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Pomegranate Fruit Extract Impairs Invasion and Motility in Human Breast Cancer

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Abstract

Purpose. Pomegranate fruit extracts (PFEs) possess polyphenolic and other compounds with antiproliferative, pro-apoptotic and anti-inflammatory effects in prostate, lung, and other cancers. Because nuclear transcription factor-κB (NF-κB) is known to regulate cell survival, proliferation, tumorigenesis, and inflammation, it was postulated that PFEs may exert anticancer effects at least in part by modulating NF-κB activity. *Experimental design*. The authors investigated the effect of a novel, defined PFE consisting of both fermented juice and seed oil on the NF-κB pathway, which is constitutively active in aggressive breast cancer cell lines. The effects of the PFE on NF-κB–regulated cellular processes such as cell survival, proliferation, and invasion were also examined. *Results*. Analytical characterization of the bioactive components of the PFE revealed active constituents, mainly ellagitannins and phenolic acids in the aqueous PFE and conjugated octadecatrienoic acids in the lipid PFE derived from seeds. The aqueous PFE dose-dependently inhibited NF-κB–dependent reporter gene expression associated with proliferation, invasion, and motility in aggressive breast cancer phenotypes while decreasing RhoC and RhoA protein expression. *Conclusion*. Inhibition of motility and invasion by PFEs, coincident with suppressed RhoC and RhoA protein expression, suggests a role for these defined extracts in lowering the metastatic potential of aggressive breast cancer species.

Keywords

breast cancer, cancer prevention, in vitro studies, *Punica granatum*

Background and Introduction

Chemopreventive agents, tamoxifen and raloxifene, have been shown to reduce the risk of estrogen receptor (ER) positive breast cancers by 50% in high-risk women but, unfortunately, their use is associated with major side effects. In addition, they do not prevent estrogen receptor negative breast cancers.1,2 Over the past decade, the use of plant-derived natural products for the prevention and treatment of cancer has increased in the United States.3-5 Pomegranate fruit extracts (PFEs) in particular appear to represent promising cancer prevention agents that could serve as potentially effective yet nontoxic alternatives or adjuncts to the use of conventional selective estrogen receptor modulators (SERMs) for breast cancer prevention.

Some of the initial studies on PFEs were conducted by Mehta and Lansky,[6] who demonstrated that extracts derived from pomegranates had significant concentration-dependent antiproliferative and pro-apoptotic effects against MCF-7 (ER+) and MD-MBA-231 or MD-MBA-435 (ER–) breast cancer cell lines. Subsequently, it was shown that treatment of highly aggressive human PC-3 prostate cancer cells with PFEs resulted in concentration-dependent inhibition of cell growth and induction of apoptosis.[7] Oral administration of PFEs to athymic nude mice implanted with androgen-sensitive prostate cancer cells, for example, resulted in a significant inhibition of tumor growth, concomitant with a significant decline in serum prostate-specific antigen (PSA) levels.[8] The same group also showed that a PFE was associated with modulation of nuclear factor-κB (NF-κB) activation in dermal keratinocytes.[9] The anticancer effects of aqueous phase PFEs are thought to result

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from polyphenols such as ellagic acid and punicalagin that contribute to >50% of the antioxidant activity of the fruit. These studies directly led to the subsequent design of a phase II clinical trial for men with rising PSA after surgery or radiotherapy. Eligible patients had a detectable, but low PSA (>0.2 and <5 ng/mL). Patients were treated with 8 oz of oral pomegranate juice daily until disease progression. Mean PSA doubling time significantly increased with treatment (ie, progression of disease was delayed), from a mean of 15 months at baseline to a remarkable 54 months posttreatment. In addition, the serum of patients treated with pomegranate juice showed antiproliferative and pro-apoptotic effects in vitro,[10] which is indirect yet convincing evidence for absorption of pomegranate-derived compounds with antiproliferative potential.

Thus, PFEs appear to be promising in cancer prevention. In particular, because NF-κB activation is linked to ER− breast cancer, we postulated that an inhibitor of NF-κB activation would be an effective chemopreventive for ER− tumors.[11] Encouraged by the recently reported effects of PFEs on prostate and lung cancer,[12] we decided to study the effects of PFEs on breast cancer cells in vitro.

Materials and Methods

Preparation and Characterization of PFEs for In Vitro Assessments

Concentrated pomegranate juice, primarily of the Wonderful cultivar, was obtained from Stieb's Pomegranate Products of Visalia, California. Pomegranate extract was obtained using a method previously described to obtain the pomegranate extract (PFEa) used in the experiments.[13]

Pomegranate seeds were obtained from Wonderful pomegranates organically grown on Kibbutz Sde Eliahu, Israel. The seeds were washed clean of debris and dried in the sun. The lipid PFE (PFE_l) was obtained from these seeds by supercritical fluid extraction at 40°C and 500 bar using pure CO_2 by Valensa, Inc. of Eustis, Florida.

Reverse-phase high-performance liquid chromatography (HPLC) electrospray ionization tandem mass spectrometry (MS) was used for detection and quantification of compounds in the ethyl acetate partition of the pomegranate product (PFE_a) by comparison with authentic standards. Corilagin, *trans-p*-coumaric acid, protocatechuic acid, punicalagin A, and punicalagin B were obtained from ChromaDex (Irvine, CA); caffeic acid, chlorogenic acid, ellagic acid, ferulic acid, and gallic acid were obtained from LKT Laboratories (St Paul, MN); and 5-(hydroxymethyl)furfural was obtained from Sigma-Aldrich (St Louis, MO). A Micromass Quatro Ultima tandem mass spectrometer (Waters Corp, Milford, MA) equipped with an Agilent 1100 HP binary pump high-pressure LC inlet was used in these studies. The mobile phase consisted of a gradient of 0.5% formic acid in H_2O and HPLC-grade methanol, obtained from Fisher Scientific (Fair Lawn, NJ). Caffeic acid, corilagin, *trans-p*-coumaric acid, gallic acid, punicalagin A, punicalagin B, and 5-hydroxymethylfurfural were separated using a Synergi 4u Polar-RP 80A (2 × 150 mm, 4 μm) LC column (Phenomenex, Torrence, CA). Ellagic acid, ferulic acid, and protocatechuic acid were separated using a Gemini 5u C18 10A (2 × 150 mm, 5 μm) LC column (Phenomenex, Torrence, CA). Compounds were detected using negative electrospray ionization in a multiple reaction monitoring mode. Calibration curves were obtained by duplicate injections of each of 6 separate concentrations. The ethyl acetate (EtOAc) partition was freeze-dried and reconstituted with 1% formic acid in water with methanol (50:50) and injected in triplicate. Quantitation of individual compounds was determined from peak areas by using the equation for linear regression obtained from the calibration curve.

The EtOAc partition used in these studies was found to contain the following: caffeic acid (0.24%), corilagin (0.10%), *trans-p*-coumaric acid (0.024%), ferulic acid (0.86%), gallic acid (16.41%), 5-hydroxymethylfurfural (5.19%), protocatechuic acid (0.034%), and punicalagins A and B (0.42%). The numbers in parentheses represent percentage of extract composition determined by relative peak area. Ellagic acid, a major constituent of pomegranate juice, was not detectable in the EtOAc acetate partition.

The PFE_l (lipid fraction, pomegranate seed oil) was analyzed in 2 steps. The first, quantification of fatty acids and assessment of the relative proportion of phospholipids, monoacylglycerols, diacylglycerols, and triacylglycerols was performed under the supervision of Dr Claire Reid (Mylnefield Lipid Analysis Unit, Scottish Crop Research Institute, Invergowrie, Dundee, Scotland) using gas chromatography/mass spectrometry (GC/MS). The second phase, quantification of sterols, was performed by Professor Zeev Wiesman (Ben Gurion University, Beersheva, Israel) using GC/MS as described. Quantification of 17-α-estradiol was accomplished with MS by Dr Mark Rogers, M-Scan, Inc (Westchester, PA). Quantification of γ-tocopherol was achieved through GC/MS by the Israel Institute of Biological Research (Nes Ziona, Israel). In short, the analysis revealed about 95% fatty acids, 99% of which occurred as triacylglycerols. Of the 95%, about 81% consisted of conjugated octadecatrienoic acids, most of which is punicic acid. In all, 12 different species of phytosterol were identified, the most prevalent being stigmasterol, α-sitosterol, and campesterol. About 1% of the oil was shown to consist of tocopherols, especially γ-tocopherol, and a significant trace amount (<1%) of α-estradiol identified.

Cell Lines

We chose to use "aggressive" breast cancer cell lines MDA-231 and SUM 149. Both cell lines are estrogen receptor/progesterone receptor negative (ER−/PR−) and are associated

with aggressive cancer phenotypes in vitro with increased invasion, motility, and constitutive activated NF-κB activity.[14] These cell lines demonstrate marked tumorigenicity with increased metastatic potential in vivo. Breast cancer cell line MDA-231 was derived from a patient with ER– metastatic breast cancer whereas the SUM 149 cell line was derived from a patient with inflammatory breast cancer. The MCF 10A line consists of spontaneously immortalized mammary epithelial cells (derived from a patient with benign fibrocystic breast disease) and was used as nonneoplastic control.

Cell Culture and Treatment

SUM 149 cells were grown in Hams F-12 media supplemented with insulin, hydrocortisone, fungizone, penicillin, gentamicin, and fetal bovine serum (FBS) at 37°C in 10% CO_2.

Cells of the MDA-231 line were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, gentamicin, amphotericin, and FBS at 37°C in 5% CO_2. Cells of the MCF 10A line were grown in DMEM/F12 supplemented with extracellular growth factor, choleratoxin, hydrocortisone, insulin, fungizone, penicillin, gentamicin, and HS at 37°C in 5% CO_2. All cells were grown to 70% confluence in Corning flasks and incubated with PFE_a at increasing concentrations from 50 to 300 μg/mL. The treated cells were then used in assays as described below.

Assessment of Apoptosis

Cells from SUM 149, MDA-231, and MCF 10A lines were plated in 100 mm^3 dishes to 70% confluence. Cells were then incubated with PFE_a for 72 hours. Untreated cells served as controls. Cells were harvested, washed with phosphate buffered saline (PBS), and subsequently stained with Annexin V and propidium iodide according to the manufacturer's protocol (Annexin V-FITC Apoptosis Kit; Clontech, Mountain View, CA). The percentage of apoptotic cells was quantified by flow cytometry.

Assessment of Proliferation

Cells from SUM 149, MDA-231, and MCF 10A lines were plated in 96-well plates in triplicate. After adherence, cells were treated with increasing concentrations of PFE_a. Triplicate samples of untreated cells served as controls. A methyl thiazolyl blue tetrazolium bromide (MTT) assay was performed at baseline (prior to PFE treatment), and on days 1, 3, and 5 to assess relative cell proliferation. An aliquot (50 μL) of the MTT solution (5 mg/mL) was added to each well. The mixture was incubated at 37°C, 5% CO_2 for 60 minutes. After 1 hour, the media with the MTT reagent was removed. Dimethylsulfoxide (DMSO, 200 μL) was added to each well and the plates placed on a shaking table at 150 rpm for 5 minutes to dissolve the formazan product. Optical density was read at 590 nM to determine viable cell numbers.

Luciferase Gene Reporter Assay for NF-κB Activity

Cells from MDA-231 and SUM 149 lines were plated in a 6-well plate to about 70% confluence. After adherence, both cell lines were transfected transiently with pNF-κB and pRL-SV40 (Clontech Laboratories, Inc.) with FuGene6 transfection reagent (Roche Biochemicals, Indianopolis, IN). The *Renilla* luciferase vector was cotransfected to normalize for transfection efficiency. After a 24-hour recovery period, media was removed and supplemented with fresh media. Cells were treated with increasing concentrations of PFE_a in duplicate, for 72 hours. Untreated cells served as controls. Samples were harvested using passive lysis buffer. Luciferase activity was quantified using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) in the dual luciferase assay system (Promega Corp, Madison, WI).

Assessment of Motility and Invasion

MDA-231 and SUM 149 cells were treated with PFE_a at increasing concentrations for 72 hours. Untreated SUM 149 and MDA-231 cells, as well as ethanol-only treated cells served as negative controls. Cell invasion was determined using a cell invasion assay kit (Chemicon International, CA). A cell suspension was prepared and an aliquot of the prepared cell suspension added into the invasion chamber and incubated for 48 hours at 37°C in a 10% CO_2 tissue culture incubator. Invasive cells were stained and quantified at a colorimetric reading of 560 nm. The treated cells were then evaluated for motility using a motility assay kit (Cellomics, Pittsburgh, PA). Untreated cells served as controls. The cells were harvested and suspended in serum-free medium. Subsequently the cell suspension was plated on top of a field of microscopic fluorescent beads. Samples were incubated for 48 hours and then fixed. Phagokinetic cell tracks corresponding to the areas of clearing in the fluorescent bead field were evaluated and quantified using a NIH ScionImager.

Protein Extraction and Western Blotting

Protein was extracted from whole cell lysates using a standard protocol. Protein (20-25 μg) was loaded into each lane and separated in a sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. The protein was then transferred to a nitrocellulose membrane, which was incubated in a bath of blocking buffer with anti-RhoC, RhoA, p65, P50, IκBα, and β-actin antibodies purchased from Sigma Chemicals (St. Louis, MO). Subsequently, the mixture was incubated with anti-mouse or anti-rabbit antibodies conjugated to horseradish peroxidase. The protein bands were detected by chemiluminescence and autoradiography.

Extraction of mRNA and QT-PCR.

Extraction of mRNA was performed using the Trizol extraction method. One microgram of total RNA was converted to cDNA using an avian myeloblastosis virus reverse transcription system (Promega, Madison, WI). A 100-µg aliquot of the resulting cDNA was amplified by polymerase chain reaction (PCR) with 25-ng RhoC or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers. Separation of PCR products on a 1.2% agarose gel was followed by imaging on an Alpha Image 950 documentation system (Alpha Innotech, San Leandro, CA).

Eicosanoid Profiling

The effects of PFEs on relative PGE_2, LTB_4, 5-HETE, 12-HETE, and 15-HETE levels in SUM 149 and MDA-231 cells were determined by a validated LC/MS/MS method. Briefly, SUM 149 and MDA-231 cells (5×10^6) were harvested by trypsinization, washed with PBS and then resuspended in 0.5 mL of PBS containing 1 mM $CaCl_2$. Samples were incubated with PFE (50-200 µg/mL) at 37°C for 10 minutes followed by addition of 2.5 µL of calcium ionophore A23187 (1 mM). An aliquot of 2.5 µL arachidonic acid (AA, 10 mM) was then added, and samples were incubated for an additional 10 minutes. The reaction was terminated by addition of aliquots of 40 µL of 1 N citric acid and 5 µL of 10% butylated hydroxytoluene. Aliquots (10 µL) of each of the deuterated relevant eicosanoids (PGE_2-d_4, LTB_4-d_4, 5-HETE-d_8, 12-HETE-d_8, and 15-HETE-d_8; 100 ng/mL) were added to the reaction mixtures as internal standards. Eicosanoids were extracted with 2 mL of hexane–EtOAc (1:1; v/v) thrice. The upper organic phases were pooled and evaporated to dryness under a stream of nitrogen at room temperature. All extraction procedures were performed under minimal light conditions. Samples were then reconstituted in 200 µL of methanol/10 mM ammonium acetate buffer (70:30, v/v), pH 8.5 and analyzed using LC/MS/MS. Reverse-phase HPLC electrospray ionization MS was used to determine eicosanoid levels using a Micromass Quattro Ultima tandem mass spectrometer (Waters Corp, Milford, MA), equipped with an Agilent 1100 HP binary pump high-pressure LC inlet for use in these studies. Eicosanoids were separated using a Luna 3 µ phenyl-hexyl (2 × 150 mm) LC column (Phenomenex, Torrence, CA). The mobile phase consisted of 10 mM ammonium acetate (pH 8.5) and methanol; the flow rate was 250 µL/min with a column temperature of 50°C. The sample injection volume was 25 µL. Samples were kept at 4°C in an autosampler prior to injection. All eicosanoids were detected using negative ionization and multiple-reaction monitoring of the transition ions for eicosanoid products and their internal standards.[15]

Results and Discussion

PFEa Treatment Results in a Selective Concentration-Dependent Decrease in the Proliferation of Aggressive Breast Cancer Cell Lines Compared With Nonneoplastic Controls

The effect of PFEa on cell proliferation was evaluated using the MTT assay. The MTT assay measures metabolic activity; and it has been shown that metabolic activity of cells is a reasonable surrogate for the cell growth. Cells of aggressive breast cancer cell lines MDA-231 and SUM 149 were incubated with PFEa at increasing concentrations from 50 to 200 mg/mL, whereas MCF 10A cells were used as nonneoplastic controls. Untreated cells in each cell line also served as negative controls. Consistent concentration-dependent and time-dependent decreases in the rates of proliferation of cells of both cancer cell lines were observed. Furthermore, at a concentration range of 50 to 200 mg/mL, these effects were highly selective in the cancer cells only and did not affect the MCF 10A immortalized normal cells. As shown in Figure 1 (top left panel), PFEa treatment of MDA-231 cells leads to a 67% reduction in growth at day 5 relative to untreated controls at a concentration of 200 mg/mL ($P = .001$). Similar effects were seen but to a lesser degree in the SUM 149 inflammatory breast cancer cells where PFEa treatment led to a 24% decrease in growth at day 5 relative to untreated controls at 200 mg/mL ($P < .05$; Figure 1, top right panel).

Of note, no effect of PFEa on the proliferation of MCF 10A breast epithelial cells was seen at these concentrations (Figure 1, bottom panel). The mechanistic basis of this selective action is unclear but may be mediated via the effects of PFEa on modulation of constitutive NF-kB activity in the MDA-231 and SUM 149 cells compared with MCF 10A cells, as the latter do not constitutively express this nuclear transcription factor.[16]

PFEa Treatment Induces a Concentration-Dependent and Selective Induction of Apoptosis in Breast Cancer Cells Compared With Nonneoplastic Controls

Because NF-kB regulates genes such as Bcl2/Bax that are implicated in apoptosis,[17,18] we evaluated the apoptotic activity of PFEa using the Annexin 5 method in SUM 149 and MDA-231 cell lines, with MCF 10A cells as nonneoplastic controls. Selective induction of apoptosis occurs in both MDA-231 and SUM 149 breast cancer cells at concentrations ranging from 100 to 300 mg/mL PFEa. In MDA-231 cells, at concentrations of 100 and 200 mg/mL, PFEa treatment leads to a selective 2-fold and 3-fold induction in apoptosis relative to MCF 10A (Figure 2, panel A). SUM 149 cells treatment with PFE revealed a 1-fold increase in apoptosis compared with MCF 10A controls (Figure 2, panel B).

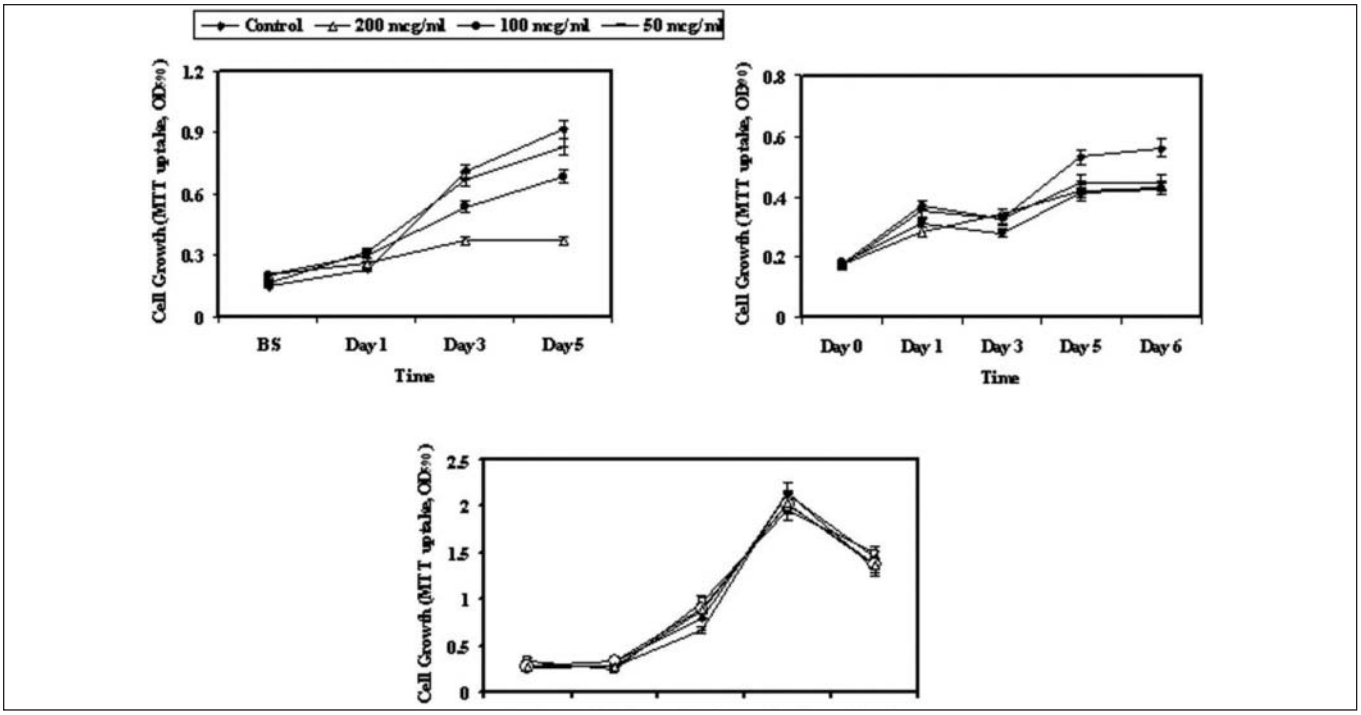

Figure 1. Dose-dependent and selective inhibition of proliferation of breast cancer cells compared to MCF 10A breast epithelial cells.
Top left panel: Effect of pomegranate fruit extract (PFE) on proliferation of MDA-231 breast cancer cells. Inhibitory effect of PFE on cell proliferation was determined using the MTT assay. Each data point indicates mean percentage viable cells of 6 replicates. At a concentration of 200 mg/mL, PFE treatment leads to a 67% reduction in growth at day 5 relative to untreated controls (mean ± SD; $P = .001$ vs untreated controls). Top right panel: Effect of PFE on proliferation of SUM 149 breast cancer cells. Each data point indicates mean percentage viable cells of 6 replicates. At a concentration of 200 mg/mL, PFEa treatment leads to a 24% reduction in growth at day 5 relative to untreated controls (mean ± SD; $P < .05$ vs untreated controls). Bottom panel: Effect of PFEa on the proliferation of MCF 10A breast epithelial cells. No significant effect of PFEa treatment was observed on the growth of MCF 10A breast epithelial cells.

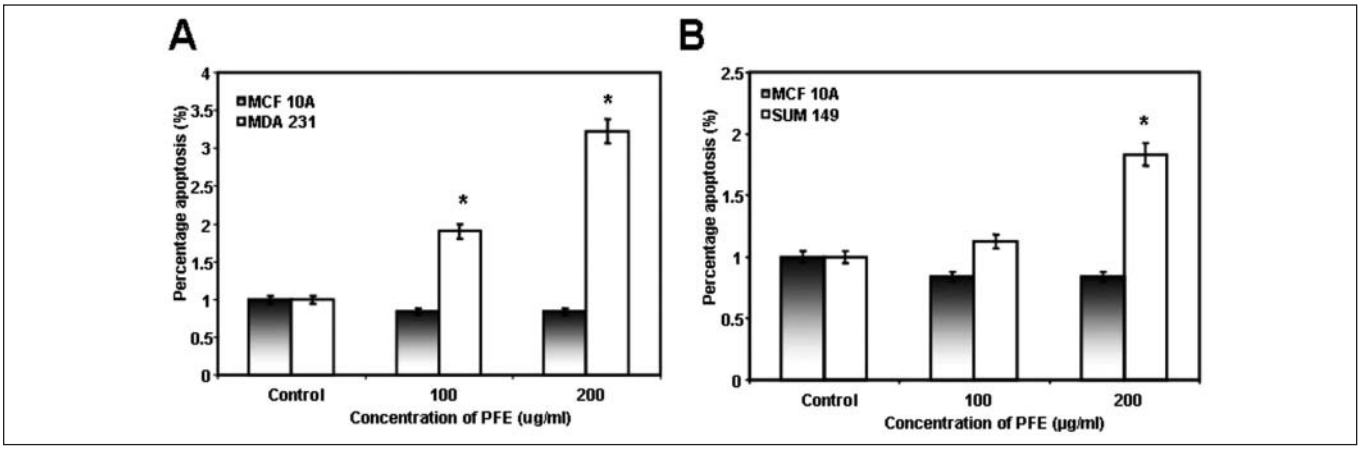

Figure 2. Pomegranate fruit extract (PFE) causes a selective and dose-dependent induction of apoptosis in breast cancer cells compared with MCF 10A breast epithelial cells.
Panel A: Effect of PFE_a on apoptosis in MDA-231 breast cancer cells. Induction of apoptotic cell death by PFE_a was determined by the Annexin-PI assay as described in Materials and Methods section. Each bar indicates the percentage apoptotic cells after incubation with PFE_a for 72 hours. At concentrations of 100 and 200 mg/mL, PFE_a treatment led to a selective 2-fold and 3-fold induction, respectively, in apoptosis relative to MCF 10A controls (mean ± SD; *$P = .001$ vs untreated controls). Panel B: Effect of PFE_a on apoptosis in SUM 149 breast cancer cells. Induction of apoptotic cell death by PFE was determined by the Annexin-PI assay. Each bar indicates the percentage apoptotic cells after incubation with PFE_a for 72 hours. At a concentration of 200 mg/mL, PFE_a treatment led to a selective 2-fold induction in apoptosis relative to MCF 10A controls (mean ± SD; *$P < .05$ vs untreated controls).

PFE Suppresses Constitutive NF-kB Activation in a Concentration-Dependent Fashion

There is increasing evidence that NF-κB activation regulates genes important for angiogenesis, invasion, and metastasis in a variety of solid tumors and hematopoietic neoplasms.[18] Because of PFEs' presumed effects on NF-κB, we focused on aggressive breast cancer cell lines with constitutive NF-κB activity such as the inflammatory breast cancer cell line, SUM 149 (derived from a patient with inflammatory breast cancer), and MDA-231 (derived from a metastatic lesion in a patient with ER–/PR– breast cancer). As controls, we used MCF 10A cells that arose by spontaneous immortalization of epithelial cells in a focus of fibrocystic breast disease.

Endogenous NF-κB activity has been shown to be 2.5-fold higher in SUM 149 and MDA-231 cells in comparison with nonmalignant human mammary epithelial cells.[19] Several studies (including our own laboratory experience) using human breast cancer cells have reported that overexpression of p50 results in constitutive NF-κB activation.[20] We investigated whether PFE_a modulates NF-κB activity in breast cancer cell lines using a luciferase gene reporter assay. We incubated MDA-231 and SUM 149 breast cancer cells with PFE_a for 72 hours. As shown in Figure 3, panels A and B, PFE_a suppressed constitutive NF-κB activity in both SUM 149 and MDA-231 cells in a concentration-dependent manner compared with untreated controls. This was independent of the effect on cell viability.

PFEa Decreases Expression of the p50 and p65 NF-kB Transcription Subunits in MDA-231 Breast Cancer Cells

In nonstimulated normal cells, NF-κB is primarily localized in the cytoplasm and is composed of transcription subunits primarily including p50, p65, and IκBα. The main event that triggers NF-κB activation is phosphorylation of the IκBα via the IκK complex. Phosphorylated IκBα dislodges from the active transcriptional units of NF-κB before undergoing proteosomal degradation. Subsequent nuclear translocation of the p50 and p65 heterodimeric units of NF-κB and binding to a specific DNA consensus sequence are followed by downstream processes, including transcription and translation of NF-κB-regulated genes controlling a variety of critical cellular processes involved in the maintenance of the malignant phenotype. These encompass cyclin D1, Bcl_2/Bax, survivin, intercellular cell adhesion molecule-1 (ICAM-1), matrix metalloproteinase (MMP), and cyclo-oxygenase type 2 (COX-2), which regulate apoptosis, proliferation, inflammation, and invasion.[21,22]

Given that PFE decreases NF-κB activation in a concentration-dependent manner, we sought to determine if this decrease was the result of decreased expression of the transcription subunits p65 and p50. Using Western blot analysis, we also found reduced p50 levels following treatment with PFE_a in both SUM 149 and MDA-231 cells, suggesting that PFE_a decreases constitutional NF-κB activation by downregulating p50 and phosphorylated IκBα. The effects of PFE_a on the p65 component were less marked and only seen at higher concentrations, ~400 μg/mL. Given that PFE_a is a multicomponent product, further investigations of bioactive constituents like ellagic acid and punicalagin might help to fully elucidate the individual roles of each component of PFE_a on NF-κB activity (Figure 4, panel C).

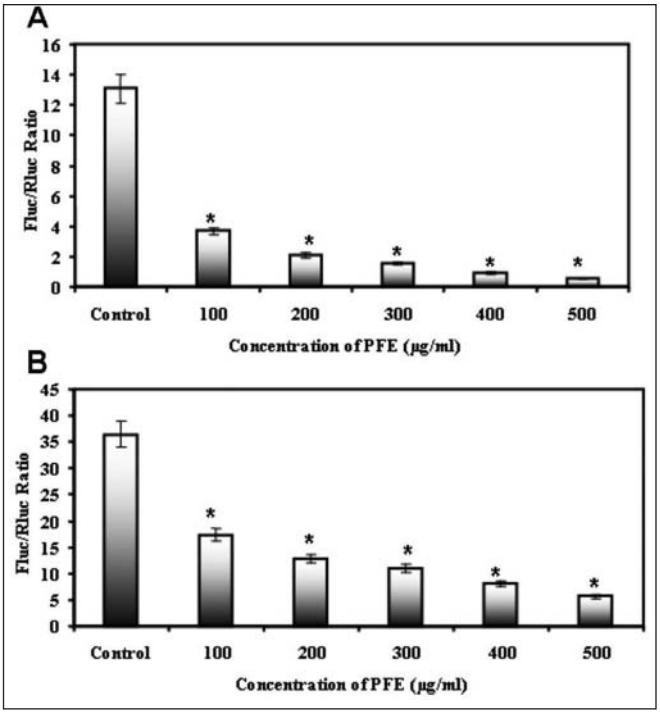

Figure 3. PFE_a (aqueous pomegranate fruit extract) suppressed constitutive NF-kB activation in breast cancer cells. SUM 149 and MDA-231 cell lines were transfected with pNF-kB vector containing 4 tandem copies of the B consensus sequence fused to a TATA-like promoter region from the SV 40 promoter. A *Renilla* luciferase vector was cotransfected into the cells to normalize for transfection efficiency. After a 24-hour recovery period, transfected cells were incubated in fresh medium with or without the addition of PFE. Untreated cells served as control. After treatment for 72 hours, cells were harvested in passive lysis buffer, and the activities of the firefly luciferase and *Renilla* luciferase quantified on a Monolight 2010 luminometer.

Panel A: PFE treatment leads to a dose-dependent decrease in constitutive NF-κB activity in MDA-231 cells compared with untreated controls. At PFE_a concentrations of 100 and 500 μg/mL, constitutive NF-κB activity decreased by 72% and 96%, respectively, compared with untreated controls (*$P < .001$ vs untreated controls). Data are presented as mean ± SD.

Panel B: PFE treatment leads to a dose-dependent decrease in constitutive NF-κB activity in SUM 149 cells compared with untreated controls. At PFE_a concentrations of 100 and 500 μg/mL, constitutive NF-κB activity decreased by 52% and 84%, respectively, compared with untreated controls (*$P < .05$ vs untreated controls). Data are presented as mean ± SD.

Effects of PFEa on Cellular Processes Regulated by NF-kB Transcription Complex

It has been demonstrated by numerous investigators that nuclear translocation and activation of the NF-κB complex

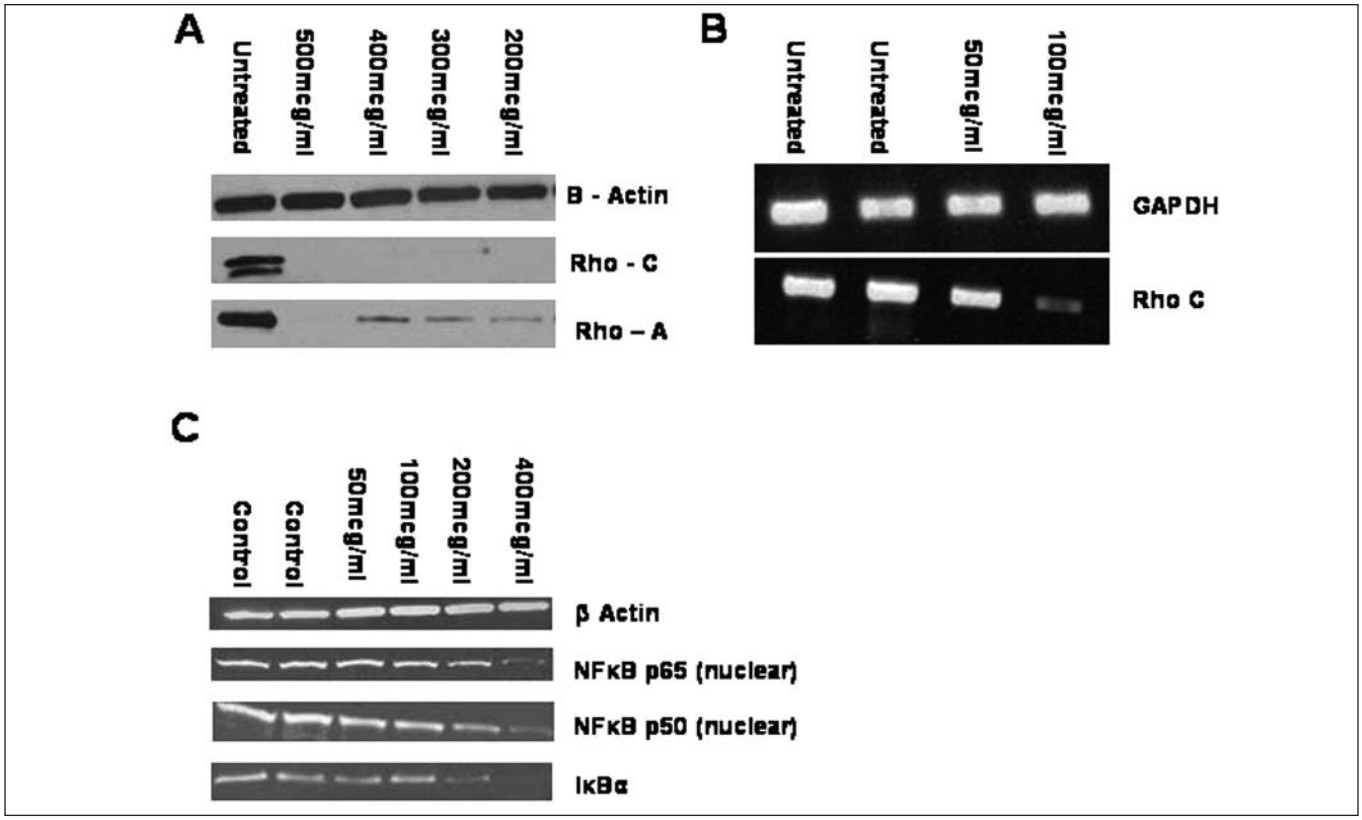

Figure 4. Inhibitory effect of pomegranate fruit extract (PFE) on RhoC and RhoA protein levels (A), decreased expression of RhoC mRNA (B), and decreased expression of the p50 and p65 NF-κB transcription subunits (C) in MDA-231 breast cancer cells.
Panel A: MDA-231 cells were treated with PFE for 72 hours. Untreated MDA-231 cells were used as controls. Western blot analysis was performed on whole-cell lysates from these cells using specific antibodies for the Rho C, Rho A, and β-actin protein. This figure is representative of 3 independent experiments. Panel B: MDA 231 cells were treated with PFE at 50 and 100 μg/mL for 72 hours. Untreated cells served as controls. mRNA was extracted using the trizol method. One microgram of total RNA was converted to cDNA. The resultant cDNA was amplified by PCR with 25 ng RhoC or GAPDH specific primers and PCR products were separated on a 1.2% agarose gel and imaged as described under Materials and Methods section. Panel C: Cells of the MB-MDA-231 line were treated with PFE_a for 72 hours. Untreated MDA-231 cells were used as controls. Western blot analysis was performed on whole-cell lysates from these cells using specific antibodies for the NF-κB p50, p65 (RelA), and phosphorylated IκBα subunits of NF-κB and β-actin protein. The β-actin remained unchanged (data not shown).

leads to the transcription of a variety of NF-κB-regulated genes such as cyclin D1, Bcl2/Bax, survivin, ICAM-1, MMP, and COX-2. These genes are important in the maintenance of the stereotypical neoplastic phenotype regulating vital cellular processes such as apoptosis, proliferation, inflammation, and invasion.[23-32] Given that PFE_a inhibits NF-κB activity, we further sought to investigate if PFE_a treatment affects invasion and motility in cancer cells compared to non-neoplastic controls in addition to its effects on proliferation and apoptosis.

PFEa Suppresses Aggressive Phenotypic Features of Invasion and Motility in MDA-231 Breast Cancer Cells

Inflammatory breast cancer and hormone receptor negative breast cancer are clinically associated with an aggressive course, including early metastasis and chemo/radiotherapeutic resistance.[33-37] The MDA-231 and SUM 149 cell lines are derived from aggressive breast cancers and have been shown in the laboratory to exhibit a highly motile and invasive phenotype in addition to high levels of constitutive NF-κB activation. We thus sought to elucidate if PGE_a affects phenotypic features of aggressive biologic behavior, including motility and invasion.

MDA-231 and SUM 149 breast cancer cells were pretreated with 100 and 300 μg/mL of PFE_a for 72 hours. Untreated MDA-231 and SUM 149 cells were used as controls. Appropriate ethanol (EtOH) controls were incorporated. Cell invasion was determined as described from a cell invasion assay kit (Chemicon International, Temecula, CA). At concentrations of 100 and 300 μg/mL, PFE_a treatment led to a decrease in invasion of MDA-231 cells by 32% and 46%, respectively ($P < .05$ vs untreated controls; Figure 5, panel F).

Random cell motility was then determined as described from the motility assay kit (Cellomics, Pittsburgh, PA). As shown in Figure 5, panel B, areas of clearing in the fluorescent bead field corresponding to phagokinetic cell tracks were quantified using NIH ScionImager.

As shown in Figure 5, panels A to E, MDA-231 cancer cells exhibit a highly motile phenotype at baseline (panel A), which

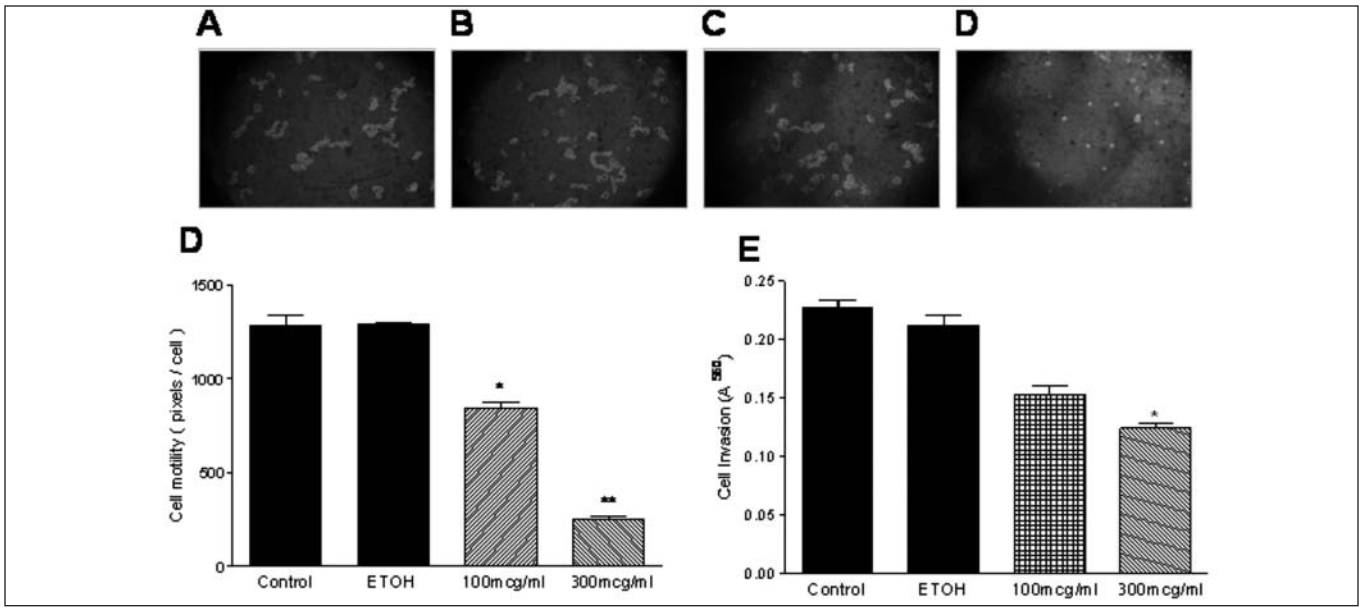

Figure 5. Investigation of the functional effects of pomegranate fruit extract (PFE) treatment on aggressive phenotypic features of cancer cells such as invasion and motility. PFE treatment leads to a decrease in motility of MDA-231 breast cancer cells. MDA-231 cells were pretreated with or without PFE for 72 hours, harvested, suspended in serum-free medium, and motility assay performed using manufacturer's protocol using a Cellomics motility assay kit as described in the Materials and Methods section.
Panel A: Untreated MDA-231 cells with the areas of clearing in the fluorescent bead field corresponding to phagokinetic cell tracks. Panel B: MDA-231 cells with EtOH controls demonstrating similar phagokinetic cell tracks to untreated controls. Panel C: MDA-231 cells treated with PFE at a concentration of 100 μg/mL, with decreased phagokinetic cell tracks. Panel D: MDA-231 cells treated with PFE at a concentration of 300 μg/mL, with markedly decreased phagokinetic cell tracks. Panel E: Quantification of phagokinetic cell tracks using NIH ScionImager. PFE treatment at concentrations of 100 and 300 μg/mL leads to a decrease in cell motility by 35% and 81%, respectively (P = .002 and P = .0001, respectively, vs untreated controls). Panel F: PFE decreases the invasive potential of MDA-231 breast cancer cells. Cells were pretreated with or without PFE for 72 hours, harvested, and resuspended in serum-free medium. An aliquot of prepared cells was taken and invasive potential of the cells was determined using a Cell Invasion assay kit as described in the Materials and Methods section. At concentrations of 100 and 300 μg/mL, PFE treatment led to a decrease in invasive potential by 32% and 46%, respectively. (P < .05 vs untreated controls). Data are presented as mean ± SD.

is unaffected in the EtOH controls (panel B), whereas PFE_a-treated cells are significantly less motile than their untreated counterparts (panels C and D). Further quantification of cell motility (panel E) revealed that PFE_a treatment at concentrations of 100 and 300 μg/mL led to a decrease in cell motility by 35% and 81%, respectively (P = .002 and P = .0001, respectively, vs untreated controls).

Further Insights Into the Mechanistic Actions of PFEa: Invasion and Motility in Aggressive Breast Cancer Cells Is Regulated by RhoC. Does PFEa Affect RhoC?

Previous reports from our laboratory have demonstrated that stable transfectants of nonneoplastic human mammary epithelial cells (HME) with RhoC led to large colony formation under anchorage-independent growth conditions. In addition, these HME-RhoC transfectants were more motile and invasive with increased tumorigenicity when xenografts were introduced into athymic nude mice. Furthermore, RhoC GTPase was found to be overexpressed in 90% of human inflammatory breast cancer (IBC) tumors compared with similar stage matched non-IBC breast cancer controls. Increased invasive potential and increased motility would translate clinically to aggressive neoplastic phenotypic features such as early metastasis and ultimately increased cancer-related mortality.[38-43]

These data led to the conclusion that Rho GTPase overexpression confers increased tumorigenicity to breast cancer with increased invasive potential. Given that we observed marked decreases in motility and invasion in breast cancer cell lines with PFE_a treatment, we thus sought to evaluate if PFE_a decreases RhoC and RhoA expression.

Cells of the MDA-231 line were incubated with PFE_a for 72 hours at concentrations that lead to a decrease in motility and invasiveness (ie, 50 and 200 μg/mL). Protein was extracted from whole cell lysates under standard protocol. Using RhoC, RhoA, and β-actin antibodies, we evaluated RhoC expression via Western blot as described in the Materials and Methods section.

As shown in Figure 4, panel A, PFE_a treatment decreases RhoC and RhoA protein expression compared with untreated controls. We further evaluated if PFE_a decreases mRNA expression of RhoC as described earlier. The housekeeping gene GAPDH was used as a loading control. Also, as shown in Figure 4, panel B, PFE_a treatment led to a decrease in mRNA expression of RhoC.

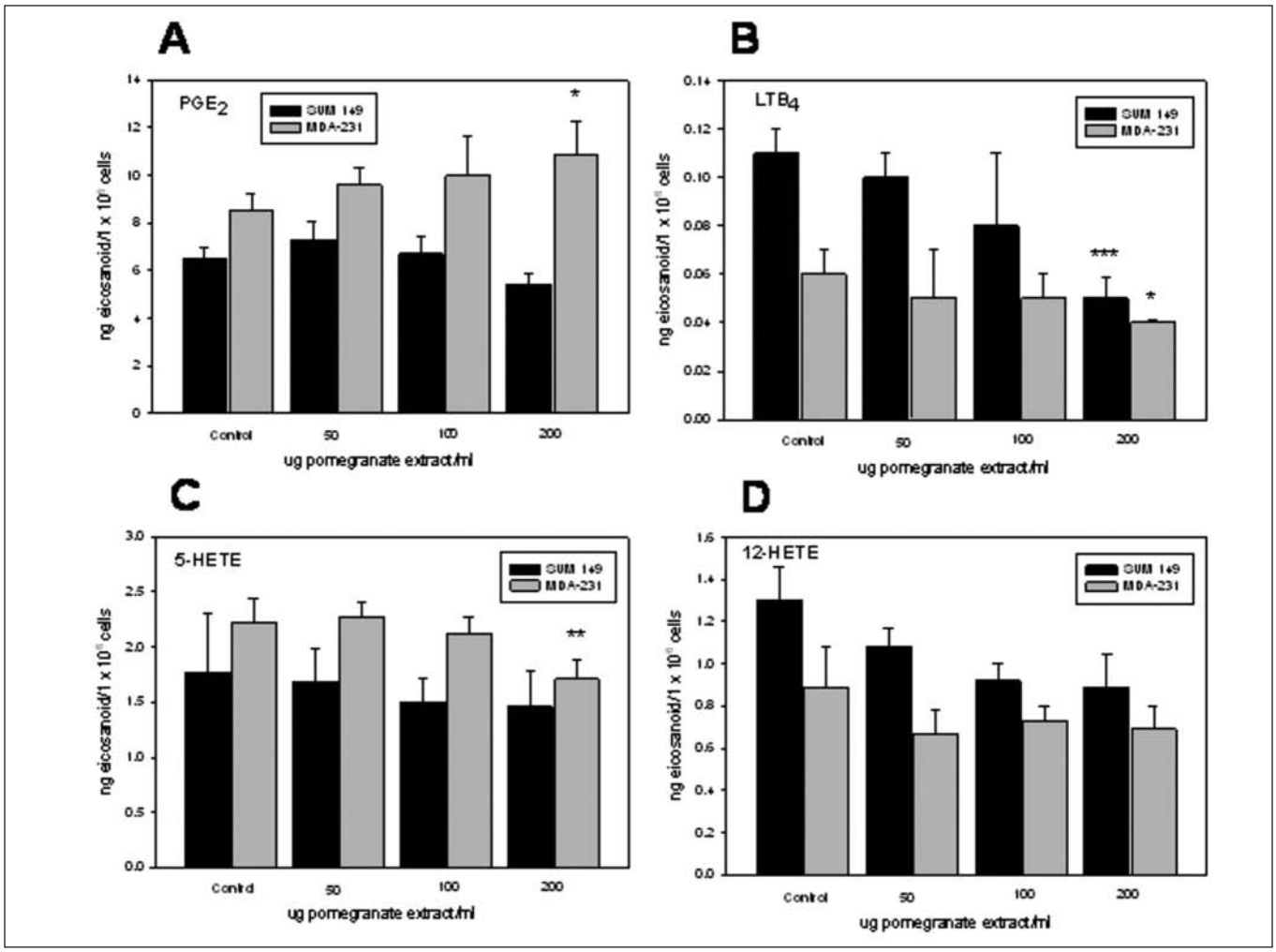

Figure 6. Alterations in (A) PGE2, (B) LTB4, (C) 5-HETE, and (D) 12-HETE concentrations in SUM 149 and MDA-231 cell lines after treatment with ethyl acetate partition of pomegranate juice in comparison with solvent (ethanol) control. Data are presented as mean ± SD; n = 3.
*P < .05.

Based on previous studies conducted at our laboratory in RhoC transfectants, it is plausible to consider that PFE_a inhibits its motility and invasion via its effect on RhoC and RhoA.

There is evolving data on the cross-talk between Rho C and NF-κB pathways. Interestingly, it has been demonstrated by Segain et al[44] in an inflammatory colitis model that RhoA activates IκK phosphorylation and proteosomal degradation, thus affecting NF-κB nuclear translocation and activation. This Rho C/NF-κB interaction could serve as a mechanism for our observations. Future detailed mechanistic studies investigating the effects of PFE_a on this cross-talk between the Rho GTPase system and NF-κB will be conducted.

Effects of PFE on Eicosanoid Profiling in SUM 149 and MDA-231 Breast Cancer Cell Lines

NF-κB has been linked to inflammation in addition to its role in carcinogenesis. It was initially proposed by Galen and Virchow that inflammation is possibly linked to cancer when it was observed that infiltrative leucocytes were found near tumor cells.[45-47] Since this initial observation, several experiments have suggested that activation of NF-κB in inflammatory cells such as macrophages and leukocytes, leads to the production of reactive oxygen species thorough induction of nitric oxide synthase (iNOS) and other enzymes. The reactive oxygen species then lead to DNA damage and thus initate the process of carcinogenesis.[48,49] It has also been found that activation of NF-κB in gastric epithelium affected by *Heliobacter pylori* infection lead to the production of inflammatory cytokines, which in turn contributes to tumorigenesis.[50,51] Considering the important role of NF-κB in inflammation and our own findings of the effects of PFE on NF-κB, we sought to evaluate the effects of PFE on inflammatory mediators in SUM 149 and MDA-231 cells.

Treatment of SUM 149 and MDA-231 cells with PFE_a had different effects on cellular eicosanoid levels. As shown in Figure 6, incubation of SUM 149 cells with PFE_a led to a

concentration-dependent decrease in LTB_4, 12-HETE, and 15-HETE levels in the inflammatory SUM 149 cells. However, there was an unexpected increase in PGE_2 levels and a decrease in 15-HETE levels in MDA-231 cells. The leukotriene, LTB4, and lipoxygenase-derived products 12-HETE and 15-HETE are all well characterized as pro-inflammatory biologically important lipids with relevance to the etiology and progression of breast cancer. Though less well studied than the COX-2 derived product PGE2, increases in LTB4 have been shown to be associated with pulmonary disease as well as oral cancer[52,53] although the role of this leukotriene in breast cancer is unclear. The LOX-12 derived product 12-HETE has been shown to be an important etiologic factor in a number of malignant diseases such as breast[54] and prostate cancers.[55] Finally, 15-HETE has been shown to be a pro-inflammatory bioactive lipid that is believed to directly contribute to breast cancer.[56] Downregulation of these pro-inflammatory lipids may also be of importance in explaining the antitumor effects of PFEs.

Conclusion

The present study was done to evaluate the effect of PFE_a on aggressive phenotypic features of breast cancer cells such as invasion and motility. The use of "biological" plant-derived products has been increasing worldwide for the treatment of various disorders such as autoimmune disease and cancer.[57-61] Increasingly, pomegranate preparations are being used as an alternative strategy toward cancer prevention and treatment. Thus it is critical to evaluate the efficacy of such preparations. PFEs have shown anticancer activity in vitro and also some activity in early phase clinical studies.[62-68] In particular, PFE_a is notable for a variety of bioactive polyphenolic compounds acting on various cellular pathways implicated in the initiation, promotion and maintenance of carcinogenesis.[9,69-71]

The role of NF-κB as an important master switch regulating several downstream mediators of carcinogenesis is now widely recognized. Our data suggest that PFE_a has important anticancer effects based on its effect on inhibition of NF-κB expression and activity. Furthermore, there is evidence that RhoC activates NF-κB via an IKK-independent mechanism.[72]

Based on the very promising preliminary data from our own laboratory and from other investigators, PFE_a appears to be an efficacious, yet nontoxic, biological agent that could become a valuable component of the breast cancer chemopreventive armamentarium. Further, there is preliminary evidence that a major mechanism of action may be via modulation of NF-κB and RhoC. The ability of PFEs to modulate downregulation of known pro-inflammatory eicosanoids is also believed to be important. Hence, we hypothesize that PFE_a, alone or in combination with PFE_l, represents a potential chemopreventive agent for ER− breast cancer in humans and propose both animal and clinical studies of this botanical complex to further gauge its safety and efficacy.

Declaration of Conflicting Interests

Ephraim P. Lansky and Robert A. Newman both have stock and ownership interests in Punisyn Pharmaceuticals. The other authors have no conflicts of interests.

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