP cells was compared to untreated controls by a commercially performed proteomic analysis of 31 different phosphorylation sites on 26 cell-signaling proteins. Following 24-h treatment of LNCaP cells with Zyflamend (0.1 µl/ml), marked changes were observed in the phosphorylation status of several prominent signaling intermediate molecules including a greater than 60% increase in c-Jun phosphorylation (Ser 73) compared to the control (Table 2). These results were reproduced in two independent screens. To certify some of these changes identified in the screen, we conducted Western blot studies of two particular molecules, Stat3 (Ser 727 phosphorylation reduced) and $PKC_{\alpha/\beta}$ (Thr 638/641 phosphorylation reduced). The Western blotting results confirmed the downregulation of these two phosphorylation events in Zyflamend-treated LNCaP cells (Fig. 5).

Discussion

Numerous epidemiological studies have supported the idea that the diets associated with an Eastern lifestyle may be protective against prostate cancer. The commercial dietary supplement Zyflamend represents a standardized herbal extract from 10 different botanicals that are commonly found in the Eastern diet. Whereas each of the herbs that are used in the formulation of Zyflamend are known to contain unique anti-inflammatory and anticancer compounds, a dietary supplement with a diverse chemical profile may better represent the benefits of an Eastern diet than any individual supplement alone.

One common property of each component of Zyflamend is the ability to influence arachidonic acid metabolism. COX, critical enzymatic mediators of the arachidonic acid metabo-

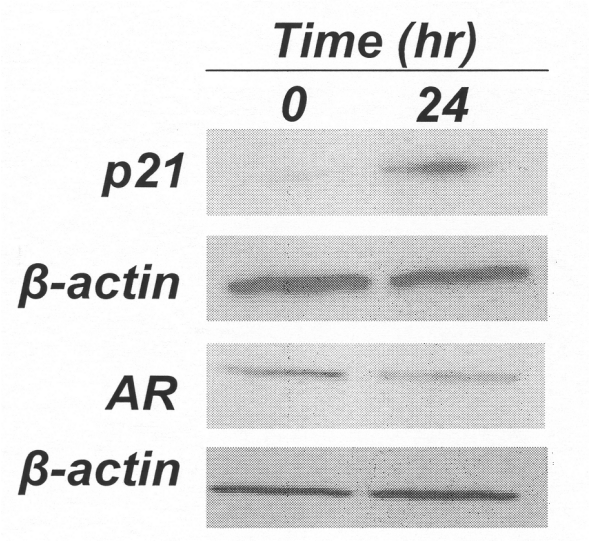

Figure 4. Expression of p21 increased and androgen receptor (AR) protein expression decreased in LNCaP cells following 24-h exposure to Zyflamend. Cells were treated with Zyflamend (0.1 µl/ml) for 24 h. Cell lysates were prepared as described previously (see **Materials and Methods**) followed by SDS PAGE and Western analysis. Membranes were probed with either p21 or AR antibody and then stripped and reprobed with β-actin antibody as a lane-loading control. Detection of β-actin was utilized for normalization. These results are representative of two individual experiments.

lism/inflammatory cascade, have been increasingly under scrutiny as targets for the development of cancer preventative or therapeutic strategies. This is particularly true for COX-2, the inducible form of this family, as its expression and activity have been associated with the development and progression of various human cancers. Data from preclinical testing of COX inhibitory agents, in particular those that selectively target COX-2, using prostate cancer models seem to support the idea that inhibition of COX enzymatic activity may also be a useful strategy in prostate cancer prevention and treatment (22,23,52,53). COX inhibitors have been shown to block cell growth and induce apoptosis in prostate cancer cells as well as suppress tumor growth in prostate cancer xenograft models (12,21–23).

However, it is also important to consider that COX and COX-2 inhibitory agents have alternative mechanistic actions that are not dependent on COX-2, as these agents have demonstrated growth inhibitory and proapoptotic activities in both COX-2 deficient cancer cell lines and tumor xenografts (28,54–57). Several different potential mechanisms of action for these COX-independent activities have been proposed and include inhibition of cGMP-specific phosphodiesterases (PDE2, PDE5) (58), reduction of antiapoptotic factor BCL-xL (59), and inhibitor of κB (IκB) kinase β (IKKβ) resulting in suppression of NF-κB signaling (60). Even though we have shown here that Zyflamend has the ability to suppress COX-1 and COX-2 enzymatic activity, the effects of this herbal agent on LNCaP cells are likely to be independent of these enzymes. This conclusion is based on both our RT-PCR analysis, which suggests that LNCaP cells do not express significant levels of the mRNA encoding the COX-2 protein, and our data indicating that PGE_2, the end product of COX-1 and COX-2 enzymatic activity, was unable to suppress the ability of Zyflamend to induce apoptosis in these cells.

Indeed, the question as to whether COX-2 plays a role in prostate cancer development and progression remains a controversial area. Whereas some studies have reported widespread expression of COX-2 in prostate cancer cell lines and in various stages of prostate tumorigenesis (18,19,52,61), other laboratories have undertaken similar studies using different antibodies to assess COX-2 expression and have come to strikingly opposing conclusions (62,20). This confusion extends to the LNCaP cell line because several investigators have described COX-2 expression in these cells, whereas a different study using both Northern and Western analysis techniques (Western conducted with three different anti-COX-2 antibodies) did not identify COX-2 immun-

Table 2. Protein Phosphorylation Screening Data From LNCaP Cells Treated With Zyflamend[a]

		% Change in Treated Samples Relative to Controls	
Protein	Phosphorylation Site	Screen 1	Screen 2
$PKC_{\alpha/\beta}$	T 638	–23	–65
Stat3	S 727	–21	–37
$Adducin_{\alpha}$	S 724	–58	–32
$GSK3_{\alpha}$	Y 279	–36	–100
c-Jun	S 73	62	102
PKB_{α}	S 473	70	55
PKB_{α}	T 308	43	16

a: Abbreviations are as follows: LNCaP, androgen-responsive lymph node-prostate cancer; PKC, protein kinase C; GSK, glycogen synthase kinase-3; PKB, protein kinase B (Akt). Alterations in phosphorylation status of signal transduction phosphoproteins in LNCaP cells treated with Zyflamend compared to untreated controls. Following treatment of LNCaP cells with 0.1 µl/ml Zyflamend or vehicle control (0.1% dimethyl sulfoxide) for 24 h, protein lysates were prepared, quantitated, and submitted for analysis. The screening assay was repeated twice using two separate batches of treated cells to verify the results. Data from both screens are listed as percent change in phosphorylation status of treated samples in comparison to vehicle controls.

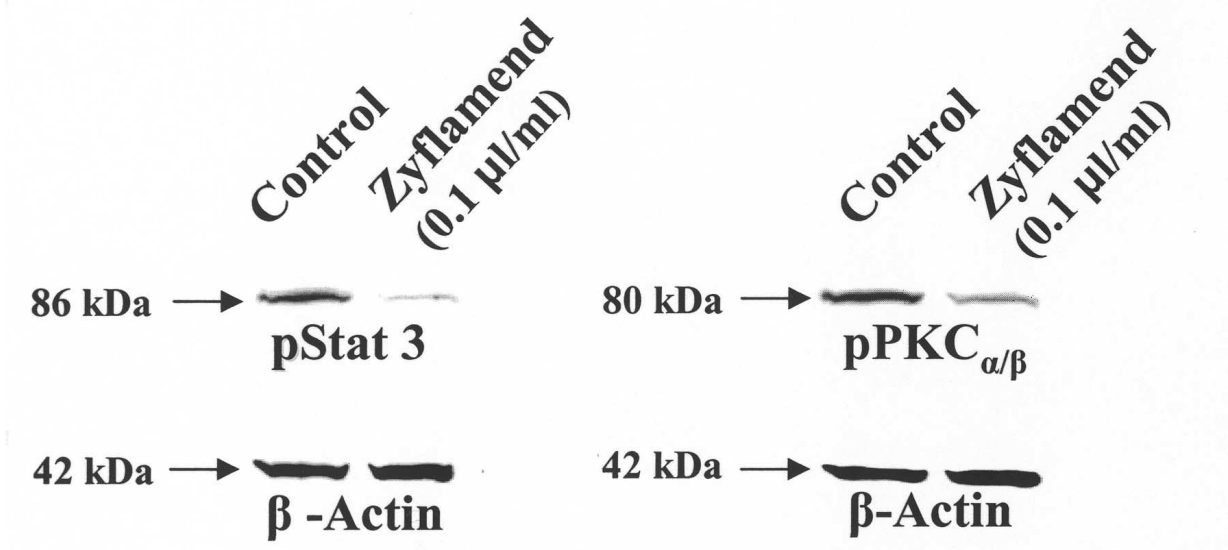

Figure 5. Western blot analysis confirms decreased protein levels of phospho-Stat3 (pStat3) and phospho-protein kinase C (p-PKC)$_{\alpha/\beta}$ in LNCaPs treated with Zyflamend (0.1 µl/ml). Cell lysates were prepared as described previously (see **Materials and Methods**) followed by SDS PAGE and Western analysis. Membranes were probed with pStat3 (Ser 727) and pPKC$_{\alpha/\beta}$ (Thr 638/641) antibodies specific for the same phosphorylation sites analyzed in the phospho-site screen then stripped and reprobed with β-actin antibody as a lane-loading control.

oreactivity (20). Our results strongly support the latter conclusion, as we were unable to amplify up fragments of the COX-2 transcript from LNCaP cDNA. Due to the apparent lack of COX-2 expression in the LNCaP cells, we believe that this cell line provides a unique prostate cancer cell model to study the potential COX-2 independent mechanisms of action of both pharmaceutical and herbal COX-2 inhibitors against prostate cancer, and Zyflamend offers a model nutriceutical agent for these kinds of studies.

In our experimental analysis of the effects of Zyflamend on gene activity in LNCaP cells, we observed an elevation in expression of the cell cycle inhibitory protein p21, which could have likely contributed to the growth inhibition observed. Our results corroborate the data reported recently by another group that demonstrated p21 expression was elevated in human colon cancer cells following exposure to the COX-2 specific inhibitor, celecoxib, in a COX-2-independent manner (29).

As well, our analysis of the effect of Zyflamend on gene expression showed that it suppressed expression of AR in LNCaP cells. Prostate cancer is a disease that is promoted by androgen action, and prostate cell growth and survival is supported by androgens. Thus, the finding that Zyflamend, like some other more specific COX inhibitory agents, downregulates AR expression also suggests a potential mechanistic effect through which Zyflamend might specifically influence prostate cancer development and growth. The exact mechanism of action involved in the reduction of AR expression in LNCaP cells following Zyflamend exposure is unknown at this time. However, nonsteroidal, anti-inflammatory drugs (NSAIDs) have been previously reported to reduce both the expression and activity of AR in LNCaP cells, and this action was suggested to be mediated through NSAID-induced c-Jun activation independent of COX (63). Data in the literature has suggested that the activated protein 1 (AP-1) transcription factor, which is comprised of a heterodimer of c-Jun and c-Fos, can inhibit AR expression via binding to AP-1 binding sites in the promoter region of the AR gene (63,64). We also observed a markedly increased level of phospho-cJun, the active form of this protein, in cells treated with Zyflamend compared to control cells, which may play such a role in the reduction of AR expression levels in Zyflamend-treated LNCaP cells.

Our phosphoprotein screening data reproducibly identified changes in the phosphorylation status of several prominent cell signaling molecules in LNCaP cells including reduction in the phosphorylation of Stat3 and PKC$_{\alpha/\beta}$ following treatment with Zyflamend. Stat3 is a latent transcription factor that mediates cytokine signals from the cell membrane to the nucleus and is activated by phosphorylation. The protooncogene Stat3 transduces interleukin (IL)-6 signaling and is required for IL-6/gp 130-mediated transformation of normal cells (65,66). Stat3 has been observed to be constitutively expressed in the majority of prostate tumors and prostate cancer cell lines, including LNCaP cells, at levels that appear to correlate to degree of malignancy, and inhibition of Stat3 induces apoptosis of LNCaP cells (67). The decrease in active Stat3 levels observed in the LNCaP cells following treatment with this herbal preparation may contribute to Zyflamend-induced apoptosis.

Active pPKC is involved in a multitude of cellular responses including alterations in cell cycle progression, survival, and transformation. The outcome of this signaling pathway appears to be dependent on many factors including the exact isozyme involved as well as the cellular environment. In fact, depending on the time of PKC activation, the result may either promote or inhibit cell cycle progression

(68,69). We observed a dramatic reduction in the level of $pPKC_{\alpha/\beta}$ in LNCaP cells following Zyflamend treatment, which was concomitant with the induction of apoptosis by this agent. The significance of this outcome is yet to be understood; however, elevated PKC expression has been correlated to the development of androgen independent prostate cancer (70,71). Additionally, patients with tumors demonstrating high levels of PKC expression have been found to have shorter survival time on relapse (71). Although further experimentation is needed, inhibition of PKC_{α} activation has been suggested to be a potentially effective drug target for the prevention of androgen insensitive disease (70).

Surprisingly, phospho-Akt levels increased in the Zyflamend-treated LNCaP cells. This was unexpected, as Akt activation is generally thought to result in a procell survival response (72). However, it is possible that the observed elevation in pAkt could be a "last attempt" of the cancer cell to survive. This sort of stress-mediated activation of the phosphoinositide 3-kinase (PI3K) pathway has been observed in several other experimental systems including renal tubular epithelial cells in which pAkt levels were found to increase on serum starvation and in mouse 3T3 fibroblasts stressed with cytotoxic agents such as H_2O_2 (73,74). Activation of proliferative signaling mechanisms under stress conditions are quite possibly an attempt of the cells to sustain cell number.

Our results demonstrate that Zyflamend, a unique herbal extract preparation, inhibits COX-1 and COX-2 activity, strongly suppresses cell growth, and induces apoptosis in LNCaP cells. Cumulatively, these results support the idea that Zyflamend might have some value in chemoprevention or adjuvant therapy for prostate cancer patients. This would be consistent with the testing of various nonselective COX and selective COX-2 inhibitory agents (sulindac, NS-398, and celecoxib) for this purpose; however, data from recent trials of selective COX-2 inhibitors suggest that use of these agents might have adverse cardiovascular effects (75). It is of interest to note that the more widely utilized pan COX inhibitor, aspirin, is not associated with these negative side effects and in fact has a well established beneficial effect for persons with cardiovascular disease (76,77). In this sense, Zyflamend has a biochemical action profile that more resembles aspirin than selective COX-2 inhibitors. Furthermore, the fact that Zyflamend is derived from natural herbal sources and is readily available in health food and nutritional supplement stores make it a potentially more convenient and desirable means to target the enormous population that is susceptible to prostate cancer. We anticipate clinical trials that might test this as well as other naturally derived pan COX inhibitory agents against prostate and other cancers.

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