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Plumbagin-Induced Apoptosis of Human Breast Cancer Cells Is Mediated by Inactivation of NF-κB and Bcl-2

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ABSTRACT

Breast cancer remains the major cause of cancer-related deaths in women world-wide. The heterogeneity of breast cancer has further complicated the progress of target-based therapies. Triple negative breast cancers, lacking estrogen receptor, progesterone receptor and the Her-2/neu (ErbB2), represent a highly aggressive breast cancer subtype, that are difficult to treat. Pleiotropic agents, such as those found in nature, can target receptor-positive as well as receptor-negative cancer cells, suggesting that such agents could have significant impact in breast cancer prevention and/or therapy. Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) is one such agent which has anti-tumor activity against several cancers. However, its mechanism of action against breast cancer is not clearly understood. We hypothesized that plumbagin may act as an effective agent against breast cancer especially triple negative breast cancer. We tested our hypothesis using ER-positive MCF-7 and ER-negative MDA-MB-231 (triple negative) breast cancer cells, and we found that plumbagin significantly inhibits the growth of breast cancer cells with no effect on normal breast epithelial cells. We also found that plumbagin induces apoptosis with concomitant inactivation of Bcl-2 and the DNA binding activity of NF-κB. Bcl-2 over-expression resulted in attenuation of plumbagin-induced effects, suggesting that the inhibition of cell growth and induction of apoptosis by plumbagin is in part due to inactivation of NF-κB/Bcl-2 pathway. To our knowledge, this is the first report, showing mechanistic and cancer cell specific apoptosis-inducing effects of plumbagin in breast cancer cells, suggesting the potential role of plumbagin in the prevention and/or treatment of breast cancer. J. Cell. Biochem. 105: 1461–1471, 2008.

KEY WORDS: PLUMBAGIN; BREAST CANCER; MDA-MB-231; MCF-7; Bcl-2; NF-κB; APOPTOSIS

Breast cancer remains the leading cause of morbidity of women with an estimated 1.2 million new cases world wide and 182,460 new cases in the United States alone in 2008 [Jemal et al., 2008]. It ranks as the second leading cause of cancer-related deaths in women in the United States [Jemal et al., 2008]. Although breast cancer can be treated in patients diagnosed with local disease as well as for those diagnosed with locally advanced disease using targeted therapy to attack breast cancer cells that express estrogen receptor (ER), the progesterone receptor (PR) and Her-2/neu (ErbB2); however a major challenge lies for those tumors that are triple negative and are known to be highly aggressive. About 15–21% of breast cancers fall under this category and most of this class of tumors are often classified as basal-type breast cancers which remain a challenge for treatment although not all basal-type breast cancers are triple negative or vice versa. Therefore, it is highly desirable to find novel agents that are multi-targeted (pleiotropic) for the treatment of all breast cancers. In search of such agents, studies have been focused on naturally occurring chemical compounds that are typically found in fruits and vegetables, and several such agents are known to have the potential for killing cancer cells irrespective of receptor-status [Russo, 2007]. These compounds are able to elicit their effects largely due to their pleiotropic activity meaning that these agents are multi-targeting [Howells and Manson, 2005; Khan et al., 2006]. Previous studies from our laboratory have characterized several natural compounds having pleiotropic activity in breast cancer cells [Rahman et al., 2006] as well as in other types of cancer cells [Alhasan et al., 2001; Li et al., 2004; Sarkar and Li, 2004] although their mechanism of action is still being investigated.

Emerging evidence suggests a negative correlation between the expression of ER and the transcription factor NF-κB in breast cancer cells, implying that the highly aggressive triple negative breast cancers cells, such as MDA-MB-231, have constitutively high NF-κB expression [Wang et al., 2007a]. NF-κB is known to play important roles in the regulation of many signaling pathways including those involved in cancer development and progression [Karin, 2006]. In

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breast cancer, ER signaling reduces the levels of functional NF-κB [Wang et al., 2007a] and thus causing reduction in the aggressiveness of breast cancer cells whereas the lack of ER induces NF-κB with concomitant increase in aggressiveness. The second mechanism by which NF-κB promotes invasiveness of ER-negative breast cancer cells is by induction of Bcl-2 [Wang et al., 2007a], leading to the survival of these cells during therapy. Bcl-2, a human proto-oncogene, and related proteins are important regulators of apoptosis. Bcl-2 family proteins are believed to constitute a critical control point in the process of apoptosis-induction, residing immediately upstream of irreversible cellular damage, and thereby influencing the induction of apoptosis [Danial, 2007]. Interestingly, it has been shown that nuclear localization of NF-κB and expression of Bcl-2 serves as a mechanism for resistance of breast cancer patients to anthracyline-based chemotherapy [Buchholz et al., 2005].

Based on the current understanding, it is evident that an agent capable of suppressing NF-κB activity as well as the expression of Bcl-2 could be a good therapeutic agent against breast cancer. Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone), a quinonoid constituent isolated from the roots of *Plumbago zeylanica* L. is one such agent showing anti-cancer activity, which is partly mediated by the inactivation of NF-κB-regulated anti-apoptotic, proliferative and angiogenic gene products [Sandur et al., 2006]. Potential role of plumbagin, as an anti-cancer agent has been recognized [Parimala and Sachdanandam, 1993; Naresh et al., 1996; Sugie et al., 1998; Hazra et al., 2002] and its anti-cancer effects have been reported in diverse cancer models such as prostate [Powolny and Singh, 2008], lung [Hsu et al., 2006; Gomathinayagam et al., 2008], cervical [Srinivas et al., 2004b; Nair et al., 2008], ovarian [Srinivas et al., 2004a] as well as the melanoma [Wang et al., 2008]. However, the effectiveness of plumbagin as an anti-cancer agent against breast cancer has not been molecularly investigated except a single report showing the growth inhibitory activity of plumbagin in human breast cancer cells [Kuo et al., 2006], which prompted us to investigate the mechanistic role of plumbagin in breast cancer cells. For our studies, we chose MDA-MB-231 (ER-negative) and MCF-7 (ER-positive) cell lines as the model and investigated the effect of plumbagin treatment. We found that plumbagin inhibited cell growth and induced apoptosis of both ER-positive as well as ER-negative breast cancer cells while there was no significant effect on MCF-10A non-tumorigenic and the so-called "normal" breast epithelial cells. This was in part due to inactivation of Bcl-2 and DNA binding activity of NF-κB. We have confirmed our results by transfection studies by forced over-expression of Bcl-2 in ER-positive, Bcl-2-low-expressing MCF-7 cells. We found that forced over-expression of Bcl-2 attenuated plumbagin induced cell growth inhibition and induction of apoptosis. Taken together, our results suggest that plumbagin could be a useful agent for the prevention and/or treatment of breast cancer especially for triple negative breast cancer, which could be due to inactivation of Bcl-2 and NF-κB activity.

MATERIALS AND METHODS

CELL LINES, REAGENTS, AND ANTIBODIES

Human breast cancer cell lines, MDA-MB-231 and MCF-7 were maintained in DMEM (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum, 100 units/ml penicillin, and 100μg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. The normal breast epithelial cell line, MCF-10A, was propagated in DMEM/F12 (Invitrogen) supplemented with 5% horse serum, 20 ng/mL EGF, 0.5 μg/mL hydrocortisone, 0.1 μg/mL cholera toxin, 10 μg/mL insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. Plumbagin was purchased from Sigma–Aldrich (St. Louis, MO) and was dissolved in DMSO to make 10 mM stock solution. Antibody against human Bcl-2 was purchased from Dako North America, Inc. (Carpinteria, CA). Antibodies against cleaved caspases, cleaved caspase-3 and cleaved caspase-9, were from Cell Signaling (Danvers, MA). Anti-LC3 antibody was purchased from MBL International (Woburn, MA). Monoclonal antibody to β-actin was purchased from Sigma–Aldrich and the polyclonal antibody against Rb (retinoblastoma) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

PLASMIDS AND TRANSFECTIONS

Bcl-2 containing vector was generated as described recently [Kong et al., 2008]. Briefly, Bcl-2 was sub-cloned into mammalian expression vector pcDNA3 from pSPUTKBcl-2 plasmid with flanking *Eco*RI and *Xho*I sites. MCF-7 cells were transfected with Bcl-2 cDNA or the vector, pCDNA3, using Lipofectamine 2000 following the transfection protocol described earlier [Wang et al., 2007b]. MCF-7 cell line, with stably transfected Bcl-2 gene, was propagated in the same medium as the parental cell plus 600 μg/ml geneticin (G418, Sigma–Aldrich). The transfected cells were treated with varying concentrations of plumbagin as described under individual experiments.

CELL GROWTH INHIBITION STUDIES BY 3-(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE ASSAY

MDA-MB-231 and MCF-7 cells were seeded at a density of 3×10^3 cells per well in 96-well microtiter culture plates. After overnight incubation, the medium was removed and replaced with a fresh medium containing DMSO (vehicle control) or different concentrations of plumbagin diluted from a 10 mM stock. After 72 h of incubation, 25 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) solution (5 mg/ml in phosphate-buffered saline, PBS) were added to each well and incubated further for 2 h at 37°C. Upon termination, the supernatant was aspirated and the MTT formazan, formed by metabolically viable cells, was dissolved in isopropanol (100 μl). The plates were mixed for 30 min on a gyratory shaker, and the absorbance was measured at 595 nm on Ultra Multifunctional Microplate Reader (TECAN, Durham, NC). Each treatment had eight replicate wells and the amount of DMSO in reaction mixture never exceeded 0.1%.

CELL VIABILITY STUDIES BY TRYPAN BLUE ASSAY

MDA-MB-231 and MCF-7 cells were seeded in 6-well culture plates and treated with plumbagin as described above. Upon completion of incubation, culture medium (with floating dead cells) was collected and pooled with the adherent cells removed from the plate by trypsinization. The cells were briefly spun and re-suspended in the normal culture medium. Cell viability was assessed by adding 50 μl

of Trypan Blue solution (0.4% in PBS) to 50 μl of the cell suspension. After 2 min, the number of living cells, which did not retain the dye was counted using a hemocytometer, and was compared to the total number of cells (living + dead) to calculate the viability percentage.

HISTONE/DNA ELISA FOR DETECTION OF APOPTOSIS

The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in breast cancer cells treated with plumbagin, according to the manufacturer's protocol. Briefly, cells (MDA-MB-231/MCF-7/ MCF-7-pCDNA3/MCF-7-Bcl-2) were treated with plumbagin or DMSO control for 72 h. After treatment, the cytoplasmic histone/DNA fragments from these cells were extracted and incubated in the microtiter plate modules coated with anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments followed by color development with ABTS substrate for peroxidase. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (TECAN) at 405 nm.

ANNEXIN V-FITC METHOD FOR ASSESSING THE PERCENTAGE OF APOPTOTIC CELLS

Cells (MDA-MB-231/MCF-7/MCF-7-pCDNA3/MCF-7-Bcl-2) were incubated in the presence or absence of varying concentrations of plumbagin for 72 h. Apoptotic cells were quantitated using Annexin V-FITC apoptosis detection kit (BD Pharmingen Biosciences, USA). Cells were trypsinized, washed twice with cold PBS and re-suspended in 1× binding buffer at a concentration of 1×10^5/ml cells in a total volume of 100 μl. To this, 5 μl of Annexin V-FITC and 5 μl of propidium iodide (PI) were added. The tubes were gently mixed and incubated in the dark for 20 min at room temperature. Four hundred microliters of 1× binding buffer was then added to each tube and the number of apoptotic cells was analyzed by flow cytometry. DMSO-treated control cells were used to serve as Annexin V-FITC- or PI-only control.

WESTERN BLOT ANALYSIS

For Western blot analysis, cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1% NP-40, 2 mM EDTA, 0.5% sodium deoxycholate and 0.1% SDS), containing complete mini EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor cocktails 1 and 2 (Sigma–Aldrich). Protein concentration was measured by BCA Protein Assay (Pierce, Rockford, IL). After the resolution of proteins on 12% polyacrylamide gels under denaturing conditions and transfer to nitrocellulose membranes, appropriate primary antibodies were added. This was followed by incubation with horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the chemiluminescence detection system (Pierce). For re-probing, membranes were incubated for 30 min at 50°C in buffer containing 2% SDS, 62.5 mM Tris (pH 6.7), and 100 mM β-mercaptoethanol, washed and incubated with desired primary followed by secondary antibodies and the signals were detected as described above.

PREPARATION OF NUCLEAR LYSATES

MDA-MB-231/MCF-7/MCF-7-Bcl-2 cells were treated with plumbagin or DMSO (control), and at the end of the treatment, nuclear protein extract was prepared and subjected to DNA binding activity of NF-κB by EMSA as described earlier [Banerjee et al., 2007]. Briefly, cell pellets were washed with cold PBS and suspended in ice-cold lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM ethylene-diamine-tetra-acetic acid (EDTA), 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin and 0.5 mg/ml benzamidine). The cells were kept on ice for 20 min and then NP-40 was added at a final concentration of 0.3%. The tubes were vigorously mixed on a vortex mixer for few seconds and centrifuged for 2 min in a microfuge at 4°C. The nuclear pellet was re-suspended in 30 μl of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 0.5 mg/ml benzamidine) and incubated on ice with intermittent mixing. The tubes were then centrifuged for 5 min in a microfuge at 4°C, and the supernatant (nuclear protein extract) was collected in a pre-chilled tube and stored at −70°C until used. The protein content was measured by BCA method.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSA was performed by incubating 8 μg of nuclear protein extract with IRDye-700-labeled nuclear factor-κB (NF-κB) oligonucleotides (LI-COR, Lincoln, NE) [Banerjee et al., 2007]. The incubation mixture included 2 μg of poly deoxyinosinic-deoxycytidylic acid (poly dI-dC) in the binding buffer. The DNA–protein complex formed was separated from free oligonucleotide on 8% native polyacrylamide gel using buffer containing 50 mM Tris, 200 mM glycine (pH 8.5), and 1 mM EDTA and then visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1 (Li-COR, Inc., Lincoln, NE). As a loading control, 8 μg of nuclear protein from each sample was subjected to Western blot analysis for Rb protein as described earlier [Wang et al., 2006].

RESULTS

EFFECT OF PLUMBAGIN TREATMENT ON PROLIFERATION AND VIABILITY OF BREAST CANCER CELLS

To assess the growth inhibitory effect of plumbagin on breast cancer cell lines, we treated MDA-MB-231 and MCF-7 cells with increasing doses of plumbagin (0–3 μM) for 72 h. As a control, we included MCF-10A non-tumorigenic so-called "normal" breast epithelial cells for this study. As observed in Figure 1A, cell proliferation was reduced in a dose-dependent manner only in MDA-MB-231 and the MCF-7 cells whereas MCF-10A cells were relatively insensitive at equivalent concentrations of the drug. In MDA-MB-231 as well as MCF-7 cells, cell growth was reduced by more than 62% and 51% respectively using 1 μM plumbagin treatment for 72 h. MCF-10A cells showed only about 25% inhibition of cell growth at this concentration and no further growth inhibition was observed with increasing doses of plumbagin. At 2 μM plumbagin concentration, cell growth was inhibited by more than 76% in MDA-MB-231 cells and by more than 60% in MCF-7 cells. Such an inhibition was not achieved in MCF-10A cells even at 10 μM of plumbagin treatment (results not shown). The inhibition of cell growth by plumbagin was significantly more in cancer cells compared with the normal

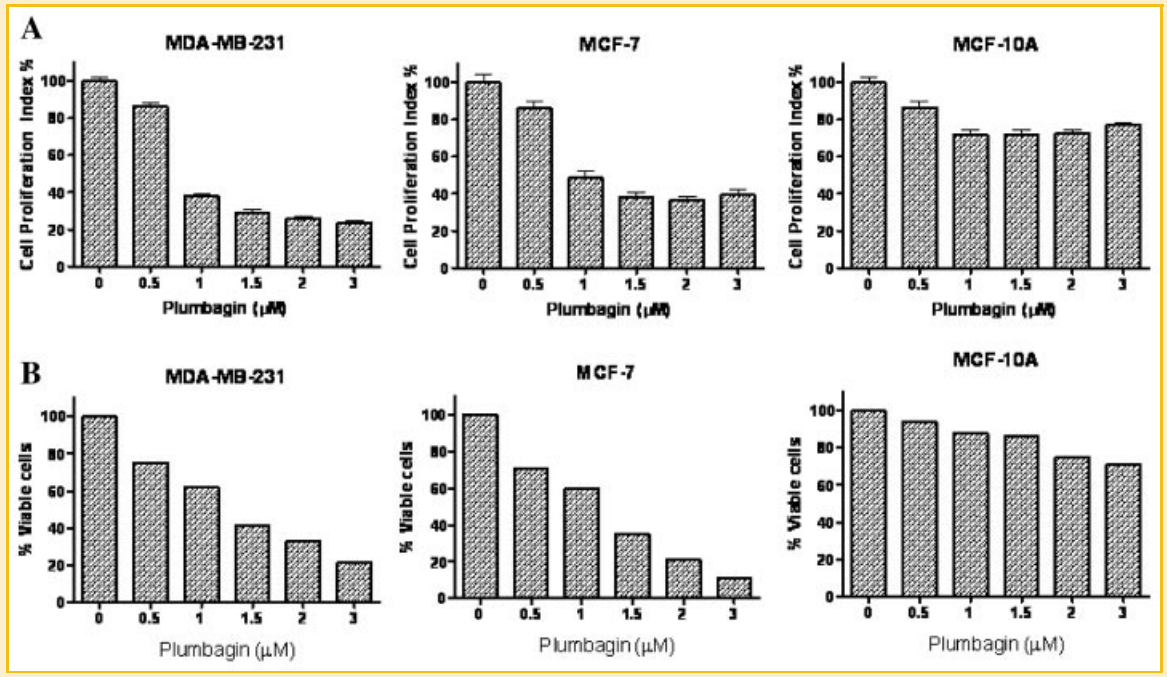

Fig. 1. Evaluation of (A) cell proliferation and (B) cell viability in plumbagin-treated MDA-MB-231, MCF-7 and MCF-10A cells by MTT and trypan blue staining, respectively. Cells were either vehicle-treated (DMSO-control) or treated with increasing concentrations of plumbagin (0.5, 1, 1.5, 2, and 3 μM) for 72 h and then analyzed as described under Materials and Methods Section. The amount of DMSO never exceeded 0.1% during the treatment. The number of cells counted/OD value in DMSO (control)-treatment was considered 100% and the number of cells in plumbagin-treated cells was calculated relative to this control as the percentage surviving cells. Each data point represents mean ± SE of eight replicates from, at least, two independent experiments.

epithelial cells, suggesting the cancer cell specific effects of plumbagin even at lower doses.

Subsequently, we tested the viability of cells treated with plumbagin using trypan blue dye exclusion method. As seen in Figure 1B, treatment with plumbagin resulted in a dose-dependent inhibition of cell viability of breast cancer cells but significantly less effect was observed on normal epithelial cells. Plumbagin at 1.5 μM induced inhibition of cell viability by 59% in MDA-MB-231 cells and 65% in MCF-7 cells whereas the same concentration resulted in only about 14% inhibition in cell viability of MCF-10A cells. At 3 μM plumbagin concentration, the inhibition of cell viability in MDA-MB-231 and MCF-7 cells was 79% and 89%, respectively, whereas in MCF-10A cells it was only about 20%. These results, combined with the MTT data as presented above (Fig. 1A), clearly suggest that plumbagin treatment results in a significant inhibition of cell growth and viability of human breast cancer cells without any substantial effects on normal breast epithelial cells. Thus the effect of plumbagin appears to be cancer cell specific, which is very important for further development of plumbagin for breast cancer prevention and/or therapy.

PLUMBAGIN INDUCES APOPTOSIS IN ER-POSITIVE AS WELL AS ER-NEGATIVE CELLS

The inhibition of overall cell growth of cancer cells by anti-cancer agents is known to be accompanied by induction of apoptosis. Moreover, plumbagin-induced apoptosis has been reported in various cancer types but never characterized in the breast cancer cells. Therefore, we assessed the ability of plumbagin for the induction of apoptosis in MDA-MB-231 and MCF-7 breast cancer cells by two different approaches, Histone/DNA ELISA method and the Annexin V/PI staining. As seen in Figure 2A, Histone/DNA ELISA method revealed a dose dependent increase in apoptotic cell death induced by plumbagin treatment in both the cell lines tested. Figure 2B shows the quantitation of apoptotic cells by plumbagin treatment as detected by Annexin V staining. The results clearly show that plumbagin caused a dose-dependent increase in the number of apoptotic cells in both the cell lines. In the DMSO-treated control cells, the number of apoptotic cells was negligible but at 3 μM plumbagin treatment, about 50% of MDA-MB-231 cells and 40% of MCF-7 cells were undergoing active apoptosis. At higher doses such as 5 μM, plumbagin resulted in a 100% cell death after 72 h treatment (data not shown). These results clearly show that plumbagin is an effective agent in the induction of apoptotic cell death of breast cancer cells, irrespective of the ER status. It is however interesting to note that the highly aggressive, triple negative breast cancer cells (MDA-MB-231 cells) are more susceptible to plumbagin-induced apoptosis compared to the ER-positive MCF-7 cells, suggesting that plumbagin could be an effective agent in the killing of triple negative breast cancer cells and, as such, could be useful for the treatment of breast cancer.

INDUCTION OF AUTOPHAGY- AND APOPTOSIS-MARKERS BY PLUMBAGIN IN TRIPLE NEGATIVE BREAST CANCER CELLS

Since plumbagin was more effective in the killing of triple negative MDA-MB-231 breast cancer cells, we further studied the mechanism of apoptosis-induction in MDA-MB-231 breast cancer cells using

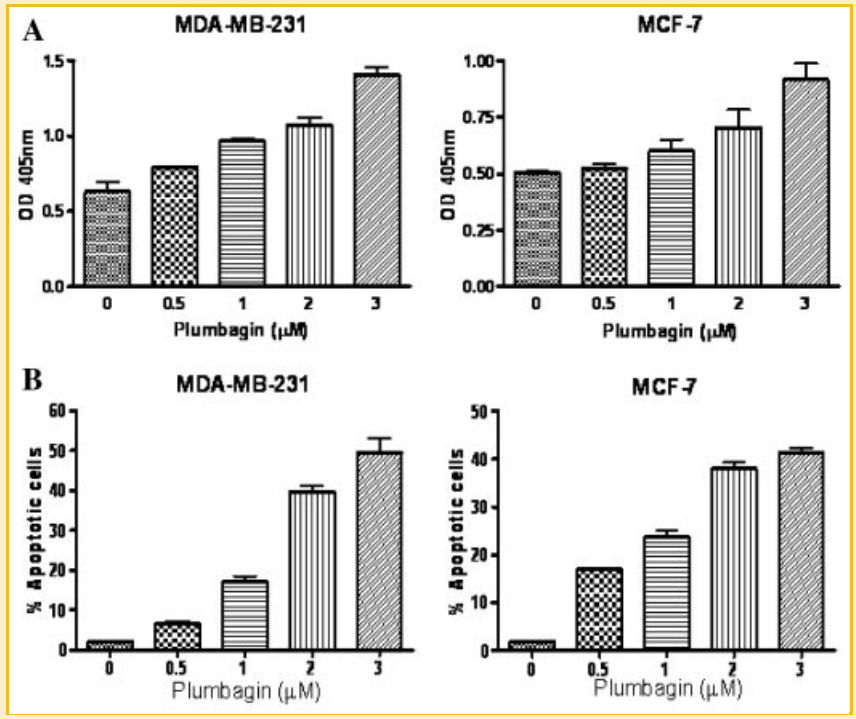

Fig. 2. Induction of apoptosis in MDA-MB-231 and MCF-7 cells by plumbagin treatment. MDA-MB-231 and MCF-7 cells were either vehicle-treated (DMSO-control) or treated with increasing concentrations of plumbagin (0.5, 1, 2, and 3 μM) for 72 h and assayed for apoptosis by (A) Histone/DNA ELISA method and (B) Annexin V-Propidium Iodide staining method as described under Materials and Methods Section. The amount of DMSO added never exceeded 0.1% and the values represent a mean ± SE of three samples from, at least, two independent experiments.

well-known markers of apoptosis. As seen in Figure 3A, MDA-MB-231 cells treated with increasing concentrations of plumbagin for 24 and 72 h showed down-regulation of anti-apoptotic protein Bcl-2 while the levels of another anti-apoptotic protein, survivin, were found to be relatively unaffected, suggesting that down-regulation of Bcl-2 may play an important role in plumbagin-induced apoptosis in human breast cancer cells. Down-regulation of Bcl-2 was evident as early as 24 h using 1 μM of plumbagin. At 72 h time-point, even 0.5 μM dose resulted in a significant decrease in the Bcl-2 protein and this was consistent with the appearance of cleaved bands for caspase-3 as well as caspase-9 (Fig. 3A) after plumbagin treatment, which further confirmed that plumbagin is an effective agent in inducing apoptotic cell death in part due to inactivation of Bcl-2 protein.

An earlier work has reported predominant induction of autophagy by plumbagin in breast cancer cells [Kuo et al., 2006]. To confirm this result, we assessed the levels of LC3 (microtubule-associated protein 1 light chain 3), a marker for autophagy [Mizushima and Yoshimori, 2007; Klionsky et al., 2008], in plumbagin-treated MDA-MB-231 cells. As seen in Figure 3B, we found that plumbagin induced LC3 at a lower dose but with increasing dose, the level of LC3 was decreased, which could be associated with massive induction of apoptotic cell death. The conversion of LC3-I to LC3-II is considered a reliable indicator of autophagic activity, and we were able to visualize both the isoforms of LC3 in our assay system, consistent with the earlier report [Kuo et al., 2006]. It appears that the induction of autophagy in breast cancer cells at lower concentration of plumbagin may be an early event followed by the induction of apoptotic cell death. Interestingly, we were unable to observe induction of LC3 in the MCF-7 cells (results not shown) whereas induction of cell death was observed in MCF-7 cells (Fig. 2A,B), suggesting that autophagy may not be the predominant process by which plumbagin induces cell death in breast cancer cells.

PLUMBAGIN INHIBITS NF-κB ACTIVITY

Next, we studied the effect of plumbagin treatment on the DNA binding activity of NF-κB as determined by EMSA because plumbagin has been shown to suppress NF-κB activation in various cancer cell lines, leading to the potentiation of apoptosis induction by cytokines and chemotherapeutic agents [Sandur et al., 2006]. We hypothesized that a similar mechanism may exist in breast cancer cell lines during anti-proliferative and pro-apoptotic effects of plumbagin mediated by the inactivation of NF-κB. This experiment was of particular significance in light of the recent evidence connecting NF-κB to the aggressive breast cancer phenotype [Wang et al., 2007a]. As seen in Figure 4, the treatment of breast cancer cells with increasing concentrations of plumbagin resulted in a dose-dependent inactivation of endogenous NF-κB. This effect was equally observed in ER-negative MDA-MB-231 cells (Fig. 4A) as well as in the ER-positive MCF-7 cells (Fig. 4B), and these results provided further proof in support of the killing of both receptor-

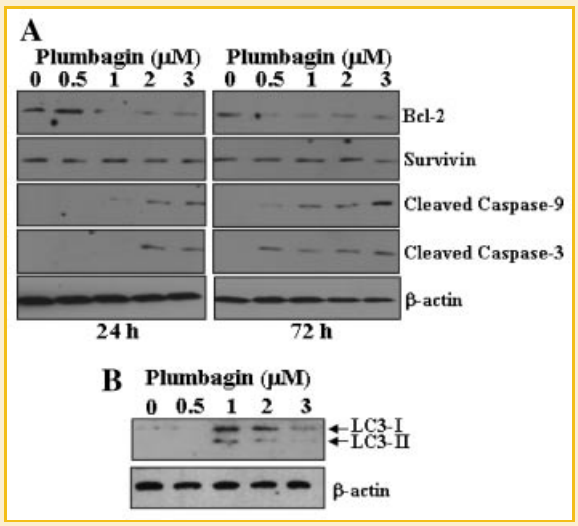

Fig. 3. Representative Western blot for analysis of protein biomarkers of apoptosis and autophagy in plumbagin-treated ER-negative MDA-MB-231 cells. A: Cells were treated with increasing amounts of plumbagin (0.5, 1, 2, and 3 μM) or DMSO control, for 24 h as well as 72 h and the expression of anti-apoptotic proteins, Bcl-2 and survivin, as well as cleaved caspase 3 and cleaved caspase 9 was studied by Western blotting, as described under Materials and Methods Section. B: To detect autophagy, MDA-MB-231 cells were treated with increasing concentrations of plumbagin (0.5, 1, 2, and 3 μM) for 72 h and the expression of LC3, an autophagy marker, was assessed by Western blotting. Cell extracts were prepared using RIPA buffer, as described under Materials and Methods Section. The β-actin protein was used as protein loading control as shown for each blot.

positive as well as receptor–negative cells. For further assessment of the equal loading of the nuclear proteins, we have done Western blot analysis for assessing the level of Rb protein expression, and we found that there was no change in the expression of Rb. Thus we believe that our result on NF-κB DNA binding activity is highly reliable showing that plumbagin is a potent agent in the inactivation of NF-κB activity. It is interesting to note that the constitutive level of NF-κB in ER-negative MDA-MB-231 cells (Fig. 4A, first lane) is higher than the corresponding level in the ER-positive MCF-7 cells (Fig. 4B, first lane) clearly suggesting that a negative correlation exists between the activity of NF-κB and the presence of ER, which is consistent with previously published report [Wang et al., 2007a].

Bcl-2 OVER-EXPRESSION SIGNIFICANTLY ABROGATES THE PLUMBAGIN-INDUCED APOPTOSIS AND NF-κB INACTIVATION

Thus far our results suggested that plumbagin treatment results in the induction of apoptotic cell death in both ER-negative as well as ER-positive cells. Experiments with the ER-negative MDA-MB-231 cells showed that such an induction in apoptosis was associated with the down-regulation of Bcl-2 expression (Fig. 3A) and inactivation of the DNA binding activity of NF-κB. We therefore believed that the induction of apoptosis in breast cancer cells by plumbagin could be mechanistically linked with the inactivation of NF-κB/Bcl-2 pathway. Such a possibility is further supported by the evidence in the literature showing that the activation of NF-κB and expression of Bcl-2 serves as a mechanism for resistance of breast cancer patients to chemotherapy [Buchholz et al., 2005]. The ER-positive, MCF-7 cells used in the current study exhibited very low endogenous levels of expression of Bcl-2 (Figs. 5 and 6A) [Wang et al., 2007a] and served as an excellent model for forced over-expression of Bcl-2 to test our hypothesis whether Bcl-2 over-expression could attenuate the plumbagin-induced killing of MCF-7 cells.

We initially tested our hypothesis by transient transfection for the over-expression of Bcl-2 in MCF-7 cells. The results shown in Figure 5 clearly document that transient transfection of MCF-7 cells

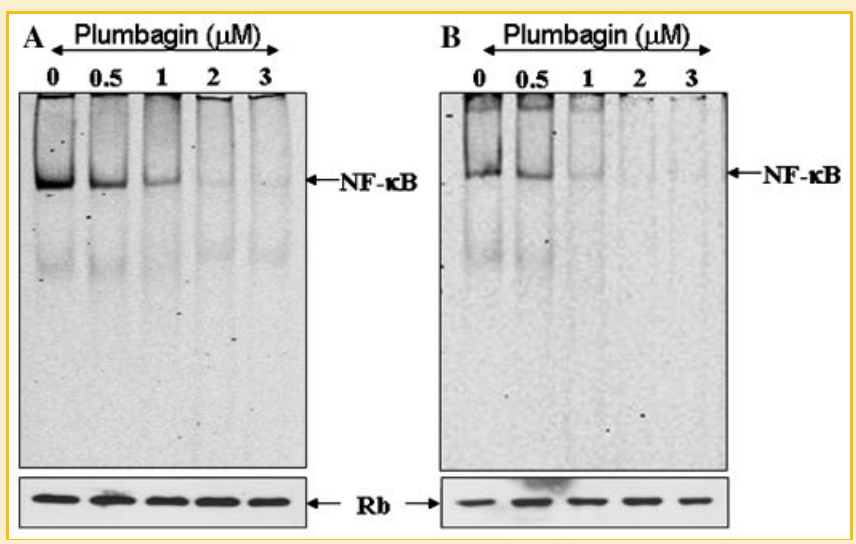

Fig. 4. Dose-dependent down-regulation of NF-κB by plumbagin in (A) MDA-MB-231 and (B) MCF-7 cells. Cells were incubated with increasing concentrations of plumbagin (0.5, 1, 2, and 3 μM) or DMSO-control for 72 h, and nuclear proteins were subjected to Gel Shift Assay for the evaluation of NF-κB DNA binding activity. Nuclear protein extracts were prepared and EMSA was performed according to the protocol described under Materials and Methods Section. Untreated (DMSO-treated) cells express basal level of NF-κB but upon plumbagin-treatment, NF-κB was down-regulated. The expression of Rb protein was used as nuclear protein loading control as shown for each EMSA.

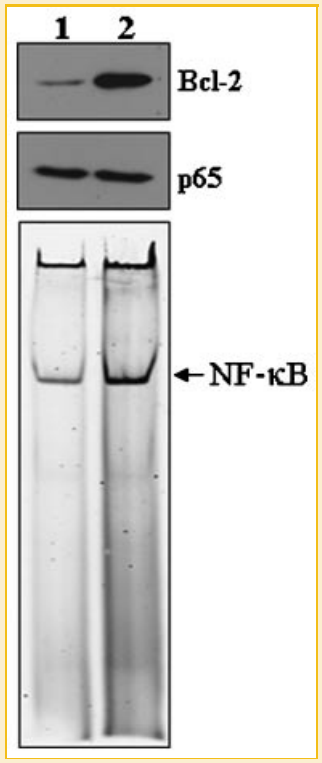

Fig. 5. Transient transfection of Bcl-2 in MCF-7 cells. MCF-7 cells were transfected with (1) pCDNA3 vector or (2) vector with Bcl-2 construct. Total cell lysates were subjected to Western blot analysis for Bcl-2 and p65 proteins, and nuclear protein extracts were subjected to EMSA, as described under Materials and Methods Section.

with Bcl-2 resulted in a marked increase in the expression of Bcl-2 protein compared to cells transfected with vector control. Since expression of NF-κB and Bcl-2 is correlated with poor prognosis [Buchholz et al., 2005], we were interested in determining the effect of Bcl-2 on NF-κB. Western blot analysis of p65 protein showed no significant effect in Bcl-2-transfected cells relative to vector-transfected cells at the protein level. However, we observed a significant increase in the NF-κB-DNA-binding activity, as revealed by EMSA (Fig. 5). These results show a positive correlation between Bcl-2 expression and NF-κB activation.

To further assess the biological effects of Bcl-2 over-expression, we established stably transfected MCF-7 cells with either the empty vector (pCDNA3) or the Bcl-2 constructs. First, we tested the expression of Bcl-2 in vector- and Bcl-2-transfected MCF-7 cells (Fig. 6A). Next, we assessed the induction of apoptosis in these cells induced by plumbagin. We employed the Histone/DNA ELISA method as well as the Annexin V/PI staining method as described above for assessing the degree of apoptosis induced by plumbagin. Figure 6B illustrates the apoptosis induction in Bcl-2 transfected MCF-7 cells compared to the vector transfected MCF-7 cells, as measured by Histone/DNA ELISA method.

Our data clearly showed that Bcl-2 transfection resulted in a remarkable inhibition of apoptosis, almost similar to that observed in the untreated control (DMSO-treated) cells. It is important to note that the level of apoptosis observed in the vector-transfected cells (Fig. 6B) is comparable to the parental MCF-7 cells (Fig. 2A). To further confirm our results, we performed Annexin V/PI staining for the quantitation of apoptotic cells. As seen in Figure 6C, Bcl-2 transfection resulted in a significant inhibition in the number of apoptotic cells by plumbagin treatment compared to the cells transfected with empty vector. Although the attenuation in the induction of apoptosis was not 100%, the degree of inhibition was highly significant compared with the empty vector-transfected control cells ($P < 0.01$) at all the concentrations of plumbagin used in this experiment. The number of apoptotic cells, generated by plumbagin treatment of vector-transfected MCF-7 cells (Fig. 6C) was found to be similar to that observed in parental MCF-7 cells as described earlier (Fig. 2B).

We next asked the question whether Bcl-2 over-expression in MCF-7 cells could also lead to a reversal of the inhibition of NF-κB DNA binding activity. As shown in Figure 6D, we found that the inactivation of NF-κB induced by plumbagin was attenuated in Bcl-2 over-expressing MCF-7 cells. Increasing concentrations of plumbagin did not result in any significant inhibition of NF-κB DNA binding activity in Bcl-2-transfected MCF-7 cells. In conclusion, Bcl-2 over-expression alone could abrogate the inhibitory effect of plumbagin on NF-κB activation.

DISCUSSION

The use of naturally occurring compounds for the treatment and/or prevention of cancer has long been advocated. A major prerequisite in the identification of such agents is that they should be physiologically non-toxic, and inert towards the normal cells. In the modern day research, studies on the components of traditional medicines have helped in the identification of some lead candidates that are being pursued for possible therapeutic purposes against human cancers. Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone), a quinonoid constituent isolated from the roots of *P. zeylanica* L. is one such compound. It is known to be part of traditional Asian medicine and has been in use for more than 2,000 years in Indian Medicine (Ayurveda). Several species of walnuts are also known to contain plumbagin and other structurally related naphthoquinones. In addition to its anti-cancer properties, plumbagin has been reported to exhibit several other biologically relevant activities such as, anti-microbial [Mossa et al., 2004], anti-bacterial [Wang and Huang, 2005], anti-atherosclerotic [Ding et al., 2005], radio-sensitizing [Ganasoundari et al., 1997; Nair et al., 2008] as well as inhibition of topoisomerase II [Kawiak et al., 2007]. Emerging evidence suggests that the anti-cancer properties of plumbagin are mediated by the inactivation of Akt/NF-κB signaling pathway as well as inactivation of MMP-9 and VEGF that are considered important for the processes of invasion, angiogenesis and metastases [Kuo et al., 2006; Sandur et al., 2006]. Therefore, we hypothesized that plumbagin could be a novel and active agent against breast cancer, and thus tested this concept in the present study.

The results described here, clearly suggest that plumbagin inhibits cell growth and viability leading to the induction of apoptotic cell death in breast cancer cells. Although the induction of apoptosis by

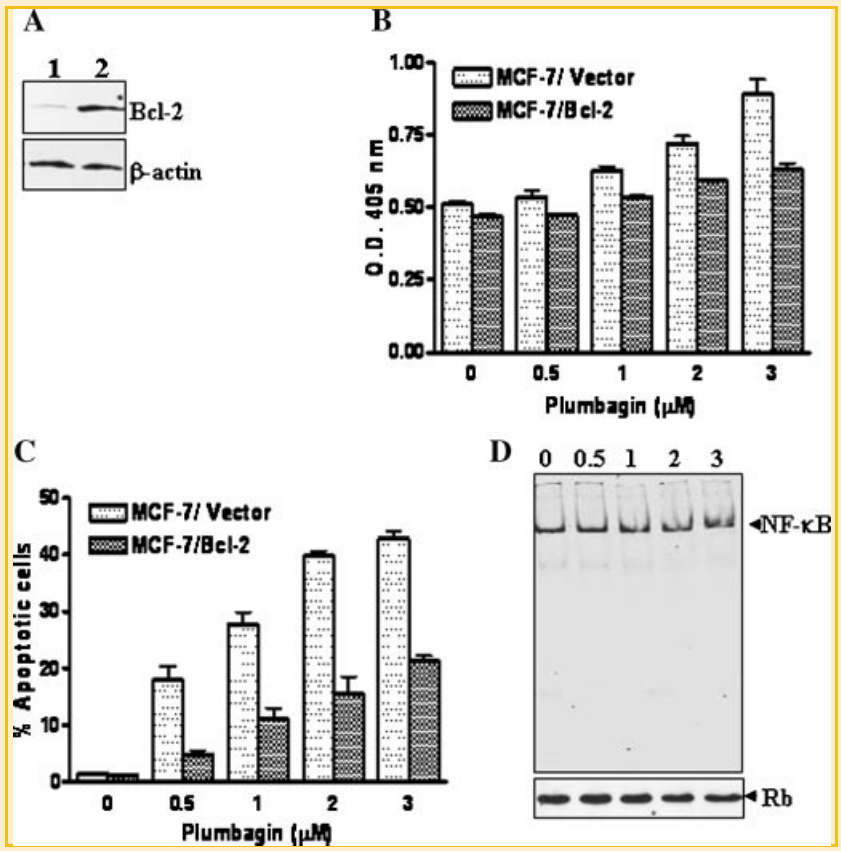

Fig. 6. Abrogation of plumbagin-induced cytotoxic effects by over-expression of Bcl-2 in MCF-7 cells. MCF-7 cells were stably transfected with either empty vector, pCDNA3, or the Bcl-2-construct. A: Western blot analysis confirmed the higher expression of Bcl-2 in Bcl-2-transfected MCF-7 cells (lane 2) compared to the expression of Bcl-2 in vector-transfected cells (lane 1). The β-Actin protein was used as protein loading control. Apoptosis-induction in empty vector- and Bcl-2-transfected MCF-7 cells by either vehicle-treatment (DMSO-control) or treatment with increasing concentrations of plumbagin (0.5, 1, 2, and 3 μM) for 72 h was assayed by (B) Histone/DNA ELISA method and (C) Annexin V-Propidium Iodide staining as described under Materials and Methods Section. D: The effect of Bcl-2 over-expression on the down-regulation of NF-κB by plumbagin (0.5, 1, 2, and 3 μM doses for 72 h) was also studied. Vector/Bcl-2-transfected MCF-7 cells were treated with DMSO (control) or increasing concentrations of plumbagin as indicated, and the nuclear proteins were subjected to Gel Shift Assay for the evaluation of NF-κB DNA binding activity, as described under Materials and Methods Section. The expression of Rb protein by Western blot analysis was used as nuclear protein loading control.

plumbagin in several cancer cells has been reported [Jaiswal et al., 2002; Srinivas et al., 2004b; Hsu et al., 2006; Sandur et al., 2006; Kawiak et al., 2007; Nair et al., 2008; Powolny and Singh, 2008; Wang et al., 2008], our results are the first to report this phenomenon in breast cancer cells. We observed apoptosis-induction in both ER-positive as well as ER-negative breast cancer cells, suggesting that plumbagin could be an effective agent for the killing of all breast cancer cell types especially the triple negative breast cancer cells for which there is no effective targeted therapy. Interestingly, a recent report has demonstrated apoptosis-induction in prostate cancer cells irrespective of their hormone-responsiveness [Powolny and Singh, 2008], which provides further support that plumbagin could be an effective agent for the killing of cancer cells irrespective of their difference in the expression status of important target genes. Here we also show that apoptosis-induction by plumbagin is accompanied by down-regulation of anti-apoptotic Bcl-2 and inactivation of NF-κB. Bcl-2 over-expression studies in MCF-7 cells further confirmed our findings; Bcl-2-transfected MCF-7 cells were resistant to plumbagin-induced killing, which was also associated with attenuation in the plumbagin-induced inactivation of the DNA binding activity of NF-κB.

There is evidence suggesting a correlation between the degree of induced-apoptosis and the response of human breast cancer to chemotherapy [Buchholz et al., 2003]. We speculated that plumbagin could be associated with better prognosis and survival rates of breast cancer patients based on its ability to induce apoptosis selectively in cancer cells. Based on our pilot studies as reported here showing that plumbagin could down-regulate Bcl-2 expression (Fig. 3) as well as inactivate the DNA binding activity of NF-κB (Fig. 4), it appears that the biological activity of plumbagin against breast cancer cell lines is in part due to its negative-modulation of NF-κB/Bcl-2 pathway which leads to apoptosis-induction in cancer cells. As mentioned above, nuclear localization of NF-κB and expression of Bcl-2 has been shown to serve as a mechanism for resistance of breast cancer patients to anthracyline-based chemotherapy [Buchholz et al., 2005], suggesting the importance of NF-κB in relation to response of breast cancer to chemotherapy. Similar positive correlation between Bcl-2

expression and poor prognosis has also been suggested in breast cancer patients [Ogston et al., 2004]. However, there are only a handful of reports on the subject, including some conflicting ones [Bonetti et al., 1998; Bottini et al., 2000], and, therefore, more clinical studies must be done to further our understanding of the subject. Nevertheless, the available data points to the involvement of NF-κB and Bcl-2 in the progression of breast cancer in clinical settings and our in vitro results describing the suppression of NF-κB and Bcl-2 expression by plumbagin treatment might be relevant to human breast cancer patients. In search for the proof of a direct relationship between NF-κB and Bcl-2, it has been shown that NF-κB can transactivate Bcl-2 promoter and that p100 over-expression in MCF-7 cells enhances endogenous Bcl-2 expression [Viatour et al., 2003]. This study also reported an association between NF-κB expression and enhanced Bcl-2 expression. Moreover, Bcl-2 has also been reported to activate NF-κB through an indirect mechanism involving the degradation of IκB, the cytoplasmic inhibitor of NF-κB [de et al., 1998]. Our results (Fig. 5) also provide support for a direct relationship between Bcl-2 expression and NF-κB activity. Similarly, a direct regulation of Bcl-2 by NF-κB is known to operate in prostate cancer [Catz and Johnson, 2001], and a correlation between enhanced Bcl-2 levels and progression to metastatic phenotype has been suggested [Mehlen and Puisieux, 2006]. Therefore, down-regulation of Bcl-2 by plumbagin in highly aggressive ER-negative cells, as reported here, may be a highly desirable activity of any such agents for breast cancer prevention and/or therapy.

Only one report exists in the literature showing the effect of plumbagin in breast cancer [Kuo et al., 2006], which was shown to be mediated by the induction of autophagy as the predominant pathway. This study also utilized MDA-MB-231 and MCF-7 cells similar to our study and reported only a small amount of apoptotic cell death after 48 h of plumbagin treatment as demonstrated by TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay. However, we observed a significant induction of apoptosis by plumbagin in both the cell lines after 72 h of treatment, as measured by two independent methods, Histone/DNA ELISA method [Kerr et al., 1994] and the Annexin V-Propidium Iodide staining method [Martin et al., 1995; Vermes et al., 1995]. Although we found induction of LC3, a marker for autophagy, in MDA-MB-231 cells, after treatment with low doses of plumbagin, which is consistent with previous findings [Kuo et al., 2006]; however the higher doses of plumbagin showed decrease in LC3 expression (Fig. 3B) which, we believe, could be due to induction of apoptotic cell death of triple negative breast cancer cells (MDA-MB-231 cells). In support of the induction of apoptosis induced by plumbagin, we found the appearance of cleaved forms of caspases 3 and 9 in MDA-MB-231 cells. Thus we believe that initially plumbagin could induce autophagy followed by the induction of apoptotic cell death of breast cancer cells, and this notion is supported by recent literature showing that a coordination exists between apoptosis (type I programmed cell death) and autophagy (type II programmed cell death) [Yu et al., 2004; Boya et al., 2005; Klionsky, 2007].

In summary, the data reported here along with the data reported by other investigators clearly show that plumbagin could be an effective agent in the killing of cancer cells. Our present pre-clinical study is highly significant in light of existing reports; and to our knowledge, this is the first report, describing the mechanism of apoptosis-induction by plumbagin in breast cancer cells showing that the effects of plumbagin are in part due to inactivation of NF-κB/Bcl-2 pathway. However, further in-depth pre-clinical animal studies are needed for advancing this field in realizing the potential role of plumbagin for the prevention and/or treatment of breast cancer.

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