Apoptosis-Inducing Effect of Garcinol Is Mediated by NF-κB Signaling in Breast Cancer Cells

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ABSTRACT

Garcinol, obtained from *Garcinia indica* in tropical regions, is used for its numerous biological effects. Its anti-cancer activity has been suggested but the mechanism of action has not been studied in-detail, especially there is no report on its action against breast cancer cells. Here we tested our hypothesis that garcinol may act as an anti-proliferative and apoptosis-inducing agent against breast cancer cell lines. Using multiple techniques such as MTT, Histone-DNA ELISA, Annexin V-PI staining, Western blot for activated caspases and cleaved PARP, homogenous caspase-3/7 fluorometric assay and EMSA, we investigated the mechanism of apoptosis-inducing effect of garcinol in ER-positive MCF-7 and ER-negative MDA-MB-231 cells. We found that garcinol exhibits dose-dependent cancer cell-specific growth inhibition in both the cell lines with a concomitant induction of apoptosis, and has no effect on non-tumorigenic MCF-10A cells. Our results suggested induction of caspase-mediated apoptosis in highly metastatic MDA-MB-231 cells by garcinol. Down-regulation of NF-κB signaling pathway was observed to be the mechanism of apoptosis-induction. Garcinol inhibited constitutive NF-κB activity, which was consistent with down-regulation of NF-κB-regulated genes. This is the first report on anti-proliferative and apoptosis-inducing action of garcinol against human breast cancer cells and the results suggest that this natural compound merits investigation as a potential chemo-preventive/-therapeutic agent, especially against breast cancer. J. Cell. Biochem. 109: 1134–1141, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: GARCINOL; BREAST CANCER; NF-κB; APOPTOSIS; MDA-MB-231; MCF-7

Garcinol is a polyisoprenylated benzophenone derivative extracted from *Garcinia indica* extracts, particularly from its rind [Padhye et al., 2009]. Genus Garcinia includes about 200 species found in the tropics and the antioxidant activity of aqueous extract of the plant has been reported, which is higher than other reported spices and fruits; thus promoting its use in cooking, home remedies and as a soft drink [Mishra et al., 2006]. The rind also contains isogarcinol, hydroxycitric acid, hydroxycitric acid lactone, citric acid, and oxalic acid while the fruit contains other compounds such as malic acid, several polyphenols, carbohydrates, anthocyanin, pigments, and ascorbic acid [Padhye et al., 2009]. Garcinol is the active principal compound of *G. indica*, which crystals out as yellow needles from the hexane extract of the fruit rind. Its strong antioxidant activity has been attributed to the presence of phenolic hydroxyl groups as well as a β-diketone moiety [Pan et al., 2001; Padhye et al., 2009].

Garcinol has been shown to possess antioxidant activity [Yamaguchi et al., 2000a,b; Sang et al., 2001, 2002; Liao et al., 2004; Hong et al., 2006] and, additionally, it also inhibits histone acetyltransferases, dysfunction of which is known to lead to cancer [Balasubramanyam et al., 2004; Arif et al., 2009]. These properties indicate a possible use of garcinol in anti-cancer research which has been evaluated in a few published reports [Tanaka et al., 2000; Pan et al., 2001; Matsumoto et al., 2003; Liao et al., 2005; Yoshida et al., 2005; Hong et al., 2006, 2007; Koeberle et al., 2009]. Despite these preliminary reports, the anti-cancer action of garcinol and its mechanism, particularly against breast cancer cells, remain poorly understood. To fill this void in our understanding, we investigated the biological effect of this compound against breast cancer cell lines such as MCF-7 and MDA-MB-231 and our results showed that garcinol can effectively inhibit the proliferation and viability of these two cell lines. As a control, we studied the non-tumorogenic cell line MCF-10A whose proliferation and viability was not inhibited, suggesting cancer cells-specific action of garcinol. To fully understand the mechanism of inhibition of growth and proliferation by garcinol, we performed detailed mechanistic studies

using multiple assays and observed that garcinol was able to induce apoptosis in both the cancer cell lines. Using the aggressive MDA-MB-231 cells as a model, we found that apoptosis-inducing effect of garcinol was caspase-dependent, and, since NF-κB activity has direct implications on progression of cancers [Karin, 2006], we also investigated the effect of garcinol on NF-κB-DNA binding activity in both the cell lines and observed a dose-dependent inhibition of NF-κB activity. A number of NF-κB-regulated genes were also found to be down-regulated by garcinol in MDA-MB-231 cells. These results suggest that garcinol could be a useful agent for the prevention and/or treatment of breast cancers especially due to its inhibitory effects on the DNA binding activity of NF-κB, resulting in the induction of apoptosis.

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 100 μg/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, Carlsbad, CA) supplemented with 5% horse serum, 20ng/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. Garcinol was purchased from Biomol/Enzo Life Sciences International, Inc. (Plymouth Meeting, PA) and was dissolved in DMSO to make 25 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); antibodies against Bcl-2 and Bcl-xL were from Santa Cruz Biotechnology (Santacruz, CA); anti-PARP antibody was from Biomol/Enzo Life Sciences International, Inc. (Plymouth Meeting, PA) while monoclonal antibody to β-actin was purchased from Sigma–Aldrich (St. Louis, MO).

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

MDA-MB-231 cells were seeded at a density of 2×10^3 cells per well while MCF-7 cells were seeded at a density of 5×10^3 cells per well in 96-well culture plates. After overnight incubation, liquid medium was removed and replaced with a fresh medium containing DMSO (vehicle control) or different concentrations of garcinol diluted from a 25 mM original stock solution. After 72 h of incubation, 25 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in phosphate-buffered saline, PBS) was 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) by mixing for 30 min on a gyratory shaker. 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%. Moreover, each experiment was repeated at least three times.

CELL VIABILITY STUDIES BY TRYPAN BLUE ASSAY

MDA-MB-231 and MCF-7 cells were seeded in six-well culture plates and treated with garcinol. 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 garcinol as described previously [Ahmad et al., 2008]. Briefly, cells were treated with garcinol 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

MDA-MB-231 and MCF-7 cells were incubated in the presence or absence of varying concentrations of garcinol 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 PI (Propidium Iodide) 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.

SOFT AGAR COLONIZATION ASSAYS

MDA-MB-231 (3×10^4) and MCF-7 (5×10^4) cells were treated with either DMSO or increasing concentrations of garcinol, allowed to grow for 72 h and then collected by trypsinization. 3×10^4 cells were then plated in 0.5 ml of culture medium containing 0.3% (w/v) top agar layered over a basal layer of 0.7% (w/v) agar (with culture medium and the supplements) in 24-well plates. At the time of seeding, the culture was supplemented with different concentrations of garcinol or the DMSO. After appropriate culture time (18 days for MDA-MB-231 and 30 days for MCF-7 cells), colonies (>50 cells) were counted. Experiments were carried out in quadruplicate, and results presented are representative of three independent observations.

CLONOGENIC ASSAY

To test the survival of breast cancer cells, clonogenic assay was performed as described previously [Ali et al., 2008; Ahmad et al., 2009b]. Briefly, cells were plated in a six-well plate, treated with garcinol for 72 h, trypsinized, and the viable cells counted (trypan blue exclusion) and plated in 100 mm petri dishes (250 MDA-MB-231 cells; 1,000 MCF-7 cells). The cells were then incubated for 20 days at 37°C in a 5% CO_2/5% O_2/90% N_2 incubator and colonies were stained with 2% crystal violet and counted.

WESTERN BLOT ASSAY

Cell lysates were obtained from cells using cold RIPA buffer as described previously [Ahmad et al., 2008]. Total proteins were fractionated using SDS–PAGE and transferred onto nitrocellulose membrane for Western blotting as described earlier [Ahmad et al., 2008, 2009a].

HOMOGENEOUS CASPASE–3/7 ASSAY FOR APOPTOSIS

Caspase-3/7 homogeneous assay was performed as described previously [Ahmad et al., 2009a] using a kit from Promega (Madison, WI). Cells were treated with garcinol or DMSO control for 72 h. After treatment, 100 μl Apo-ONE® caspase-3/7 reagent was added and plates were shaken for 2 min, followed by incubation at room temperature for 3 h. The fluorescence was then evaluated using ULTRA Multifunctional Microplate Reader (TECAN) at excitation/emission wavelengths of 485/530 nm.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

EMSA was performed by incubating 5 μg of nuclear protein extract with IRDye-700-labeled nuclear factor-κB (NF-κB) oligonucleotides (LI-COR, Lincoln, NE) [Ahmad et al., 2008]. The incubation mixture included 2 μg of poly dI–dC (poly deoxyinosinic–deoxycytidylic acid) 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).

DATA ANALYSIS

The experimental results presented in the figures are representative of three or more independent observations. The data are presented as the mean values ± SE. Statistical comparisons between groups were done using one-way ANOVA. Values of $P < 0.05$ were considered to be statistically significant and individual P-values are reported in the figures, as appropriate.

RESULTS

GARCINOL INHIBITS PROLIFERATION AND VIABILITY OF BREAST CANCER CELLS

We first investigated the anti-proliferative properties of garcinol by exposing breast cancer cell lines, MDA-MB-231, and MCF-7 to increasing doses of garcinol for 72 h followed by MTT assay to assess the effect of treatment on cell proliferation. Our results (Fig. 1A) show that garcinol had a dose-dependent inhibitory effect on the
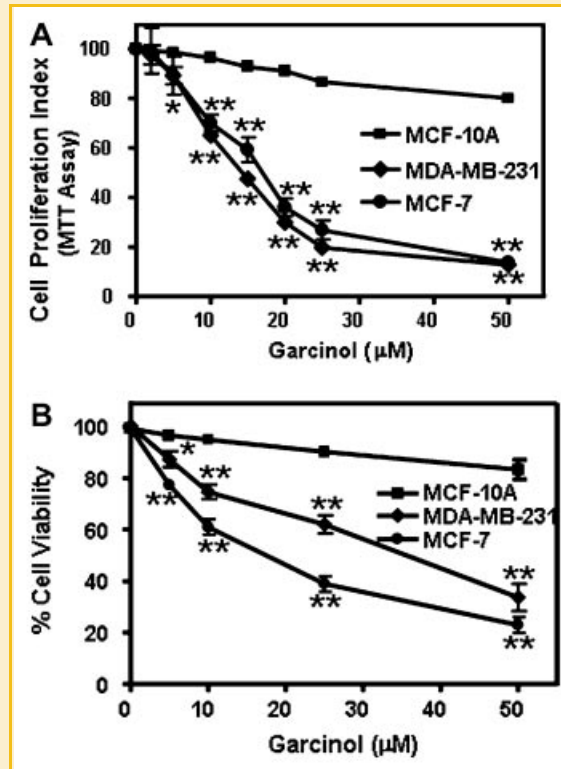

Fig. 1. Evaluation of cell proliferation (A) and cell viability (B) in garcinol-treated breast cancer cells by MTT and trypan blue staining, respectively. Cells were either vehicle-treated (DMSO-control) or treated with increasing concentrations of garcinol for 72 h and then analyzed. The amount of DMSO never exceeded 0.1% during the treatment. The number of cells counted or O.D. value in DMSO (control)-treatment was considered 100% and the number of cells in garcinol-treated cells was calculated relative to this control. Each data point represents mean ± SE of eight replicates from, at least, three independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ versus respective DMSO-treated controls (0 μM garcinol).

proliferation of both the cell lines. As a control, we included MCF-10A cells in our experiments. MCF-10A is a non-tumorogenic breast epithelial cell line, which is considered "normal breast epithelial cell line." Garcinol had a minimal inhibitory effect on MCF-10A cells; a 25 μM dose could inhibit the proliferation of these cells by only about 10% while the same dose was found to inhibit the proliferation of cancer cells (MDA-MB-231 and MCF-7) by ~80%. We also conducted cell viability assays, using trypan blue, and observed similar effects of garcinol (Fig. 1B). Again, garcinol had no/minimal effect on the viability of MCF-10A cells but it resulted in a dose-dependent killing/loss of viability of cancer cells. These results suggest that garcinol has anti-proliferative and growth inhibitory effects against breast cancer cells but, at the same time, it has no detrimental effect against the normal breast epithelial cells.

INDUCTION OF APOPTOSIS BY GARCINOL

The inhibition of overall cell growth of cancer cells by anti-cancer agents is known to be accompanied by induction of apoptosis. Therefore, we assessed the ability of garcinol to induce apoptosis in MDA-MB-231 and MCF-7 breast cancer cells using two different
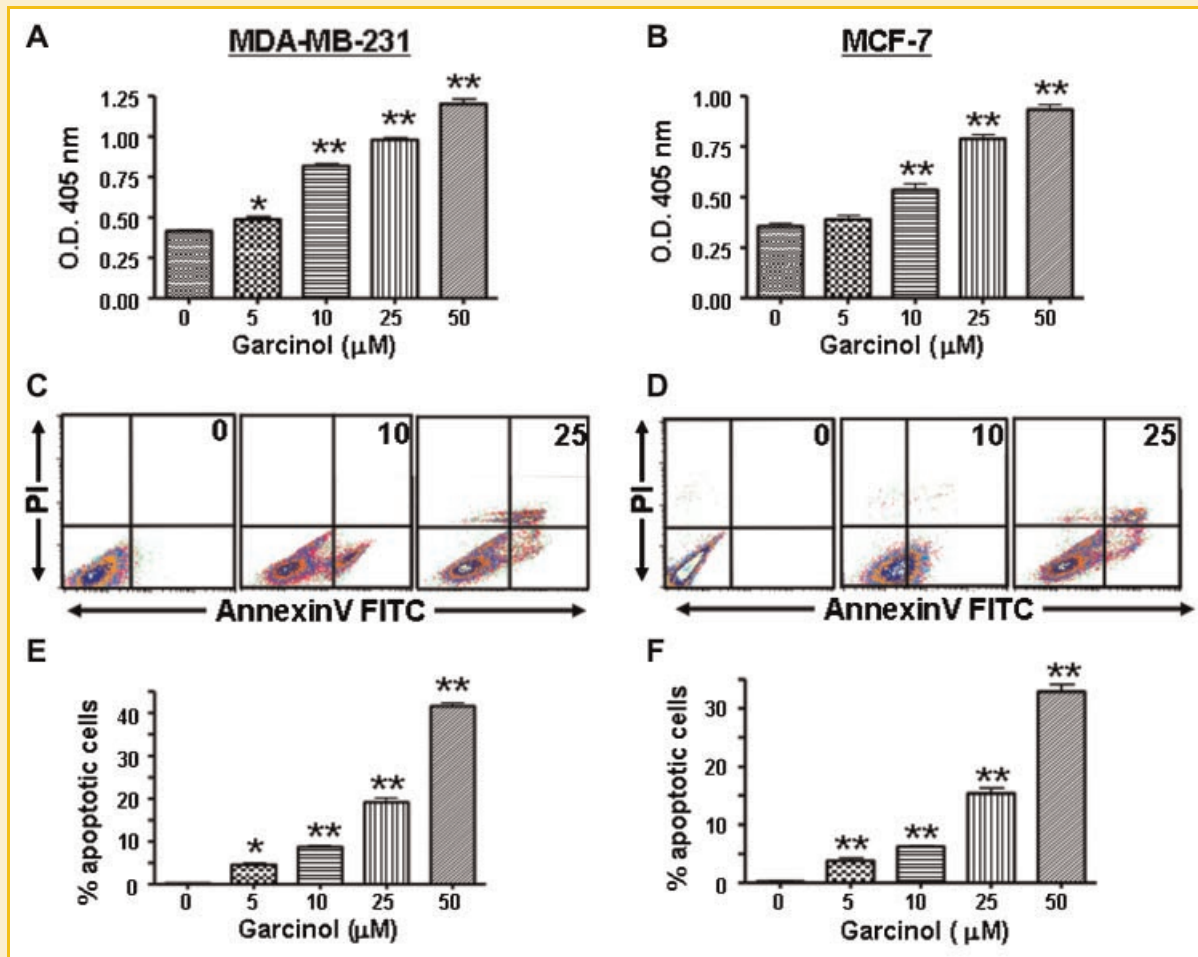
Fig. 2. Induction of apoptosis in MDA-MB-231 and MCF-7 cells by garcinol treatment. MDA-MB-231 and MCF-7 cells were either vehicle-treated (DMSO-control) or treated with increasing concentrations of garcinol for 72 h and assayed for apoptosis by Histone/DNA ELISA method (A,B) and Annexin V-PI staining method (C,D) as described under Materials and Methods Section. C,D: Representative pictures from the flow cytometry data for MDA-MB-231 and MCF-7 cells, respectively. E,F: Analysis of the Annexin V-PI flow cytometry data, respectively, showing exact % of cells undergoing apoptosis. The values represent mean $\pm$ SE of three samples from, at least, three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus DMSO-treated controls (0 μM garcinol). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

approaches, Histone/DNA ELISA method and the Annexin V/PI staining. As documented in Figure 2A,B, Histone/DNA ELISA method revealed a dose dependent increase in apoptotic cell death induced by garcinol treatment in both the cell lines, MDA-MB-231 (Fig. 2A), and MCF-7 (Fig. 2B). Annexin V/PI staining also revealed a dose-dependent apoptosis-inducing effect of garcinol in both the cell lines tested (Fig. 2C,D). Figure 2E,F represents quantitation of apoptotic cells, as detected by Annexin V staining after garcinol treatment. The results clearly show that garcinol treatment resulted in a dose-dependent increase in the number of apoptotic cells in both breast cancer cell lines. The results were found to be highly significant for almost all the concentrations of garcinol tested, compared with DMSO-treated controls, in both the cell lines.

EFFECT OF GARCINOL ON THE CLONOGENICITY OF BREAST CANCER CELLS

To determine the effect of garcinol treatment on clonogenic potential of breast cancer cells, anchorage-dependent as well as anchorage-independent (soft agar) clonogenic assays were performed. Treatment of MDA-MB-231 and MCF-7 cells with 25 μM garcinol resulted in a marked reduction in the number of colonies, as determined by crystal violet staining (Fig. 3A). In soft agar assay, we found a dose-dependent inhibition by garcinol on the clonogenic potential of both the cell lines (Fig. 3B). Although garcinol treatment resulted in a slightly more inhibitory effect on MCF-7 cells; the effect on MDA-MB-231 cells, the more aggressive cells, was almost equally significant. In soft agar anchorage-independent assay, 25 μM garcinol was found to inhibit the colony forming ability of both the cell lines by more than 50%. The results obtained from the clonogenic assays support our observations of MTT assay, as shown in Figure 1, clearly demonstrating that garcinol significantly inhibits the growth and proliferation of breast cancer cells.

GARCINOL INDUCES A CASPASE-DEPENDENT APOPTOSIS

In an attempt to further characterize the garcinol-induced apoptosis, we assessed the activation of caspases using more aggressive breast
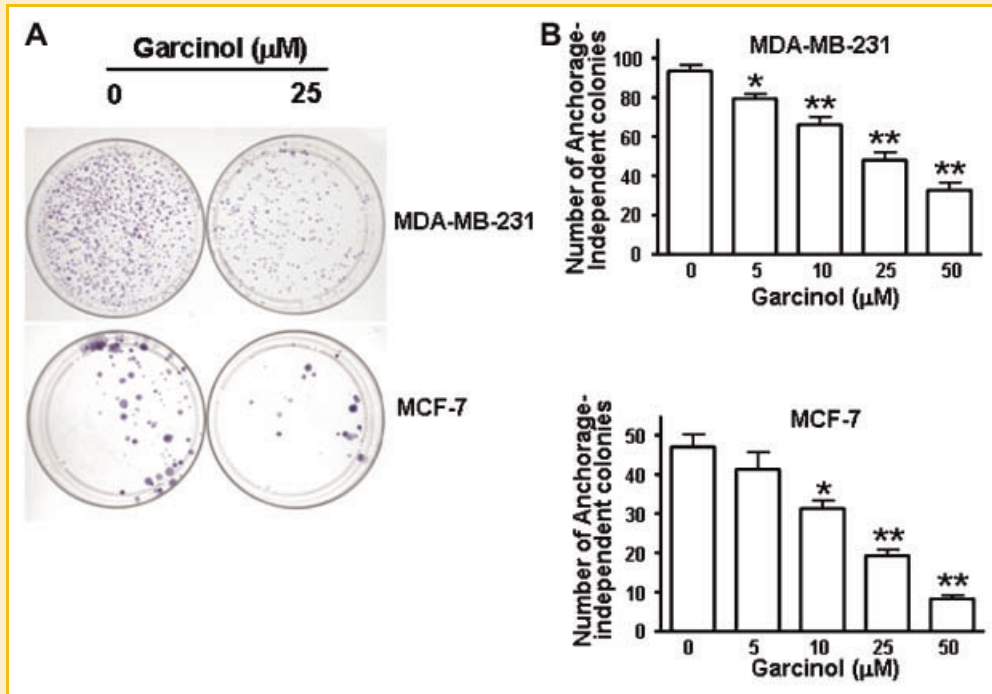
Fig. 3. Clonogenic assays for assessing the effects of garcinol on breast cancer cells. A: Anchorage–dependent and (B) Anchorage–independent assays were performed as described under Materials and Methods Section. Experiments were repeated at least three times and representative data is presented. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus DMSO–treated controls (0 μM garcinol). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

cancer cell line, MDA-MB-231, which showed detectable expression of caspases-3 as well as -9. Activation of these caspases involves a cleavage resulting in the production of active caspases from their pro-forms. Thus, the presence of activated caspases in the cells is an acceptable indicator of the induction of caspase-dependent apoptosis. In our Western blot analysis, we used antibodies that recognize only the cleaved (active) form of these caspases. We found a dose-dependent increase in the cleaved form of caspase-3 as well as caspase-9 in MDA-MB-231 cells (Fig. 4A), suggesting that garcinol treatment results in the activation of these caspases, which

eventually leads to the induction of apoptosis. Cleavage of poly (ADP-ribose) polymerase (PARP) is also studied widely as an early marker of apoptosis, and we found that garcinol treatment resulted in a dose-dependent cleavage of PARP in MDA-MB-231 cells (Fig. 4A). To further confirm our results, we performed another assay for apoptosis, the caspase-3/7 homogeneous assay. This assay determines the levels of active caspase-3 and/or caspase-7 by fluorescence as a measure of apoptosis. Results from this assay (Fig. 4B) also suggested a dose-dependent induction of apoptosis induced by garcinol in MDA-MB-231 cells, as evidenced by
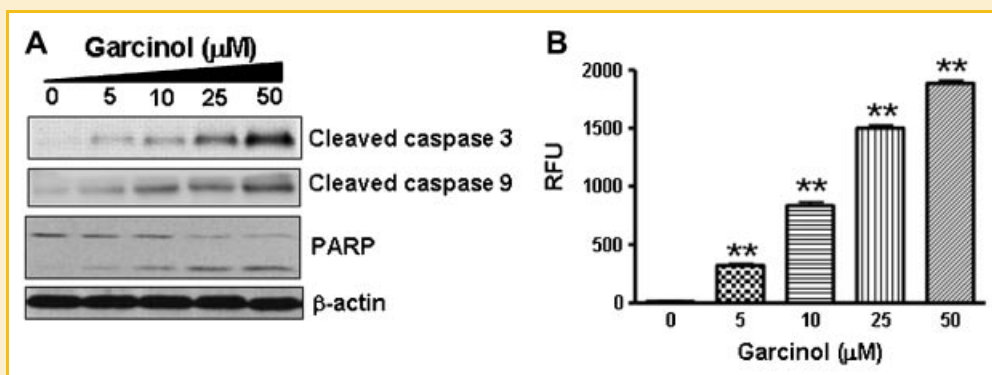

Fig. 4. Representative Western blots (A) showing the effects of increasing concentrations of garcinol on the status of caspases and PARP cleavage in MDA–MB–231 cells after 72 h of treatment. The β–actin protein was used as protein loading control for the blot. Detection of apoptosis by homogeneous caspase–3/7 assay in MDA–MB–231 cells (B) as described under Materials and Methods Section. Experiments were repeated at least three times and representative data are presented. $^{**}P < 0.01$ versus DMSO–treated controls (0 μM garcinol).

increased fluorescence that is reminiscent of active caspase-3/7. Thus, the use of different methodologies confirmed our findings that garcinol is a potent inducer of apoptosis of breast cancer cells and apoptosis-inducing effect of garcinol is dependent on the activation of caspases in MDA-MB-231 cells.

EFFECT OF GARCINOL ON ENDOGENOUS NF-κB AND ITS TARGET GENES

Next, we studied the effect of garcinol treatment on endogenous DNA-binding activity of NF-κB. Exposure of MDA-MB-231 and MCF-7 cells to increasing doses of garcinol followed by EMSA of the nuclear extracts revealed that garcinol significantly inhibits the DNA-binding activity of NF-κB (Fig. 5). NF-κB is a master transcription factor that influences a number of genes that are involved in proliferation, survival, and metastasis of cancer cells [Sarkar and Li, 2008; Sarkar et al., 2008]. Thus, inhibition of NF-κB activity by garcinol is a further proof that this compound has high potential for its use as an anti-cancer agent. Moreover, these results also provide the mechanism by which garcinol may exert its anti-proliferative and apoptosis-inducing effects in breast cancer cells. To further investigate the influence of garcinol on NF-κB signaling pathway, we studied the effect of garcinol on the expression of several gene products that are positively modulated by NF-κB (Bcl-2, Bcl-Xl, and IκBα). Interestingly, the NF-κB signaling is inversely related to ER expression [Wang et al., 2007] and is particularly active in MDA-MB-231 cells. We, therefore, investigated the status of the molecular targets of NF-κB signaling in MDA-MB-231 cells, and found a dose-dependent inhibition of these NF-κB-target genes (Bcl-2, Bcl-xL, and IκBα) after treatment with increasing doses of garcinol (Fig. 6). Since NF-κB itself was found to be inhibited by garcinol (Fig. 5), these results were expected; however these results
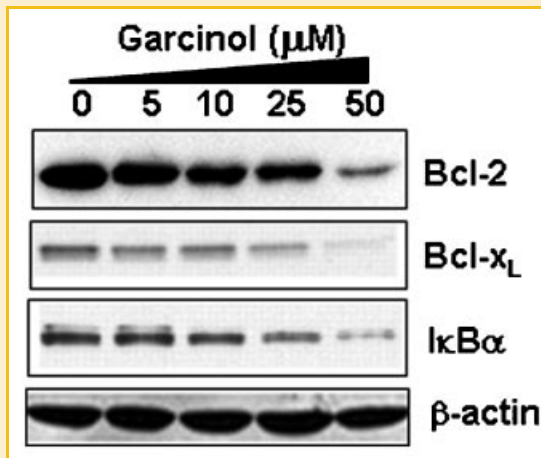
Fig. 6. Representative Western blot analysis showing the effects of increasing concentrations of garcinol on NF-κB-regulated proteins in MDA-MB-231 cells after 72 h. The β-actin protein was used as protein loading control for this experiment.

provided convincing proof in support of the functional role of NF-κB down-regulation by garcinol.

DISCUSSION

Breast cancer remains a major cause of cancer-related deaths in women world-wide [Jemal et al., 2009]. Triple negative breast cancers, the one lacking ER, progesterone receptor (PR), and the Her-2/neu (ErbB2), represent a highly aggressive breast cancer subtype, which is difficult to treat. MDA-MB-231 cells are a representative of such triple negative phenotype and are highly metastatic. 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. Garcinol, extracted from *G. indica*, has emerged in recent times as a putative candidate for anti-cancer studies. Although there is some literature available on its anti-cancer effects in a few cancer models [Pan et al., 2001; Sang et al., 2001, 2002; Liao et al., 2005; Hong et al., 2006, 2007], there is no such report in breast cancer especially nothing is known regarding the molecular mechanism by which garcinol may induce apoptosis in breast cancer cells. We hypothesized that this compound might be effective against breast cancer cells and to test this hypothesis, we investigated the effects of garcinol in ER-positive as well as ER-negative breast cancer cells. We found that garcinol was equally efficient in inhibiting the growth and proliferation of cells representing both the classes. In the cell proliferation MTT assay (Fig. 1A), we found that garcinol was more effective against the ER-negative MDA-MB-231 cells at almost all the doses tested, compared to the ER-positive MCF-7 cells. These results are really encouraging because garcinol was effective in inhibiting the growth and proliferation of breast cancer cells in general, and, moreover, garcinol was more effective against the aggressive and metastatic MDA-MB-231 cells having the phenotype
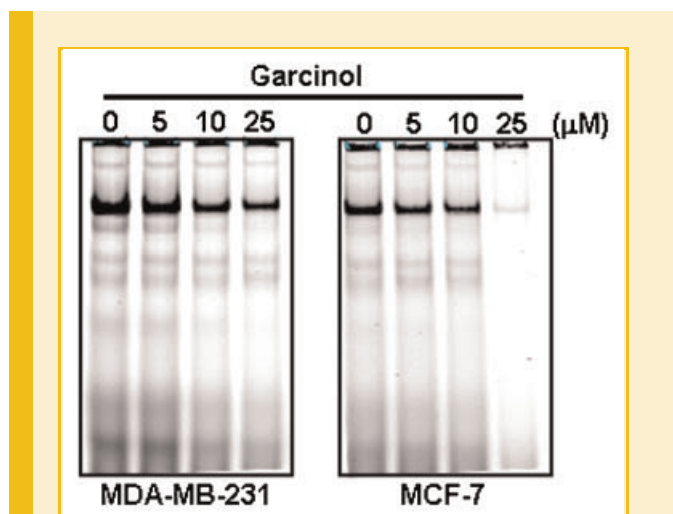

Fig. 5. Dose-dependent down-regulation of NF-κB by garcinol in MDA-MB-231 and MCF-7 cells. Cells were incubated with increasing concentrations of garcinol or DMSO-control for 72 h, and nuclear proteins were subjected to gel shift assay for the evaluation of NF-κB DNA binding activity, as described under Materials and Methods Section.

of triple negative breast cancer (TNBC). These results clearly suggest that garcinol could be useful for the treatment of TNBC.

In an earlier study, the effects of garcinol and its oxidative derivatives were investigated on the growth of HT-29 and HCT-116 colon cancer cells, as well as IEC-6 and INT-407 which are normal immortalized intestinal cells [Hong et al., 2007]. Garcinol showed potent growth-inhibitory effects on all intestinal cells and was found to be more effective in inhibiting the growth of cancer cells than that of normal immortalized cells. Our results with breast cancer cells, especially when we compared our results with so-called "normal" breast epithelial cells (MCF-10A) showed cancer cell-selective killing, which is consistent with the data described above. Moreover, we found significant decrease in the colony forming ability of both the cancer cell lines, which suggests that garcinol could be useful for inhibiting metastasis. This finding is likely to have major clinical implications towards the development of garcinol as a therapeutic agent in the future.

Anti-cancer agents, including the natural compounds, have been traditionally evaluated for their apoptosis-inducing action which is also consistent with the overall growth inhibition of cancer cells and inhibition of tumor progression [Sarkar and Li, 2009]. Therefore, we assessed apoptosis-inducing effects of garcinol, which revealed a dose-dependent induction of apoptosis by garcinol in both ER-positive as well as ER-negative cells. In an earlier published report on human leukemia HL-60 cells, garcinol was reported to display strong growth inhibitory activity through induction of caspase-3 activity in a dose- and time-dependent manner and degradation of PARP protein [Pan et al., 2001]. In the current study, we found activation of caspase-3 and caspase-9, and further confirmed caspase-3/7 activation. These results, along with the dose-dependent PARP cleavage clearly indicate the involvement of caspases in the induction of apoptosis by garcinol. Caspase-9 belongs to the intrinsic apoptotic pathway triggered within the cell by mitochondria [Allan and Clarke, 2009] while the effector caspase-3 can be activated by intrinsic as well as extrinsic pathways leading to degradation of cellular proteins [Porter, 2006]. Caspases-3 and -9 share an interesting relationship; an activated caspase-9 brings about activation of caspase-3 [Allan and Clarke, 2009] but there is also evidence to suggest a feedback activation of caspase-9 by caspase-3 [Zou et al., 2003]. Our present results are not sufficient to establish whether garcinol-induced apoptosis involves one or both of the apoptotic pathways—the intrinsic or the extrinsic, and thus more studies would be needed to address this phenomenon.

It is well known that the regulation of cell growth and apoptosis is part of a normal process for the maintenance of homeostasis in which NF-κB plays a very crucial and important role by regulating many signaling pathways including those that are involved in cancer development and progression [Karin, 2006]. There are scanty reports on the inhibitory action of garcinol on NF-κB [Liao et al., 2004; Hong et al., 2006] documenting that garcinol could be an effective inhibitor of lipopolysaccharide-induced activation of NF-κB. Moreover, recent evidence suggests a negative correlation between the expression of ER and the activity of NF-κB in breast cancer cells, implying that the highly aggressive TNBC, such as MDA-MB-231, have constitutively higher NF-κB activity [Wang et al., 2007]. Thus, we have tested whether garcinol could inhibit the constitutive NF-κB activity in breast cancer cells. Our current results confirmed this inverse correlation because the basal NF-κB activity in MDA-MB-231 cells was greater than those in less aggressive, ER-positive MCF-7 cells, which is consistent with our previously published data [Ahmad et al., 2008]. These results clearly support the pleiotropic effects of garcinol and it is clear that the apoptosis inducing effect of garcinol could, in part, be due to inactivation of NF-κB.

Another mechanism by which NF-κB promotes invasiveness of ER-negative breast cancer cells is by induction of Bcl-2 [Wang et al., 2007; Ahmad et al., 2008], leading to the survival of these cells during therapy. It has been shown that nuclear localization (activation) of NF-κB and the expression of Bcl-2 serve as a mechanism for resistance of breast cancer patients to anthracyline-based chemotherapy [Buchholz et al., 2005]. It has also been reported that activated NF-κB signaling [Wu and Bonavida, 2009] as well as over-expression of anti-apoptotic Bcl-2 family members leads to resistance to therapy in human cancers [Buchholz et al., 2005; Kostanova-Poliakova and Sabova, 2005; Kang and Reynolds, 2009]. Interestingly, preclinical studies have shown that agents targeting anti-apoptotic Bcl-2 family members have preclinical activity as single agents and in combination with other anti-neoplastic agents [Kang and Reynolds, 2009]. In view of such a central role of Bcl-2 family members in pro-survival pathways, their down-regulation by garcinol, as observed in the present study, provides another mechanism through which this compound modulates the cellular apoptotic machinery leading to an efficient induction of apoptosis.

The results presented here clearly show that garcinol is an effective inhibitor of cell growth/proliferation and inducer of apoptosis in human breast cancer cells. Inactivation of NF-κB signaling and down-regulation of its target genes appears to be one potential mechanism by which garcinol exerts its apoptosis-inducing effects. This is the first report describing such an activity of garcinol in breast cancer cells although further in-depth research including animal experiments are needed in order to fully appreciate the value of garcinol in the inhibition of tumor progression and/or treatment of breast cancer and other human malignancies.

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