Inactivation of NF-κB by 3,3′-diindolylmethane contributes to increased apoptosis induced by chemotherapeutic agent in breast cancer cells

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Abstract

Constitutive activation of Akt or nuclear factor-κB (NF-κB) has been reported to play a role in de novo resistance of cancer cells to chemotherapeutic agents, which is a major cause of treatment failure in cancer chemotherapy. Previous studies have shown that 3,3′-diindolylmethane (DIM), a major in vivo acid-catalyzed condensation product of indole-3-carbinol, is a potent inducer of apoptosis, inhibitor of tumor angiogenesis, and inactivator of Akt/NF-κB signaling in breast cancer cells. However, little is known regarding the inactivation of Akt/NF-κB signaling by DIM, such as Taxotere. Therefore, we examined whether the inactivation Akt/NF-κB signaling caused by B-DIM could sensitize breast cancer cells to chemotherapeutic agents both in vitro and in vivo. MDA-MB-231 cells were simultaneously treated with 15 to 45 μmol/L B-DIM and 0.5 to 1.0 nmol/L Taxotere for 24 to 72 h. Cell growth inhibition assay, apoptosis assay, electrophoretic mobility shift assay, and Western blotting were done. The combination treatment of 30 μmol/L B-DIM with 1.0 nmol/L Taxotere elicited significantly greater inhibition of cell growth compared with either agent alone. The combination treatment induced greater apoptosis in MDA-MB-231 cells compared with single agents. Moreover, we found that NF-κB activity was significantly decreased in cells treated with B-DIM and Taxotere. We also have tested our hypothesis using transfection studies, followed by combination treatment with B-DIM/Taxotere, and found that combination treatment significantly inhibited cell growth and induced apoptosis in MDA-MB-231 breast cancer cells mediated by the inactivation of NF-κB, a specific target in vitro and in vivo. These results were also supported by animal experiments, which clearly showed that B-DIM sensitized the breast tumors to Taxotere, which resulted in greater antitumor activity mediated by the inhibition of Akt and NF-κB. Collectively, our results clearly suggest that inhibition of Akt/NF-κB signaling by B-DIM leads to chemosensitization of breast cancer cells to Taxotere, which may contribute to increased growth inhibition and apoptosis in breast cancer cells. The data obtained from our studies could be a novel breakthrough in cancer therapeutics by using nontoxic agents, such as B-DIM, in combination with other conventional therapeutic agents, such as Taxotere.

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Introduction

Carcinoma of the breast is the most common cancer in American women and remains the second leading cause of cancer-related female deaths in the United States (1). Currently, breast cancer is treated with surgery, chemotherapy, radiation therapy, or combined modalities with remarkable success. In addition, patients with breast cancer or preneoplastic lesions are also treated with hormonal therapy, either for treatment or prevention purposes. Although these treatment modalities are successful, a significant number of patients either do not respond to commonly used chemotherapeutic agents or have tumor recurrence during therapy and develop metastasis, for which there is no curative therapy. The failure in the treatment is largely contributed by de novo (intrinsic) chemoresistance and or acquired chemoresistance of tumor cells (2, 3). Therefore, this study was designed to eliminate chemoresistant pathways of tumor cells before therapy with a conventional therapeutic agent to achieve optimal results.

Nuclear factor-κB (NF-κB) is a key regulator and transcription factor of genes, which mediate apoptotic signaling pathways; it also plays critical roles in cell proliferation, cell adhesion, inflammation, differentiation, angiogenesis, and tumor cell invasion (3–7). It has been reported that activated NF-κB blocks cellular apoptosis in several different cell types (5–9) and that the inactivation of NF-κB makes cells more sensitive to apoptosis-inducing agents (10). Studies have shown that the activation of Akt and NF-κB is responsible for the resistance of cancer cells to chemotherapeutic agents and contributes to treatment failure in cancer chemotherapy (2, 3, 11–14). Because NF-κB plays important roles in many cellular processes, research on the interaction between NF-κB activation and...
other cell signal transduction pathways, including the phosphoinositide-3 kinase/Akt pathway, has received increased attention in recent years. Recently, constitutive activation of NF-κB was found in human breast cancer cells, possibly due to the activation of different signaling pathways, such as phosphoinositide-3 kinase/Akt and mitogen-activated protein kinase, which are also known to increase the expression of Bcl-2/Bcl-XL and other NF-κB-targeted genes (2, 3, 11–17). It has also been shown that expression of antiapoptotic protein Bcl-2/Bcl-XL is associated with cancer cell viability and drug resistance (15); unfortunately, the clinical importance of NF-κB expression remains unclear in patients with breast cancer. We, therefore, hypothesize that the down-regulation of Akt/NF-κB signaling in breast cancer cells could be a novel therapeutic approach for achieving optimal results in patients with chemoresistant breast cancer. However, very little is known regarding the inactivation of Akt/NF-κB that leads to sensitization of breast cancers to conventional therapeutic agents, such as Taxotere.

Several studies have shown that inhibition of Akt activation by phosphoinositide-3 kinase inhibitor (LY294002 or Wortmannin) sensitizes cancer cells, particularly breast cancer cells, to undergo apoptotic cell death induced by Adriamycin and Taxotere (2, 18, 19). These results strongly suggest that the inactivation of the Akt pathway, which in turn will inactivate the NF-κB pathway, will also sensitize breast cancer cells to Adriamycin and Taxotere. Recent studies from our laboratory have indicated that inactivation of NF-κB by pretreatment with genistein leads to chemosensitization of pancreatic cancer cells to apoptosis induced by docetaxel and cisplatin (20). Another study also indicated that inactivation of NF-κB can directly chemosensitize pancreatic cancer cells to gemcitabine (21). A recent study has indicated that DIM and Paclitaxel can promote apoptosis in MDA-MB-435eb1 (Her2/Neu positive, ER-ve) human breast cancer cells (22). Moreover studies from our laboratory and others have shown that B-DIM is a potent inhibitor of cell growth, inducer of apoptotic cell death and inhibitor of tumor angiogenesis, which is believed to be partly due to inactivation of Akt and NF-κB signaling pathways in several cancer cells (5, 6, 23–26). More recently, we found that B-DIM can inhibit Akt and NF-κB in breast cancer cells, suggesting that B-DIM could also sensitize breast cancer cells to Taxotere (6). Our recent results also show that the inactivation of NF-κB down-regulates its transcriptional downstream signaling molecules, such as Bcl-2, Bcl-XL, and survivin (5–7, 27, 28), leading us to believe that B-DIM not only inactivates NF-κB but also down-regulates its important downstream regulatory genes. This effect of B-DIM could, in turn, sensitize chemoresistant breast cancer cells to Taxotere-induced killing. In this study, we tested our hypothesis using MDA-MB-231 breast cancer cell line (ER-ve) in vitro and in animal model in vivo (MDA-MB-231–induced tumors in SCID mouse) exposed to B-DIM alone, Taxotere alone, or their combinations. Because we strongly believe that the inactivation of NF-κB pathways could be successfully exploited for the development of novel therapeutic strategies for estrogen-independent breast cancer using B-DIM in combination with Taxotere. In this study, we specifically investigated how B-DIM could sensitize breast cancer cells to conventional therapeutic agents, such as Taxotere. We found that B-DIM could sensitize breast tumor cells to Taxotere, resulting in greater antitumor activity by inactivating Akt, NF-κB, and other targeted genes. Our results suggest that B-DIM is an effective agent in sensitizing breast cancer cells to Taxotere, which is likely a novel breakthrough for devising optimal therapies for breast cancer.

**Materials and Methods**

**Cell Culture and Reagents**

MDA-MB-231 human breast cancer cells (invasive and ER-ve; American Type Culture Collection) were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 5% CO2 atmosphere at 37°C. DIM (Bio Response; commonly known as BR-DIM and hereafter termed as B-DIM) was dissolved in DMSO to make 20 mmol/L stock solution and was added directly to the media at different concentrations. Taxotere (Aventis Pharmaceuticals) was dissolved in DMSO to make a 4 μmol/L stock solution.

**Cell Growth Inhibition by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay**

MDA-MB-231 cells were seeded in 96-well culture dishes. After 24 h of incubation, MDA-MB-231 were treated with 15, 30, or 45 μmol/L B-DIM and then exposed to chemotherapeutic agents (0.5, 0.75, or 1.0 nmol/L Taxotere) for 24, 48, or 72 h. For single-agent treatment, MDA-MB-231 cells were treated with B-DIM (15, 30, or 45 μmol/L) and/or Taxotere (0.5, 0.75, or 1.0 nmol/L) alone for 24 to 72 h. After treatment, cancer cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (0.5 mg/mL; Sigma) at 37°C for 2 h and then with isopropyl alcohol at room temperature for 1 h. The spectrophotometric absorbance of the samples was determined by ULTRA Multifunctional Microplate Reader (TECAN) at 595 nm.

**Histone/DNA ELISA for Detecting Apoptosis**

Cell apoptosis ELISA detection kit (Roche) was used to detect apoptosis according to the manufacturer’s protocol (6, 29–32). Briefly, MDA-MB-231 cells were treated with B-DIM and/or chemotherapeutic agents (Taxotere) as described above. After treatment, the cytoplasmic histone/DNA fragments from cancer cells with different treatments were extracted and bound to immobilize antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined by ULTRA Multifunctional Microplate Reader (TECAN) at 405 nm.

**DNA Ladder Analysis for Detecting Apoptosis**

MDA-MB-231 cells were treated with 30 μmol/L DIM and then exposed to chemotherapeutic agents (1.0 nmol/L Taxotere) as indicated for peroxidase, the spectrophotometric absorbance of the samples was determined by ULTRA Multifunctional Microplate Reader (TECAN) at 595 nm.

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Taxotere) for 72 h. For single-agent treatment, MDA-MB-231 cells were treated with 30 μmol/L B-DIM, 1.0 nmol/L Taxotere alone for 72 h. After treatment, cellular cytoplasmic DNA from MDA-MB-231 cells with different treatments was extracted, and the DNA ladder was visualized as described previously (5, 6).

**NF-κB DNA-Binding Activity Measurement**

MDA-MB-231 cells were plated at a density of 1 × 10⁵ cells in 100-mm dishes and were incubated. After 24 h, the cells were treated with a combination of B-DIM and chemotherapeutic agents as described above. For single-agent treatment, cancer cells were treated with B-DIM (30 or 45 μmol/L) and 1 nmol/L Taxotere alone for 72 h. After treatment, nuclear protein in the cells was extracted as described previously (5, 6). Using randomly selected frozen tumor tissue, nuclear proteins were also extracted as described previously (7). For electrophoretic mobility shift assay (EMSA), 10 μg of nuclear protein was assembled with 5× gel shift-binding buffer (20% glycerol, 5 mmol/L MgCl₂, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl, 0.25 mg/mL poly(dI)-poly(dC), and IRDye 700–labeled NF-κB oligonucleotide (LI-COR). After incubation at room temperature for 30 min, the samples were loaded on a prerun 8% polyacrylamide gel, and electrophoresis was continued at 30 mA for 90 min. The signal was then detected and quantified with Odyssey IR imaging system (LI-COR). For loading control, 10 μg of nuclear proteins from each sample was subjected to Western blot analysis for retinoblastoma protein, which showed no alternation after B-DIM or Taxotere treatment.

**Western Blot Analysis**

Twenty-five micrograms of cell extract from p65 cDNA transfection or 50 μg of tumor lysate from animal experiments were subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Membranes were then incubated with monoclonal anti–phosphorylated Akt Ser⁴⁷³ (1:1,000; Cell Signaling), Akt (1:500; Santa Cruz Biotech), polyclonal anti–NF-κB p65 (1:1,000; Upstate), antisurvivin (1:200; Santa Cruz), anti–Bcl-X₁ (1:200; Santa Cruz), anti–poly(ADP-ribose) polymerase (PARP; 1:5,000; Biomol), or anti–β-actin (1:5,000; Sigma) antibodies, washed with Tween 20–TBS, and incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (Pierce).

**Animal Studies**

Female homozygous ICR SCID/SCID mice, ages 4 weeks, were purchased from Taconic Farms. The mice were maintained according to the NIH standards established in the Guidelines for the Care and Use of Experimental Animals. All experimental protocols were approved by the Animal Investigation Committee of Wayne State University. To initiate the xenografts, 5 × 10⁶ MDA-MB-231 cells (in serum-free medium) were injected s.c. bilaterally in the flank areas of two SCID mice. Animals were observed for development of s.c. tumors at the sites of injection (usually in 2–3 weeks). When the tumor size reached 1,500 mg, animals were treated...
were euthanized and the tumors were removed and dissected into small pieces (~30 mg). Small pieces of tumors were then transplanted s.c. into right and left flanks of a new group of SCID mice using 12-gauge trocar. Animals were examined thrice per week for the development of palpable tumors (usually in 2 weeks). Once palpable tumors developed (0.5 cm or 0.5 cm, 63 mg), animals were randomly divided into four groups of 10 animals in each group. Group I was assigned as control, and group II mice were given B-DIM by oral gavage (3.5 mg/day/animal by oral gavage). In addition, the mice from group III received three doses of Taxotere (5 mg/kg, i.v.) every 72 h after 24 h of B-DIM gavaging (33, 34). Group IV was exposed to B-DIM and also treated with Taxotere as shown for groups II and III. The dose of B-DIM selected for this experiment was based on our previous studies that showed antitumor activity (6, 28, 35–37). Sesame seed oil, shown as safe by other researchers, was used to facilitate gavage and avoid irritation of the esophagus (7, 38). The control mice received only sesame seed oil without B-DIM. Mice from all experimental groups were sacrificed 3 weeks after the start of all treatments, and the tumors were harvested from each animal and processed for preparation of nuclear proteins, as well as total protein for Western blot analysis. The activity of NF-\(\kappa\)B in tumor cells was measured by EMSA, and the PARP cleavage in tumor cells was assessed by Western blot analysis. The volume of the tumor in each group was determined by weekly caliper measurement according to the formula \(\frac{a b^2}{2}\), wherein \(a\) is length and \(b\) is the shortest measurement.

**Tissue Collection, Fixation, and H&E Staining**

Freshly harvested tumors grown were fixed in 10% buffered formalin for 48 h and then embedded and sectioned. Samples were then washed with tap water and soaked in a graded series of 50%, 60%, 70%, 80%, and 90% ethanol for 30 min, and then in 90% and 100% ethanol for 1 h. They were then held in a solution of 100% ethanol and xylene at a 1:1 ratio for 30 min before being embedded in paraffin and held at 60°C for 1 h to make paraffin blocks. Transverse sections (5 μm) were taken from the blocks and prepared for histochemical staining. H&E staining was used for histologic observation.

**Results**

**B-DIM – Potentiated Breast Cancer Cell Growth Inhibition Caused by Taxotere**

MDA-MB-231 breast cancer cells were treated with B-DIM, Taxotere, or B-DIM in combination with lower doses of Taxotere. The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MDA-MB-231 breast cancer cells were treated with B-DIM, Taxotere, or B-DIM in combination with lower doses of Taxotere. The cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

**Figure 2.** Induction of apoptosis in breast cancer cells tested by ELISA, DNA ladder, and PARP cleavage assay. A, histone/DNA fragment analysis by ELISA. MDA-MB-231 breast cancer cells were treated 30 μM B-DIM and then simultaneously exposed to 1.0 nmol/L Taxotere for 72 h. B, MDA-MB-231 breast cancer cells treated with B-DIM and Taxotere for 72 h as illustrated by the DNA ladder. Lane 1, DNA marker; lane 2, control; lanes 3, 4, and 5, cells treated with B-DIM (30 μmol/L), Taxotere (1 nmol/L), B-DIM (30 μmol/L) + Taxotere (1 nmol/L), respectively. PARP cleavage assay showed that combination treatment with B-DIM and Taxotere induced significantly greater apoptosis both in vitro (C) and in vivo (D). Lane 1, control; lanes 2, 3, and 4, treated with B-DIM (30 μmol/L), Taxotere (1 nmol/L), B-DIM (30 μmol/L) + Taxotere (1 nmol/L), respectively. ***, \(P < 0.05\) compared with control.

**Figure 3.** Western blot analysis for Akt, phosphorylated Akt, survivin, and Bcl-X\(_{L}\) in NF-\(\kappa\)B p65 cDNA transfected and parental MDA-MB-231 breast cancer cells. Control, parental; Tax, Taxotere. The signal of each protein expression was quantified with Gel Doc 1000 image system (Bio-Rad).
assay, and the effect of B-DIM or Taxotere on the growth of different cancer cells is shown in Fig. 1. We have tested several doses of B-DIM and Taxotere with different time points, and we found that treatment of cells with B-DIM or Taxotere alone for 72 h typically caused 30% to 40% growth inhibition in cancer cells using the doses tested (Fig. 1A and B). However, B-DIM in combination with lower doses of Taxotere resulted in ~50% growth inhibition in this cancer cells, suggesting the greater inhibitory effect of combination treatment (Fig. 1C). These results showed that combination of B-DIM along with lower doses of Taxotere elicited significantly greater inhibition of cancer cell growth compared with either agent alone. The lower dose of Taxotere in inhibiting cell growth when combined with a nontoxic agent (B-DIM) will have significant ramification for extending our studies for human breast cancer treatment.

**B-DIM Sensitized Breast Cancer Cells to Apoptosis Induced by Taxotere**

By three independent measurement of apoptotic assays, we observed induction of apoptosis in breast cancer cells treated with B-DIM and Taxotere. By apoptotic cell death ELISA analysis and DNA ladder analysis, we observed similar results showing that both B-DIM and Taxotere alone induced apoptosis in the tested breast cancer cells (Fig. 2A and B). Moreover, we found that 30 μmol/L B-DIM combined with lower doses of Taxotere induced greater apoptosis in the cancer cells compared with single-agent treatment (Fig. 2A and B). We also observed that the B-DIM and Taxotere combination treatment *in vitro* and *in vivo* produced PARP cleavages compared with monotherapy (Fig. 2C and D), suggesting greater apoptosis induced by the combination treatment. Using Western blot analysis, we found that B-DIM alone or in combination with chemotherapeutic agents down-regulated the expression of phosphorylated Akt, survivin, and Bcl-XL in NF-κB p65 cDNA-transfected or parental breast cancer cells (Fig. 3). These results are consistent with the cell growth inhibition observed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, suggesting that greater cell growth inhibition resulting from the combination treatment is partly mediated through the induction of greater apoptosis in breast cancer cells.

**Inhibition of Tumor Growth *in vivo* by B-DIM Alone or in Combination with Taxotere**

To test whether B-DIM has any antitumor effect *in vivo*, we conducted an animal experiment using a xenograft model of breast cancer. Under our experimental conditions described earlier, we found that B-DIM significantly inhibited MDA-MB-231 tumor growth and potentiated MDA-MB-231 tumor growth inhibition induced by Taxotere, demonstrating an enhanced inhibitory effect of B-DIM/Taxotere combination treatment on the *in vivo* model of breast cancer (Fig. 4A). H&E histology evaluation showed the maximum degree of necrosis in B-DIM/Taxotere combination treatment group (Fig. 4B). The body weight of mice in each group did not show any significant difference, suggesting no apparent toxicity due to B-DIM and Taxotere treatment. To explore the

![Figure 4](https://mct.aacrjournals.org/content/6/10/2761/F4.large.jpg)

**Figure 4.** Inhibition of MDA-MB-231 tumor growth by B-DIM and Taxotere using xenograft model of breast cancer. Under the experimental conditions, treatment of animals with B-DIM in combination with Taxotere caused 50% reduction in tumor volume compared with control group (A). MDA-MB-231 cells were grown subcutaneously in the SCID animals. After the termination, the entire xenograft "tumor mass" was harvested from each animal and totally embedded for histologic processing. Histologic evaluation represents the largest "two-dimensional" tumor mass measurements of the four study groups with a significant decline in these measurements from the control to the B-DIM (3.5 mg/day/animal by oral gavage), Taxotere (5 mg/kg, i.v.), and B-DIM + Taxotere combination treatment groups, respectively. Microscopically, the viable tumor in all groups is composed of solid sheets of malignant tumor cells with moderate to significant nuclear polymorphism and high mitotic rate, including abnormal figures. Variable degrees of tumor necrosis was evident in all sections, ranging from 5% to 90%, and the B-DIM plus Taxotere group showed the maximum degree of necrosis (B). Gel shift assay for NF-κB was done on randomly selected frozen tumor tissues obtained from each treatment groups. Lanes 1 and 2, control; lanes 3 and 4, B-DIM; lanes 5 and 6, Taxotere; lanes 7 and 8, B-DIM and Taxotere (combination treatment). Results showed that B-DIM in combination with Taxotere was effective in down-regulating NF-κB in treated animals relative to control tumors (C). *, P < 0.05 compared with control.
molecular mechanism by which B-DIM potentiated the antitumor and antimetastatic activity of Taxotere, we further analyzed the NF-κB expression altered by B-DIM or Taxotere treatment.

**B-DIM Causes Inactivation of NF-κB DNA-Binding Activity In vivo**

Using EMSA, we found that B-DIM in vivo significantly inhibited NF-κB DNA-binding activity (Fig. 4C). Importantly, our animal studies showed that dietary B-DIM in combination with Taxotere inhibited NF-κB DNA-binding activity in SCID s.c. tumors (Fig. 4C). These results show that B-DIM in combination with Taxotere in vitro and in vivo significantly reduces NF-κB DNA-binding activity, resulting in breast cancer cell killing.

**Effect of B-DIM and Taxotere on NF-κB p65 Expression and NF-κB DNA-Binding Activity**

Nuclear proteins from cultured cancer cells treated with Taxotere were subjected to analysis for NF-κB DNA-binding activity as measured by EMSA. The results showed that 2 nmol/L Taxotere treatment for 2 h significantly induced NF-κB DNA-binding activity in cancer cells compared with the untreated cells (data not shown), whereas 1 nmol/L Taxotere treatment for 72 h did not change NF-κB DNA-binding activity in cancer cells compared with the untreated cells (Fig. 5A). Importantly, NF-κB p65 cDNA transfection enhanced the NF-κB DNA-binding activity, whereas B-DIM alone or in combination with Taxotere abrogated the expression and activation of NF-κB p65 (Fig. 5A). These results clearly suggest that B-DIM in combination with Taxotere exerted inhibitory effects by abrogating NF-κB DNA-binding activity through decreasing the expression and activation of NF-κB p65. Furthermore, it has been indicated that inhibition of NF-κB potentiated the anticancer effect of chemotherapeutic agents (39). In the present study, we found that NF-κB p65 cDNA transfection enhanced the expression of nuclear and cytoplasmic NF-κB p65 in breast cancer cells, whereas B-DIM in combination with Taxotere significantly...
abrogated this expression, which was measured by Western blot analysis (Fig. 5B), suggesting that B-DIM and Taxotere treatment inactivates NF-κB p65 expression, which may contribute to increased growth inhibition and apoptosis in breast cancer cells.

**Apoptosis-Enhancing Effect of B-DIM Is Mediated Through the NF-κB Pathway**

We transfected NF-κB p65 cDNA into MDA-MB-231 cells, treated the transfected cells with B-DIM or Taxotere, and detected apoptosis. We found that p65 cDNA transfection inhibited apoptosis in B-DIM−treated and untreated breast cancer cells (Fig. 5C). In contrast, B-DIM combined with Taxotere showed a greater inhibitory effect on the induction of apoptosis in p65 cDNA-transfected breast cancer cells compared with Taxotere monotreatment (Fig. 5C). These results provide mechanistic support in favor of our claim that the apoptosis-inducing effect of Taxotere is enhanced by B-DIM and that it is partly mediated through the NF-κB pathway.

**Statistical Analysis**

The statistical significance was determined using Student’s t test, and P < 0.05 was considered significant.

**Discussion**

Current reports have shown that plant-derived dietary compounds provide protection against the development of certain cancers, including breast cancer (40–42). We and others have previously shown that dietary indole-3-carbinol, a natural compound present in cruciferous vegetables, inhibits proliferation and induces apoptosis in several tumor cell lines (5–7, 23, 27, 28, 43–45). B-DIM has also been found to possess anticarcinogenic effects in experimental animals and to inhibit the growth of human cancer cells (6, 22, 23, 25, 28, 36). More recently, we found that B-DIM can inhibit Akt and NF-κB in breast cancer cells, suggesting that B-DIM could sensitize the breast cancer cells to Taxotere (6). Taxotere has been frequently used for treatment of various cancers, including breast cancer, alone or in combination with other agents (46, 47). Each of these compounds is useful against breast cancer cells with clearly different mechanism(s) of action. Here, we report that suppression of constitutively active NF-κB in MDA-MB-231 cells is a major event by which B-DIM sensitizes breast cancer cells to a chemotherapeutic agent, and as such, Taxotere could induce more apoptosis in breast cancer cells when combined with B-DIM compared with either agents alone.

Apoptosis is one of the most vital pathways through which chemopreventive agents inhibit the overall growth of cancer cells. Thus, it is important to investigate whether inhibition of cell proliferation and induction of apoptosis are associated with the down-regulation of apoptosis-related genes, such as Akt, Bcl-XL, and survivin, by B-DIM, which could lead to chemosensitization of breast cancer cell to chemotherapeutic agents, such as Taxotere (6, 28, 48, 49). Previously, we and others have shown that B-DIM down-regulates NF-κB and its targeted genes, such as Akt, Bcl-XL, and survivin (5, 6, 26, 28). In the present study, our results showed that B-DIM and Taxotere, as single agents, did induce apoptosis in MDA-MB-231 breast cancer cells. However, treatment of cells with B-DIM in combination with a lower dose of Taxotere resulted in a significantly greater induction of apoptosis in MDA-MB-231 breast cancer cells as documented by ELISA, DNA ladder, and PARP cleavage assay, in conjuction with a more significant inhibition of cell growth as observed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. These results suggest that more significant inhibition of MDA-MB-231 cell growth caused by a low dose of combination treatment may be mediated by increased induction of apoptosis.

Constitutively, activated NF-κB has been found in many tumors (3, 50). Therefore, to determine the mechanism(s) of increased apoptosis induced by B-DIM and Taxotere, we investigated the activity of NF-κB, which plays a critical role in the inhibition of apoptotic response. NF-κB also regulates the expression of a large number of genes that play critical roles in apoptosis, viral replication, tumorigenesis, various autoimmune diseases, and inflammation (3). NF-κB is composed of a heterodimer of p65 and p50 subunits in most cell types and is sequestered in the cytoplasm by its inhibitory proteins, the IκBs (3, 5, 6, 51). During the phosphorylation and degradation of IκBs, NF-κB p65 is activated and rapidly transported from the cytoplasm to the nucleus in cancer cells (3, 5, 6, 51). Therefore, NF-κB p65 has been described as a major culprit and important therapeutic target in cancer (3, 4, 52). It has been reported that inhibition of NF-κB can enhance the anticancer effect of chemotherapeutic agents (39). It is known that Taxotere, a chemotherapeutic agent, is commonly used to treat patients with breast cancer; however, the use of high-dose Taxotere in these patients always leads to various levels of toxicity and thus reduces its therapeutic benefit (53, 54). In the present study, we conducted NF-κB p65 cDNA transfection and found that p65 cDNA transfection increased the expression of NF-κB. However, B-DIM inhibited the expression of NF-κB DNA-binding activity, whereas Taxotere showed a lesser degree of apoptosis, supporting the observation that NF-κB causes inhibition of apoptosis. We have previously reported that DIM inhibits NF-κB activity in breast cancer cells (6).

**Figure 6.** Schematic representation of potential anticancer effect of chemotherapeutic agents mediated by B-DIM−induced inhibition of Akt and NF-κB activity.
Importantly, our present results showed that B-DIM in combination with a low dose of Taxotere was significantly associated with the inhibition of cell proliferation and induction of apoptosis, which could be due to the inhibition of NF-κB p65 expression and NF-κB DNA-binding activity by B-DIM, which in turn sensitizes breast cancer cells to Taxotere-induced killing. Several clinical trails have also suggested that the combination treatments may have greater anticancer activity than single agents (55, 56). Therefore, our findings provide a possible mechanism for the greater induction of apoptosis by a low dose of Taxotere in combination with B-DIM, suggesting that B-DIM could be potentially useful when combined with chemotherapeutic agents for the treatment of breast cancer and, as such, will have lower systemic toxicity compared with current modalities.

Furthermore, to investigate whether B-DIM could sensitize breast cancer cells to Taxotere, we conducted in vitro study. We found that B-DIM in combination with Taxotere could inhibit tumor growth in MDA-MB-231 tumor xenograft model that could be mechanistically linked with inactivation of NFκB activity. Our present observation also showed that the maximum degree of necrosis occurred in B-DIM plus Taxotere treatment group. These results further support the conclusion that inhibition of NFκB activation promotes Taxotere-induced apoptosis in vivo. Overall, our results showed that blocking NFκB signaling by B-DIM could lead to chemosensitization of breast cancer cells to chemotherapeutic agents, such as Taxotere. Importantly, the data obtained from our in vitro and in vivo experiments could be a novel breakthrough approach in cancer therapeutics using a nontoxic agent, such as B-DIM, in combination with lower dose of other conventional chemotherapeutic agents.

It is known that resistance to chemotherapeutic agents is the major cause for treatment failure in cancer chemotherapy. Inhibition of NFκB was found to potentiate the anticancer effect of chemotherapeutic agents (39). However, to achieve greater inhibitory effect, combination treatment with other chemopreventive agents could be a better therapeutic approach. It has been reported that Taxotere is one of the most potent chemotherapeutic agents for the treatment of patients with metastatic and early-stage breast cancer (57), and several studies have shown that histone deacetylase inhibitor LAQ824 and genistein sensitize cancer cells to undergo apoptotic cell death induced by Taxotere (18, 20, 57). It is also known that the use of high-dose Taxotere for patients with breast cancer leads to various levels of toxicity, thereby reducing its therapeutic benefit (54). Several clinical trails have also suggested that combination treatments may have greater anticancer activity than single agents (56). Our findings also showed that B-DIM can sensitize breast cancer cells to Taxotere. Our results, along with previous reports, strongly suggest that the effect of B-DIM may be mediated through the inhibition of NFκB signaling, resulting in the inhibition of cell proliferation and induction of apoptosis, which could lead to sensitization of breast cancer cells to conventional therapeutics agents, such as Taxotere (Fig. 6). However, the combination treatment may result in different levels of systematic toxicities (58, 59). Thus, optimization of combination chemotherapy based on molecular mechanism may improve therapeutic indexes that are critically needed for the treatment of patients with breast cancer. In the present study, our results clearly showed that combination of lower dose of a chemotherapeutic agent with a nontoxic dietary compound (B-DIM) with no side effect in humans could be a novel therapeutic strategy against breast cancers.

In summary, our results show that B-DIM in combination with low doses of Taxotere enhances the inhibition of cell growth and the induction of apoptosis in MDA-MB-231 breast cancer cells through inhibition of the NFκB signaling pathway both in vitro and in vivo. Our results open a new avenue and challenge the current paradigm for the prevention and/or treatment of breast cancer; however, further investigation, including clinical trials, are warranted to prove or disprove the usefulness of B-DIM in combination with conventional therapeutic agents for the treatment of breast cancer.

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References
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Inactivation of NF-κB by 3,3′-diindolylmethane contributes to increased apoptosis induced by chemotherapeutic agent in breast cancer cells


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