

# Blueberry Phytochemicals Inhibit Growth and Metastatic Potential of MDA-MB-231 Breast Cancer Cells through Modulation of the Phosphatidylinositol 3-Kinase Pathway

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## Abstract

Dietary phytochemicals are known to exhibit a variety of anticarcinogenic properties. This study investigated the chemopreventive activity of blueberry extract in triple-negative breast cancer cell lines *in vitro* and *in vivo*. Blueberry decreased cell proliferation in HCC38, HCC1937, and MDA-MB-231 cells with no effect on the nontumorigenic MCF-10A cell line. Decreased metastatic potential of MDA-MB-231 cells by blueberry was shown through inhibition of cell motility using wound-healing assays and migration through a polyethylene terephthalate membrane. Blueberry treatment decreased the activity of matrix metalloproteinase-9 and the secretion of urokinase-type plasminogen activator while increasing tissue inhibitor of metalloproteinase-1 and plasminogen activator inhibitor-1 secretion in MDA-MB-231 conditioned medium as shown by Western blotting. Cell signaling pathways that control the expression/activation of these processes were investigated via Western blotting and reporter gene assay. Treatment with blueberry decreased phosphatidylinositol 3-kinase (PI3K)/AKT and NF $\kappa$ B activation in MDA-MB-231 cells, where protein kinase C and extracellular signal-regulated kinase (ERK) were not affected. *In vivo*, the efficacy of blueberry to inhibit triple-negative breast tumor growth was evaluated using the MDA-MB-231 xenograft model. Tumor weight and proliferation (Ki-67 expression) were decreased in blueberry-treated mice, where apoptosis (caspase-3 expression) was increased compared with controls. Immunohistochemical analysis of tumors from blueberry-fed mice showed decreased activation of AKT and p65 NF $\kappa$ B signaling proteins with no effect on the phosphorylation of ERK. These data illustrate the inhibitory effect of blueberry phytochemicals on the growth and metastatic potential of MDA-MB-231 cells through modulation of the PI3K/AKT/NF $\kappa$ B pathway. *Cancer Res*; 70(9): 3594–605. ©2010 AACR.

## Introduction

The triple-negative breast cancer phenotype, so named due to its lack of expression of estrogen receptor, progesterone receptor, and the HER-2 epidermal growth factor receptor (1), comprises 10% to 15% of all breast cancer cases (2). These breast cancers are characterized by an aggressive clinical history with poor disease-free and overall survival (3, 4) and show a high rate of metastasis to the cerebrum (5) and visceral sites compared with patients with other types of breast cancers. Currently, there is no defined standard treatment strategy for prevention of reoccurrence for this disease other than traditional chemotherapy, to which it is highly

resistant. Furthermore, there exist a limited amount of data on which to base treatment and prevention strategies, and no substantial information is available on targeted preventive measures for occurrence and reoccurrence of this disease.

Tumor metastasis is a multistep process involving cell adhesion, degradation of the extracellular matrix, and cell migration. The metastatic potential of tumor cells is influenced by a balance of the expression of proteases and their inhibitory proteins. Matrix metalloproteinases (MMP) are a family of 24 secreted and membrane-type proteases that mediate this process. MMP-2 and MMP-9 are highly expressed in the uterus and breast and play an important role in the invasive stages of cancer (6). MMPs are directly activated by the serine protease plasmin, which is activated from its proenzyme form (plasminogen) by the serine protease urokinase-type plasminogen activator (uPA; ref. 7). Overexpression of uPA has been found in many tumor types (8) and is correlated with poor prognosis (9). The expression of uPA is positively regulated by growth factors, such as hepatocyte growth factor (HGF), and cell signaling pathways, such as the phosphatidylinositol 3-kinase (PI3K)/AKT pathway (10). Direct inhibition of uPA activity is modulated by plasminogen activator inhibitor (PAI), a secreted protein of the serpin family, and tissue inhibitors of metalloproteinases (TIMP; refs. 11–13).

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The motility and invasive potential of many metastatic cancer cell lines has been inhibited by phytochemicals such as [6]-gingerol (14), isothiocyanates from broccoli and watercress (15), tea catechins (16, 17), genistein, apigenin (18), *Ganoderma lucidum* (19), ganoderic acid from the *G. lucidum* (20) mushroom, and *Phellinus linteus* (21). Inhibition of MMP and uPA expression and activity was also shown in a number of these studies along with upregulation of the expression of TIMPs and PAI.

Bioactive substances in berries exhibit a variety of anticancer effects, such as inhibition of cell proliferation, induction of apoptosis, modulation of cell signaling, and effects on gene expression (22–24). Mulberry anthocyanins decreased MMP-2 and uPA and increased TIMP-2 and PAI expression in lung cancer cells (25). Flavonoid-enriched fractions from lowbush blueberries (*Vaccinium angustifolium*) downregulated the activity of MMP-2 and MMP-9 and increased the activity of TIMP-1 and TIMP-2 in prostate cancer cells (26). In addition, black raspberries inhibited esophageal tumors in rats (27) and modulated NF $\kappa$ B, activator protein-1 (AP-1), nuclear factor of activated T cells, and the expression of a number of genes associated with cellular matrix, cell signaling, and apoptosis (28–30).

Preliminary data showed that of a panel of vegetable and fruit extracts, blueberry inhibited the proliferation of triple-negative breast cancer cell lines with no effect on the nontransformed MCF-10A cell line. This observation led us to postulate whether this activity extends to modulation of metastatic potential as well. In this study, effects on the motility and migration of cells along with the modulation of regulatory proteins MMP-2, MMP-9, uPA, TIMP, and PAI *in vitro* were evaluated. The effect of daily oral administration of whole blueberry juice against tumor growth in mice was also evaluated. The results of these studies provide us with important information on the role of blueberry phytochemicals as preventive agents against the occurrence or recurrence (metastasis) of triple-negative breast cancers.

## Materials and Methods

**Preparation of blueberry extract.** Whole fresh highbush blueberries (*V. corymbosum*) were weighed and juiced, and the remaining solids were separated by centrifugation (4,000 rpm) for 15 minutes. The volume was measured, sterile filtered using 0.3- $\mu$ m Millipore filters, and frozen at  $-20^{\circ}\text{C}$  until use. Total phenolics were determined according to the Folin-Ciocalteu method (31) and measured as gallic acid equivalents. Samples and phenolic standard (gallic acid) were processed identically. The absorbance was determined at 755 nm, and final results were calculated from the standard curve. Residual solvents [methanol (MeOH), ethyl acetate (EA), etc.] were removed from the extracts *in vacuo* by using a Buchi rotary evaporator. Dried extracts were reconstituted in vehicle (DMSO) for cell culture studies.

**Cell culture.** MDA-MB-231, HCC38, HCC1937, and MCF-10A cells were obtained from the American Type Culture Collection. MDA-MB-231, HCC38, and HCC1937 cells were cultured in RPMI 1640 containing 10% fetal bovine serum

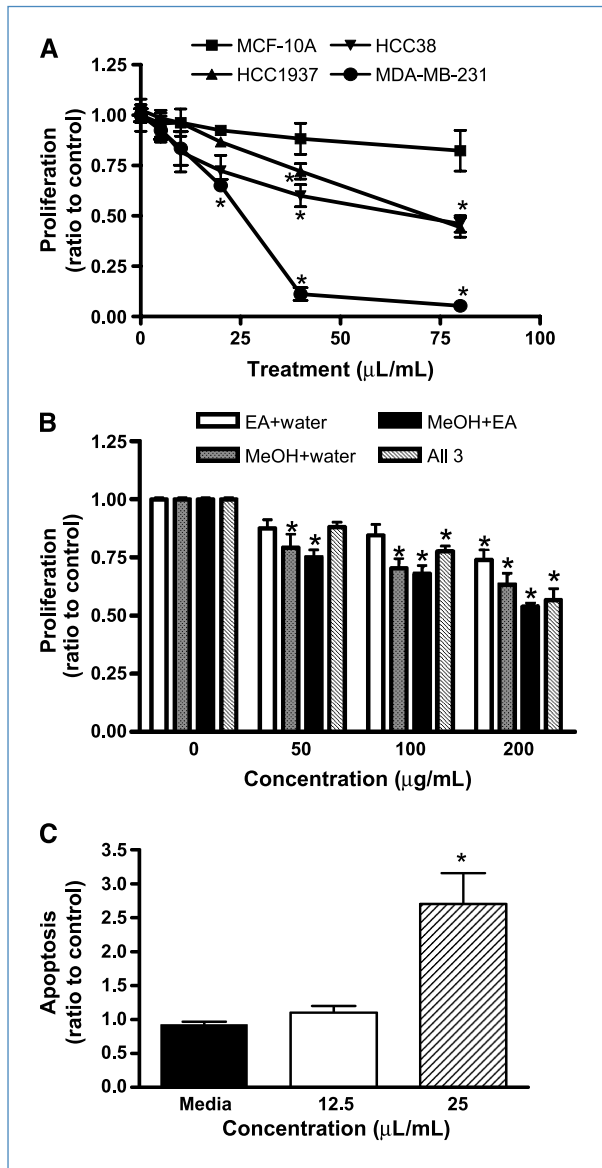
in the presence of 100 units/mL penicillin and 0.1 g/L streptomycin. MCF-10A cells were cultured in DMEM F-12 50/50 medium containing 5% horse serum in the presence of 10  $\mu$ g/mL insulin, 20 ng/mL epidermal growth factor, 100 ng/mL cholera toxin, 0.5  $\mu$ g/mL hydrocortisone, 100 units/mL penicillin, and 0.1 g/L streptomycin. Cells were incubated at  $37^{\circ}\text{C}$  with 95% air and 5% carbon dioxide. All cells were used in experiments during the linear phase of growth.

**Cell viability assay.** Cells were treated with 100  $\mu$ L media plus water or blueberry extract and incubated for 72 hours. Extract was tested at 0, 5, 10, 20, 40, and 80  $\mu$ L/mL concentrations. At the end of the drug exposure duration, cell viability was measured using the CellTiter-Glo Luminescent Cell Viability Assay according to the manufacturer's instructions (Technical Bulletin No. 288; Promega Corp.) as previously reported (22). All plates had control wells containing medium without cells to obtain a value for background luminescence, which was subtracted from the test sample readings. Data are expressed as a ratio of treated to untreated cells (mean  $\pm$  SE for three replications).

**Apoptosis ELISA.** Cells were plated in 60-mm dishes at a density of 100,000 per dish and allowed to attach for 24 hours. Cells were treated with media + water control (blueberry extract is water soluble) or blueberry extract at a concentration of 12.5  $\mu$ L/mL or 25  $\mu$ L/mL for 72 hours. Following treatments, nonadherent and adherent cells were collected and apoptosis was assessed using the Cell Death Detection ELISA<sup>PLUS</sup> Assay (Boehringer Mannheim) according to the manufacturer's instructions as previously reported (32). Background values were subtracted from readings (media plus reagent, no cells) and expressed as absorbance of dye bound to antibodies bound to mononucleosomes and oligonucleosomes at 405 nm of each treated sample divided by media controls.

**Scratch motility (wound-healing) assay.** MDA-MB-231 cells were plated in a six-well plate at a concentration of  $5 \times 10^5$  per well and allowed to form a confluent monolayer for 24 hours. Cells were then serum starved for 24 hours, and the monolayer was scratched with a pipette tip, washed with serum-free medium (SFM) to remove floating cells, and photographed (time 0). Cells were treated with either SFM or HGF (40 ng/mL) in the presence or absence of various concentrations of blueberry extract or wortmannin (0.2  $\mu$ mol/L), UO126 (10  $\mu$ mol/L), or Bis-I (20  $\mu$ mol/L) for 24 hours. Cells were then photographed again at three randomly selected sites per well.

**Migration assay.** The migration assay was performed on a polyethylene terephthalate (PET) membrane (8- $\mu$ m pore size) in a Tissue Culture (TC) insert (Becton Dickinson). MDA-MB-231 cells were plated into the upper chamber of the TC insert containing SFM, and the insert was placed into a well of a six-well plate containing 2 mL of either SFM + HGF (40 ng/mL) alone or SFM + HGF with either wortmannin (0.2  $\mu$ mol/L), UO126 (10  $\mu$ mol/L), or Bis-I (20  $\mu$ mol/L) or various concentrations of blueberry extract. The control well contained SFM only. After 24 hours, the top surface of the TC insert was scraped using a cotton swab and the cells on the lower surface of the membrane were fixed for 15 minutes



**Figure 1.** MDA-MB-231 cell proliferation and apoptosis. A, breast cell lines were treated with increasing concentrations of blueberry extract for 72 h. B, MDA-MB-231 cells were treated with increasing concentrations of blueberry fractions (EA, MeOH, and water fractions) for 72 h. Proliferation was assessed using the CellTiter-Glo Luminescent Cell Viability Assay. Data are expressed as a ratio of treated samples to untreated controls. Columns, mean; bars, SE. \*,  $P \leq 0.01$  versus untreated controls ( $n \geq 9$ ). C, cells were exposed to blueberry extract at indicated concentrations for 72 h, harvested, and analyzed using the Cell Death Detection ELISA<sup>PLUS</sup> Assay. Columns, mean; bars, SE. Data are expressed as absorbance at 405 nm of each treated sample divided by control. \*,  $P \leq 0.01$  versus untreated controls ( $n \geq 12$ ).

with MeOH and stained with crystal violet. Cells that had migrated to the bottom of the membrane were visualized and counted using an inverted microscope. For each replicate ( $n = 3$ ), cells in four randomly selected fields were counted and averaged. Data are expressed as a ratio to the HGF-treated group.

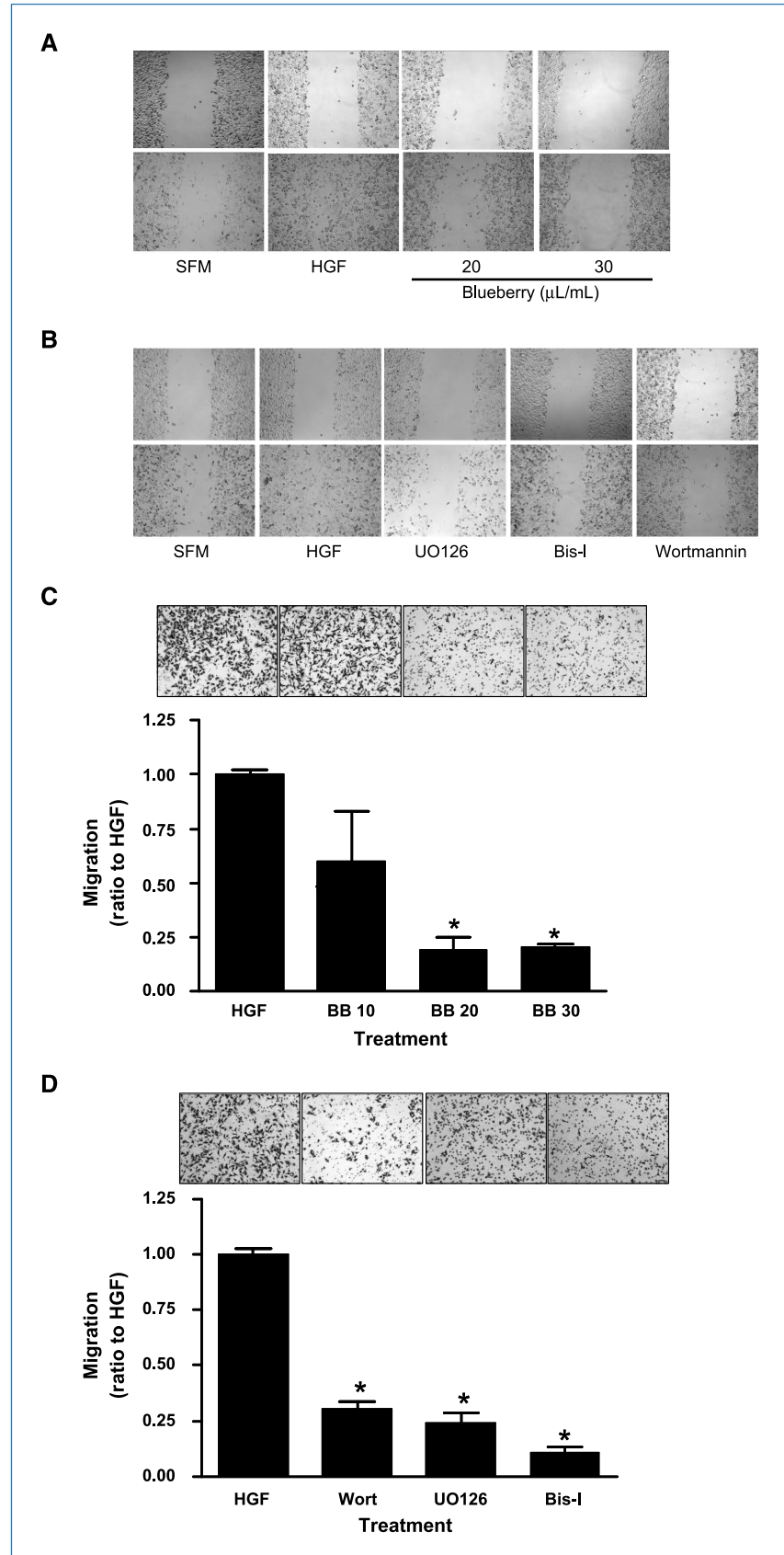
**Western blotting.** MDA-MB-231 cells were serum starved for 24 hours and then treated with either vehicle control, appropriate cell signaling inhibitors, or various concentrations of blueberry extract for 2 hours before stimulation with HGF (40 ng/mL) for 15 minutes. Cell lysates were run on a 10% acrylamide gel, transferred to a nitrocellulose membrane, and probed with either anti-phosphorylated and total AKT and ERK antibodies or phospho-protein kinase C (PKC) plus  $\beta$ -actin (Cell Signaling Technology, Inc.). Bands were visualized via chemiluminescence using horseradish peroxidase-conjugated secondary antibodies. Bands were quantified using Bio-Rad Quantity One software.

**Reporter gene assay.** MDA-MB-231 cells were transiently transfected with the pNF $\kappa$ B-luciferase expression construct (Stratagene) using the Lipofectamine Plus reagent system (Invitrogen) according to manufacturer's protocol. Six hours posttransfection, cells were treated with SFM, HGF (40 ng/mL) alone or in combination with blueberry (20 or 30  $\mu$ L/mL) or signaling inhibitors for 24 hours. Cell lysate was collected following passive lysis and NF $\kappa$ B activity assayed on a TD 20/20 luminometer (Turner Designs). Protein concentration was also assayed using the BCA Assay (Pierce). Data is expressed as relative luciferase units/protein content as a ratio of treated samples to SFM samples.

**Animal experiments.** Five-week-old intact female BALB/c *nu/nu* athymic mice were purchased (Charles River Laboratories) and randomly divided into two groups of eight mice each. At 6 weeks of age, mice were gavaged fed with either 100  $\mu$ L water control or 100  $\mu$ L blueberry. Animals were gavaged daily for the duration of the experiment. At 7 weeks of age, mice were injected s.c. with MDA-MB-231 cells ( $3 \times 10^6$ ) in Matrigel (BD Biosciences). Body weights were monitored weekly as an indicator of overall health. At the end of 7 weeks of gavage treatment, mice were euthanized via CO<sub>2</sub> asphyxiation; tumors were removed, weighed, and sent for H&E histologic staining through the City of Hope Pathology Department Core Facility. Tumor specimens were stained using cleaved caspase-3 antibody (Cell Signaling Technology) for apoptosis and Ki-67 antibody (Dakocytomation) staining for cell proliferation, and phospho-ERK, phospho-AKT, and phospho-p65 NF $\kappa$ B (Cell Signaling Technology). Immunohistochemical slides were quantified by taking six random fields and counting stained and unstained cells and dividing each by the total number of cells counted to generate the percent of positive cells. The protocol for this study was approved by the City of Hope Research Animal Care Committee.

**Zymogram analysis of MMP activity.** MDA-MB-23 cells were treated with SFM for 24 hours before treatment with HGF (40 ng/mL) or HGF plus either wortmannin (0.2  $\mu$ mol/L) or various concentrations of blueberry extract. Cells were incubated for 24 hours, and the medium was collected and concentrated using Microcon YM-10 filters (Millipore). Concentrated medium was mixed with electrophoresis buffer without reducing agent, incubated at room temperature for 15 minutes, and loaded on 10% acrylamide gels containing gelatin (Bio-Rad). Gels were run at 120 V for 90 minutes and incubated in renaturing solution (Bio-Rad) for 30 minutes at room temperature with agitation. Gels

**Figure 2.** HGF-induced motility and migration in MDA-MB-231 cells. Confluent monolayers were scratched with a plastic pipette tip and incubated in SFM or in the presence of either HGF (40 ng/mL), or HGF plus blueberry (BB) extract (20 and 30  $\mu$ L/mL; A), or various cell signaling inhibitors (B) for 24 h. Migration of MDA-MB-231 cells through a PET membrane (0.8- $\mu$ m Transwell culture inserts) was evaluated in the presence or absence of 10–30  $\mu$ L/mL blueberry (C) or various cell signaling inhibitors (D) after 24 h of treatment. Quantification of the number of migrating cells from three separate experiments. Data are expressed as a ratio to HGF-treated cells. Columns, mean; bars, SE. \*,  $P \leq 0.01$ , significant difference from HGF-treated cells ( $n \geq 3$ ).



were rinsed once in water and incubated in developing buffer (Bio-Rad) for 30 minutes at room temperature with agitation before incubation in fresh developing buffer at 37°C overnight. Developing buffer was decanted, and the gels were stained with 0.3% Coomassie blue solution for 1 hour and destained until clear bands were visible. Bands were visualized on a Kodak Gel Logic 200 imaging system with the Kodak 1D software. Loading control included equal amounts of sample run on 10% acrylamide gels and stained with Coomassie blue.

**uPA, PAI, and TIMP-1 secretion.** Conditioned medium (see above) was also evaluated for secretion of uPA, PAI, and TIMP-1 via Western blot analysis with anti-uPA, anti-PAI, and anti-TIMP-1 antibodies (Santa Cruz Biotechnology).

**Statistical analysis.** To assess statistical significance, values were compared with controls with either Student's *t* test or one-way ANOVA, followed by Dunnett's multiple range test ( $\alpha = 0.05$ ), using Prism GraphPad 4 software (GraphPad Software, Inc.).

## Results

**Polyphenol content of blueberry extract and isolated fractions.** Water, MeOH, and EA fractions of the original blueberry extract were created. Total polyphenol content of blueberry extract and the fractions was determined using the Folin-Ciocalteu method and expressed as gallic acid equivalents. Results showed that whole blueberry extract contained 1.59% polyphenols, EA contained 7.16%, MeOH contained 1.45%, and water extract contained no detectable polyphenolic compounds (based on dry matter). Therefore, polyphenolic compounds were more highly concentrated in the EA fraction compared with whole blueberry or the MeOH and water fractions.

**Antiproliferative effects of blueberry extracts.** Blueberry extract was tested in triple-negative breast cancer cell lines (MDA-MB-231, HCC38, and HCC1937) and one untransformed breast cell line (MCF-10A). The pH of medium plus blueberry extract was tested at each concentration, and pH was not allowed to go below 7.0. Results showed that blueberry was most effective in the most aggressive cell line (MDA-MB-231) while having no significant effect on the untransformed MCF-10A cells (Fig. 1A). Water, MeOH, and EA fractions of the blueberry extract were also tested against the proliferation of MDA-MB-231 cells in increasing concentrations. Results showed that the activity of the fractions was lower in comparison with the whole blueberry extract despite the increased polyphenolic content of the EA fraction. Two-by-two and a combination of all three fractions did not improve the activity (Fig. 1B). Therefore, we may conclude that whole blueberry was more active than its isolated fractions. However, it is also possible that the active components may get destroyed or lost during the fractionation process. Further development of methods to more efficiently recover individual fractions is needed.

**Induction of apoptosis by blueberry extract.** To determine whether the decreased cell number was due to the induction of cell death, apoptosis was quantitated using the

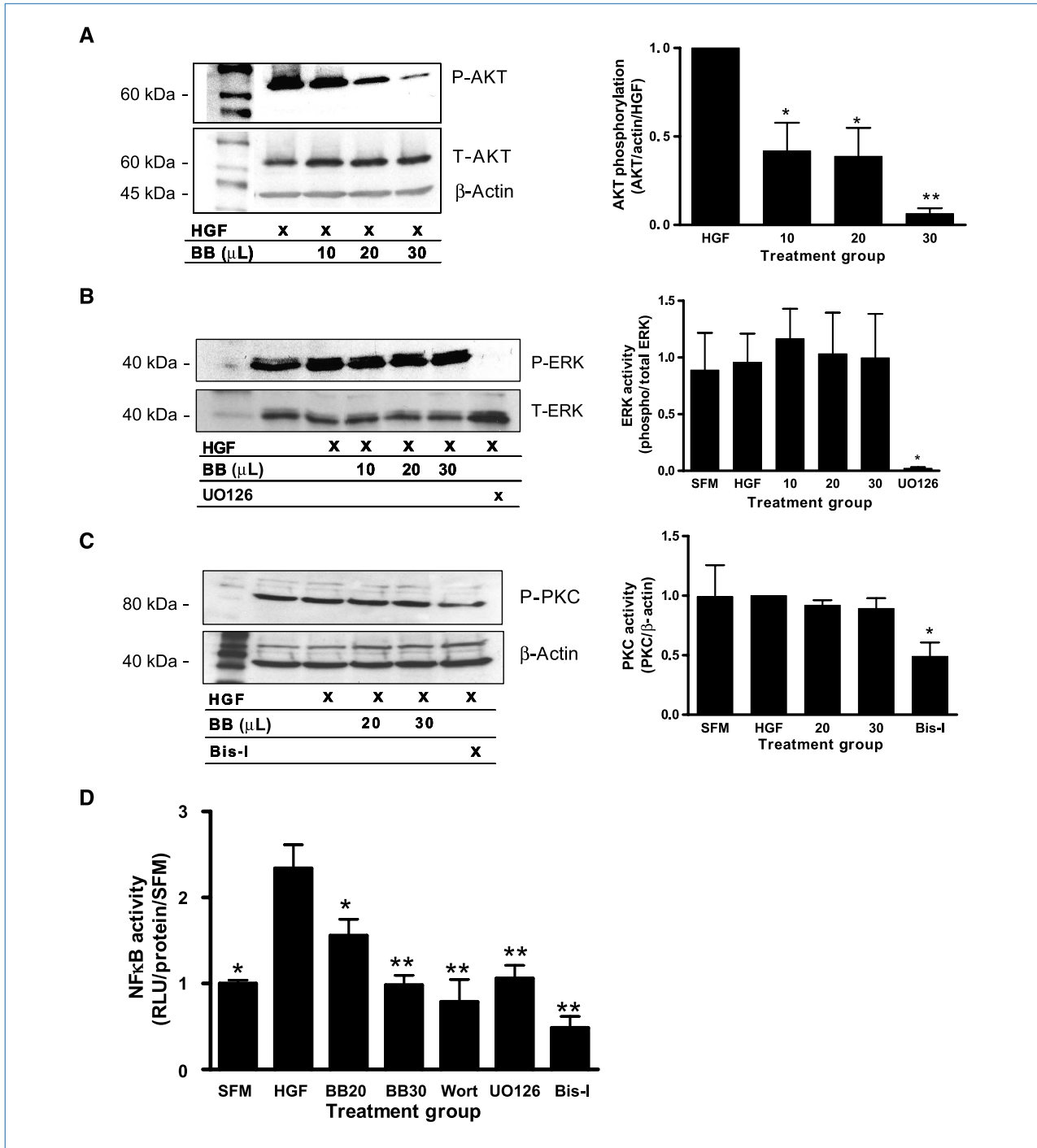
Cell Death Detection ELISA<sup>PLUS</sup> assay (Roche Diagnostics). Following treatment with blueberry extract (12.5 and 25  $\mu\text{L}/\text{mL}$ ) for 72 hours, a 1.5-fold increase in DNA fragmentation was detected compared with water-treated control cells. This small amount of apoptosis in the blueberry-treated cells does not account for the observed decrease seen in the cell viability assay. Therefore, the results of the viability assay can largely be attributed to an inhibition of proliferation.

**Effects of blueberry extract on the metastatic potential of MDA-MB-231 cells.** Due to the highly invasive nature of triple-negative breast cancers, we investigated the effects of blueberry extract on the invasive potential of MDA-MB-231 cells *in vitro*. Wound-healing assays were used to determine whether blueberry extract could inhibit HGF-induced migration of MDA-MB-231 cells. Twenty-four hours after cell monolayers were wounded, HGF-treated cells had completely filled in the cleared area. Treatment with 20 and 30  $\mu\text{L}/\text{mL}$  of blueberry extract inhibited HGF-induced migration of cells (Fig. 2A). To determine the importance of the signaling pathways PI3K/AKT, ERK, and PKC in the migration of MDA-MB-231 cells, the cell signaling inhibitors wortmannin, UO126, and Bis-I were used. Treatment with all three inhibitors also inhibited the migration of cells in this assay (Fig. 2B), showing the involvement of these signaling pathways in the migration of triple-negative breast cancer cells.

Inhibition of HGF-induced migration by blueberry was investigated using the PET membrane method. MDA-MB-231 cells treated with HGF alone migrated to the bottom side of the membrane. Blueberry extract (20 or 30  $\mu\text{L}/\text{mL}$ ) significantly reduced cell migration (Fig. 2C) as did treatment with wortmannin, UO126, or Bis-I (Fig. 2D).

**Inhibition of cell signaling pathways by blueberry in vitro.** Due to the importance of the PI3K/AKT, ERK, and PKC signaling pathways to the activation of MMPs, we determined the effect of blueberry extract on the activation of these pathways. Results revealed that AKT was phosphorylated in response to HGF treatment; however, pretreatment with blueberry extract at 10, 20, and 30  $\mu\text{L}/\text{mL}$  significantly reduced HGF-induced AKT activation ( $P \leq 0.05$  and  $0.01$ ; Fig. 3A). Blueberry also decreased HGF-induced transcriptional activity of NF $\kappa$ B at 20  $\mu\text{L}/\text{mL}$  ( $P \leq 0.05$ ) and 30  $\mu\text{L}/\text{mL}$  ( $P \leq 0.01$ ; Fig. 3D). Alternatively, the phosphorylation of ERK and PKC was unaffected (Fig. 3B and C). Although inhibition of ERK and PKC decreased cell motility and migration in our assays, blueberry does not affect migration through inhibition of these pathways. On the other hand, inhibition of PI3K/AKT leading to downregulation of NF $\kappa$ B transcriptional activity may be one mechanism behind the action of blueberry extract against MDA-MB-231 cell migration.

**Inhibition of MMP activity and the secretion of its regulatory proteins.** The activity of blueberry extract against gelatinase (MMP-2 and MMP-9) activity was assayed using gelatin zymogram gels. Zymography identifies MMPs based on their preferential substrate (gelatin in the case of MMP-2 and MMP-9) and molecular weight. Once the sample is separated on the gel, it is incubated in an activation buffer and the MMPs present in the sample digest the substrate present in the gel, showing the activity of the enzymes. When the gel is



**Figure 3.** ERK, NFκB, and AKT activation in MDA-MB-231 tumor cells. The phosphorylation of AKT (A), ERK (B), and PKC (C) in cell lysates from cells treated with blueberry, wortmannin (Wort), UO126, or Bis-I was determined via Western blotting and chemiluminescence. Bar graphs indicate quantification of at least three separate blots using Quantity One software. D, NFκB activity was evaluated via reporter gene assay. Luciferase activity was assayed after 24 h and normalized to total protein. Data are expressed as relative luciferase units (RLU) as a ratio of treated samples to SFM samples. Columns, mean; bars, SE. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.01$ , significant difference from HGF-treated controls ( $n \geq 3$ ).

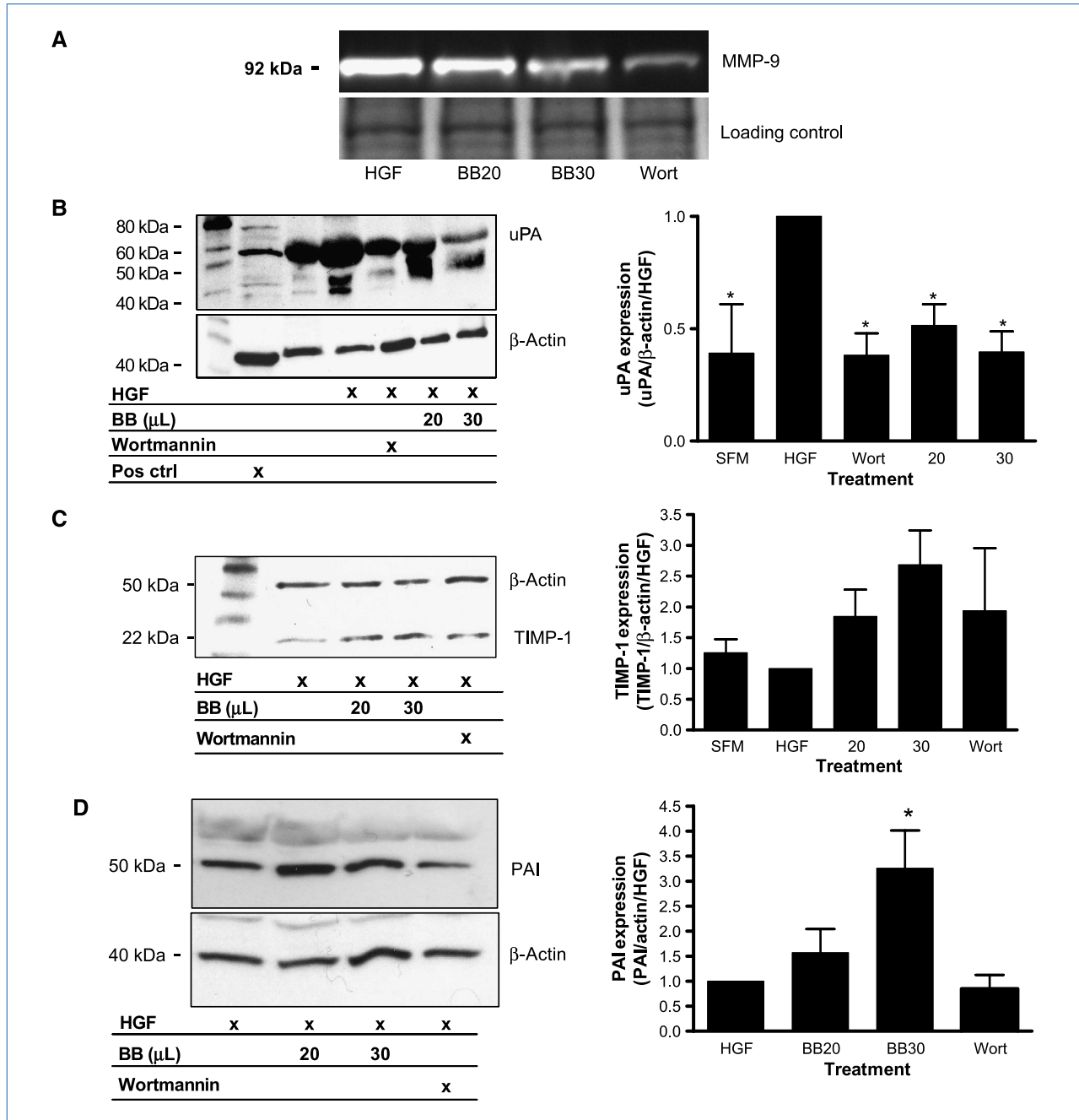
stained, activity is shown as a cleared region (or a clear band) in the gel.

In conditioned medium from cells treated with HGF, a band was identified at 92 kDa (corresponding to MMP-9).

Treatment with blueberry (30 μL/mL) extract or wortmannin decreased the intensity of the band, indicating inhibition of MMP-9 activity (Fig. 4A). This suggests that the inhibitory activity of blueberry on the migration of MDA-MB-231 cells

may be due to inhibition of MMP-9 activity and that inhibition of the PI3K/AKT signaling pathway is also important to MMP-9 activation in this cell line. The activity of other MMP family members was also investigated using casein gels. Casein is the preferential substrate for the detection

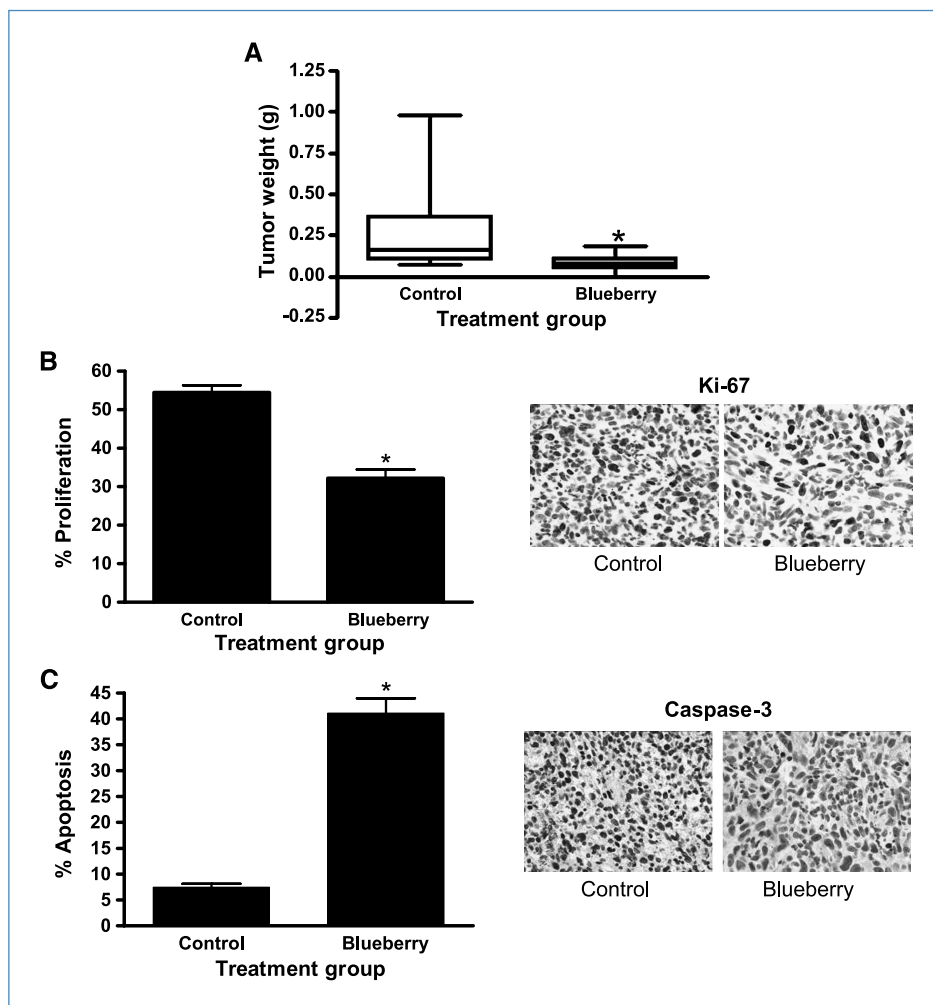
of MMP-11, MMP-1, MMP-7, MMP-12, MMP-13, and MMP-9 (at very high concentrations). No cleared zones (or bands) were seen in this assay, suggesting a lack of activity of these MMPs in the MDA-MB-231 cells (data not shown).



**Figure 4.** Modulation of metastatic proteins. A, inhibition of MMP-9 activity in conditioned medium from MDA-MB-231 cells treated with HGF (40 ng/mL) and blueberry extract (20 and 30  $\mu$ L/mL) or wortmannin (0.2  $\mu$ mol/L) for 24 h was evaluated via gelatin zymography. Bands were visualized on a Kodak Gel Logic 200 imaging system with the Kodak 1D software. Equal amounts of sample run on 10% acrylamide gels and stained with Coomassie blue as loading control. Conditioned medium from cells was evaluated for modulation of uPA (B), TIMP-1 (C), and PAI (D) secretion via Western blotting and chemiluminescence. Bar graphs indicate quantification of at least three separate blots using Quantity One software. Columns, mean in each group ( $n \geq 3$ ); bars, SE. \*,  $P \leq 0.05$ , statistical significance from HGF-treated cells.

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**Figure 5.** Inhibition of breast tumor growth. Seven- to 8-week-old athymic nude female mice were gavage fed daily with 100  $\mu$ L blueberry extract or water. One week after the start of gavage, mice were s.c. injected in the right flank with MDA-MB-231 cells ( $1 \times 10^6$ ). After 6 wks, mice were euthanized and tumors were removed, weighed, and sent for histologic staining. A, tumor weights. B, Ki-67 antibody staining for cell proliferation. C, cleaved caspase-3 for apoptosis was evaluated via immunohistochemistry. Bar graphs indicate quantification of six random fields; stained and unstained cells were counted and divided by the total number of cells counted to generate the percentage of positive cells in each group. Columns, mean in each group ( $n \geq 6$ ); bars, SE. \*,  $P \leq 0.01$ , statistical significance from control group.



The secretion of the MMP inhibitors uPA, TIMP-1, and PAI-1 in conditioned medium from MDA-MB-231 cells was also evaluated, as this is one possible mechanism of blueberry-induced inhibition of MMP activity. Western blot analysis revealed that HGF increased and blueberry extract decreased secretion of uPA in conditioned medium (Fig. 4B). A trend for increased TIMP-1 expression was observed in blueberry- and wortmannin-treated cells compared with HGF-treated cells, although this was not statistically significant when quantified (Fig. 4C). In addition, PAI expression was significantly increased in blueberry-treated cells (Fig. 4D).

**In vivo chemopreventive effects of blueberry extract.** To evaluate the chemopreventive potential of the blueberry extract *in vivo*, we used the MDA-MB-231 xenograft model. Blueberry treatment was initiated 1 week before and continued 6 weeks following tumor cell injection. Results showed significantly smaller tumor weights in the mice treated with blueberry extract versus control mice ( $P \leq 0.01$ ; Fig. 5A). Consistent with the *in vitro* experiments, proliferation (Ki-67 staining) of tumor specimens was significantly decreased in blueberry-treated mice ( $P \leq 0.01$ ; Fig. 5B) and apoptosis

(caspase-3 staining) of tumor specimens was significantly increased ( $P \leq 0.01$ ; Fig. 5C). These results illustrate that blueberry juice is orally active and that the intake of blueberry may have an inhibitory effect on estrogen-independent and HER-2-independent tumor growth *in vivo*.

Tumor specimens from the blueberry- and water-treated mice were stained via immunohistochemistry for active (phosphorylated) AKT and ERK and the p65 subunit of NF $\kappa$ B. The activation of NF $\kappa$ B and AKT has been identified as important to triple-negative breast cancer growth and metastasis, as has the ERK pathway. Results showed that both p65 NF $\kappa$ B and AKT phosphorylation were significantly reduced in tumors from blueberry-treated mice ( $P \leq 0.01$ ; Fig. 6A and B). Alternatively, there was no significant difference in the amount of phospho-ERK staining between untreated and treated tumor specimens (Fig. 6C), which is consistent with our *in vitro* experiments.

## Discussion

The aims of this study were to determine the antitumor activity of a whole blueberry preparation against MDA-MB-231

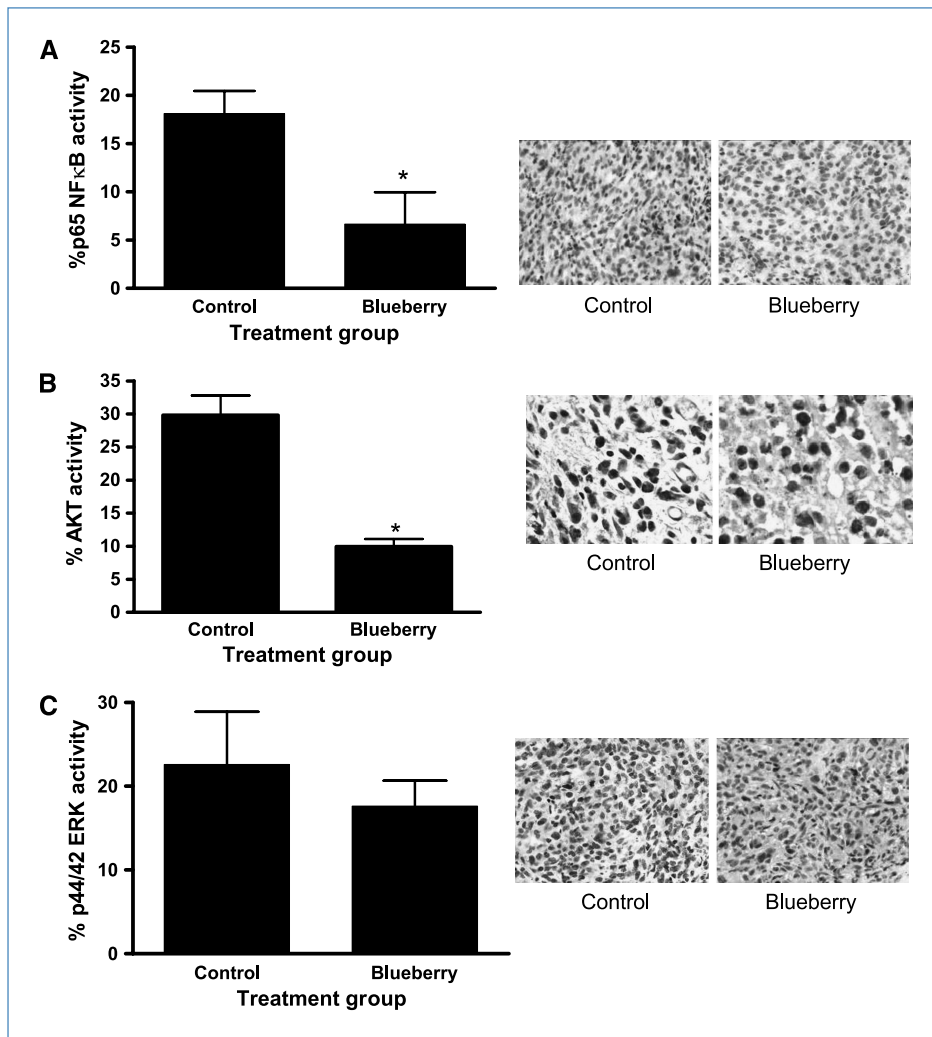


cells in a xenograft mouse model and to investigate the modulation of the metastatic process *in vitro*. Preliminary results showed that among a panel of whole food preparations, blueberry exhibited the highest antiproliferative effect against MDA-MB-231 cells while having no effect on the nontransformed MCF-10A cell line. One aim in developing chemopreventive agents is to identify those that are easily accessible and nontoxic; therefore, blueberry was chosen for further study.

The failure of the isolated blueberry fractions to equal or exceed the antiproliferative activity of the whole blueberry extract is similar to results seen with the fractionation of cranberry juice. In that study, whole cranberry juice had higher antiproliferative activity than isolated anthocyanin and proanthocyanidin fractions (33). Because the isolated compounds from blueberry and cranberry are phenolic in nature, they are prone to oxidation/decomposition when purified and taken out of the matrix of the whole fruit/juice. Therefore, we conclude that the isolated fractions are less active than whole blueberry due to their separation from

the whole, and whole blueberry extract was used for further investigations.

*In vivo* experiments showed no adverse effects of blueberry when ingested by mice. In addition, oral activity was observed through decreased growth of MDA-MB-231-derived tumors in a xenograft mouse model. The decreased tumor size was attributed to decreased cell proliferation and increased apoptosis, as measured by Ki-67 and caspase-3 immunohistochemical staining of tumor tissues. A recent study by Aiyer and colleagues (34) showed comparable results in rats, where oral administration of whole blueberry powder decreased breast tumor volume by 40% compared with untreated control animals. Similar studies showed that oral ingestion of blueberry extract decreased hemangioma tumor size in mice (35). In contrast, studies by Stoner (36) showed that diets containing 5% and 10% of whole blueberry powder did not inhibit *N*-nitrosomethylbenzylamine-induced esophageal tumors in rats. The differing inhibitory effects on breast and esophageal tumor growth observed in these two studies are likely due to the



**Figure 6.** ERK, NF $\kappa$ B, and AKT activation in MDA-MB-231 tumor xenografts. Tumors from blueberry- or water-treated mice were antibody stained for phospho-NF $\kappa$ B (A), phospho-AKT (B), or phospho-ERK (C) and evaluated via immunohistochemistry. Bar graphs indicate quantification of six random fields; stained and unstained cells were counted and divided by the total number of cells counted to generate the percentage of positive cells in each group. Columns, mean in each group ( $n \geq 6$ ); bars, SE. \*,  $P \leq 0.01$ , statistical significance from control group.

different tumor models used. Overall, studies suggest that the oral intake of blueberries may convey chemopreventive benefits *in vivo*.

Inhibition of invasive potential is important to the prevention of tumor reoccurrence. *In vitro* experiments showed that treatment with blueberry extract inhibited HGF-induced migration of MDA-MB-231 cells and decreased MMP-9 activity. A large number of natural products, including berries, have been shown to inhibit metastatic potential in cancer cell lines, including prostate, lung, breast, and fibrosarcoma. These products were found to target key proteins, including MMP-2, MMP-9, uPA, and the MMP inhibitors TIMP-1 and PAI. In addition, the AKT, NF $\kappa$ B, AP-1, c-Jun NH<sub>2</sub>-terminal kinase, and ERK signaling pathways were also regulated in a number of these studies, illustrating the importance of these signaling pathways (18, 25, 26, 37–41). The secretion of metastasis-related proteins is, at least in part, under the control of the above-mentioned cell signaling pathways, although their involvement can vary by tissue/cell type. For example, our *in vitro* experiments showed that blueberry treatment of MDA-MB-231 cells had no effect on the activation of PKC and ERK, where it inhibited the activation of the PI3K/AKT/NF $\kappa$ B pathway. Therefore, in further studies on the effects of blueberry on metastasis-related proteins, we focused on the role of this pathway.

In our experiments with blueberry, the modulation of metastatic proteins was shown to be through a decrease in uPA secretion and increased secretion of the MMP inhibitor PAI, the expression of which can be controlled by the PI3K/AKT/NF $\kappa$ B pathway. These cell signaling proteins are also important to the proliferation and survival of tumor cells (42, 43). Therefore, one mechanism by which blueberry may alter tumor growth and metastatic potential of MDA-MB-231 cells is through modulation of the PI3K/AKT/NF $\kappa$ B pathway. In contrast, Huang and colleagues (30) reported that a MeOH fraction of blueberry failed to inhibit UVB-induced activation of NF $\kappa$ B and AP-1 in mouse epidermal cells. The authors speculated that these observations may be due to the different anthocyanin profile of blueberries compared with other berries. The Huang study induced skin carcinogenesis in mice via exposure to UV radiation. In addition, the authors treated the mice with a MeOH fraction of blueberry. Therefore, the different cancer type, route of induction, and blueberry treatment (our study used blueberry juice that contains both water and lipid-soluble compounds that could work synergistically to inhibit NF $\kappa$ B activity) likely account for the differing inhibitory effect of blueberry on NF $\kappa$ B between the Huang study and ours.

The results of our studies suggest that the oral intake of blueberries could be a key component of long-term breast

cancer prevention strategies. We showed that blueberry ingestion in mice decreased not only tumor growth but also the activity of AKT and NF $\kappa$ B, which are markers for metastatic potential in breast tumors. Several studies of tissues from breast cancer patients have shown that the activity of PI3K and AKT is significantly increased in the triple-negative subset of breast cancers (44–46). This illustrates the importance of these signaling pathways to the pathology of this disease.

A limitation of this study is that *in vitro* studies cannot be extrapolated to possible activity *in vivo*. The role of *in vitro* studies is to screen for activity against biomarkers that can then be tested *in vivo* using animal studies. Our intent with the *in vitro* studies was to evaluate mechanisms through which blueberry may modulate the metastatic potential of MDA-MB-231 cells, which are now under investigation *in vivo*.

It was suggested that the antiproliferative activity of fruit extracts against cancer cell lines is due to the production of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and resultant oxidative stress (47). However, Liu and Sun (48) showed that H<sub>2</sub>O<sub>2</sub> was not produced after addition of apple extracts to medium and that there is no correlation between phenolic content and antiproliferative activity, and suggested that the previous results were due to incorrect measurement methods. Therefore, the results of our proliferation studies are likely a consequence of the effects of the phytochemicals in blueberries, not the production of H<sub>2</sub>O<sub>2</sub> in the cell medium.

The dose of blueberry in our *in vivo* study is equal to a fresh blueberry intake of 25 g/kg. With a conversion to human dose (based on surface area; ref. 49), this is equal to 2.03 g/kg human or 122 g (4.3 oz) of fresh blueberries per day for a 60-kg person. A single serving of fresh blueberries is 6 oz, which is an attainable intake for the average person. Therefore, blueberry intake could be an important part of dietary cancer prevention strategies.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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