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3,3′-Diindolylmethane and Paclitaxel Act Synergistically to Promote Apoptosis in HER2/Neu Human Breast Cancer Cells

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Background. HER2/neu positive breast tumors are difficult to treat. About 25 to 30% of invasive breast tumors overexpress the HER2/neu oncogene. These tumors are aggressive and become resistant to chemotherapeutic drugs. 3,3′-diindolylmethane (DIM), the active metabolite of indole-3-carbinol, a naturally occurring compound found in cruciferous vegetables, has been found to have anti-cancer properties in both humans and animals. DIM has been shown to induce cell cycle arrest and apoptosis in animal breast cancer models. Because HER2/neu overexpression confers resistance to paclitaxel, and DIM has anti-tumor effects, we hypothesized that DIM will enhance the cytotoxic effects of paclitaxel, a common taxane drug, on human Her2/neu breast cancer cells by potentiating its effect on cell cycle and stimulating apoptosis.

Methods. The MDA-MB-435eB1 human Her2/neu breast cancer cells were treated with varying concentrations of DIM and paclitaxel. The cells were analyzed at different time points (24, 48, and 72 h). Proliferation was measured by a commercial cell proliferation assay (Promega Procheck Assay). Cell-cycle analysis and apoptosis were determined by flow cytometry. Western blot analysis was performed on to determine the effect of DIM and/or paclitaxel on the proteins involved in apoptosis, and epidermal growth factor-induced activation of HER2/neu and ERK1/2 signaling proteins.

Results. Both DIM and paclitaxel exhibited time and concentration dependent inhibition of cell proliferation. TUNEL assay indicated that the combination also increased the number of apoptotic cells more than either agent alone. The presence of cleaved poly (ADPRibose) polymerase (PARP) significantly increased in the combination treatment, whereas Bcl-2 is decreased. DIM alone decreased the activation of the Her2/neu receptor; the combination decreased the activation of ERK1/ERK2.

Conclusions. DIM in combination with paclitaxel synergistically inhibits growth of Her2/neu human breast cancer cells through G2M phase cell-cycle arrest and induction of apoptosis/necrosis. The Her2/neu receptor and its downstream signaling protein ERK1/2 appear to be involved in DIM's affect on cell growth and differentiation, whereas apoptosis appears to be mediated through the mitochondrial pathway (Bcl-2/ PARP). It appears DIM, a naturally occurring, nontoxic compound, may be a beneficial addition to a traditional (taxane-based) chemotherapy regimen.

Key Words: breast cancer; HER2/neu; 3,3′-diindolylmethane; paclitaxel; apoptosis.

INTRODUCTION

Women with breast tumors that are positive for the HER2/neu gene are difficult to treat [1, 2]. About 25 to 30% of all invasive breast tumors overexpress Her2/neu/erbB2 [1]. This gene increases the aggressiveness of breast cancer and resistance to chemotherapeutic drugs, including paclitaxel (Taxol) and other taxanes [2–5]. Chemotherapeutic resistance leads to a high reoccurrence rate and ultimately affects

long-term survival. Attempts to improve chemosensitization of the HER2/neu positive tumors to chemotherapeutic agents have met with limited success. Thus, there is a need to develop treatment strategies that will enhance the effectiveness of these drugs for this patient population.

Most tested strategies for improving chemotherapeutic effect involve combining traditional chemotherapeutic agents in varying dose strengths, time courses, and drug combinations. However, very rarely have non-traditional agents been used with more traditional chemotherapies to study their effect. Numerous studies have demonstrated the anti-cancer effects of food components such as quercetin, curcumin, and resveratrol [6]. 3,3′-diindolylmethane (DIM) is a naturally occurring active component of cruciferous vegetables that is formed from its precursor indole 3 carbinol (I3C). I3C and DIM have been shown to protect against many neoplasms, including prostate, cervical, and breast cancer [7–10]. In human breast cancer cells, I3C inhibits proliferation [11, 12], blocks cells in the G1 phase of the cell cycle [21], and induces apoptosis [13]. I3C and tamoxifen were found to act separately and/or cooperatively in inhibiting growth of estrogen receptor positive cells [14]. Recently, DIM was also shown to decrease proliferation of estrogen receptor-positive and estrogen receptor-negative breast cancer cells by inducing a G1 cell cycle arrest [15] and by stimulating apoptosis [16]. DIM induced apoptosis in breast cancer cells through the mitochrondrial pathway [17] by inhibiting Akt and nuclear factor-_B pathway [17–20].

Studies have shown that HER2/neu overexpression in breast cancer cells confers resistance to the mitotic effects of paclitaxel [21–24]. DIM induces cell death in breast cancer cells. However, it is not known whether DIM can overcome the chemoresistance of cancer cells. Therefore, we investigated whether DIM will enhance the cytotoxic effects of paclitaxel by through alterations in ERK1/ERK2 signaling. In this study, we used MDA-MB-435 human breast cancer cells transfected with HER2/neu/erbB2 (MDA-MB-435eB1) to determine the individual and combined effects of DIM and paclitaxel on cell growth, cell cycle, and apoptosis. We also investigated apoptotic pathways that may be involved in the interactions of DIM and paclitaxel.

MATERIALS AND METHODS

Cell Culture

Dr. Dihua Yu at the University of Texas M.D. Anderson Cancer Center (Houston, TX) kindly provided 435eb1 Her2/neu positive, estrogen/progesterone receptor negative human breast carcinoma cells. Cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS). Every 4 weeks the cells were passaged with media supplemented with G418 (800 ug/ml).

Materials

Bioresponse DIM was kindly provided by Dr. Michael Zeligs (BioResponse, LLC, Boulder, CO). Polyclonal antibody to BCL-2, bax, and PARP were obtained from Santa Cruz Biotechnology. Anti-ERK1/ERK2, and anti-phospho ERK1/ERK2 were obtained from Cell Signaling.

Cell Survival Assay

435eb1 cells were plated in 96-well plates and incubated in DMEM/F12 with 5% FBS for 24 h. Cells were then treated with 0, 5, 10, and 15 uM DIM for 0, 24, 48, and 72 h. The number of viable cells was determined with ProCheck cell viability assay.

Cell Cycle/Flow Cytometric Analysis

435eb1 cells were plated in T-175 flasks and incubated in DMEM/F12 with 5% FBS for 24 h. Cells were then treated with vehicle, 15 uM DIM, 10 nM Paclitaxel, and 15 uM DIM +10 nM Paclitaxel and incubated for an additional 72 h. Attached and floating cells were harvested, fixed in 70% ethanol, digested with RNase A, stained with propidium iodide, and analyzed by fluorescence-activated cell sorting using a Beckman Coulter XL-MCLTM flow cytometer with System II Software.

Apoptosis Assay

435eb1 cells were plated in T-175 flasks and incubated in DMEM/F12 with 5% FBS for 24 h. Cells were then treated with vehicle, 15 uM DIM, 10 nM Paclitaxel, and 15 uM DIM +10 nM Paclitaxel and incubated for an additional 72 h. Cells were then harvested and underwent TUNEL analysis using the ApopTag plus peroxidase *in situ* apoptosis detection kit.

Western Blot Analysis

435eb1 cells were plated in T-75 flasks and incubated in DMEM/F12 with 10% FBS for 24 h. Cells were then treated with vehicle, 15 uM DIM, 10 nM Paclitaxel, and 15 uM DIM +10 nM Paclitaxel and incubated for an additional 72 h. For determination of HER2/neu, phospho-HER2/neu, ERK1/2, and phospho-ERK1/2, cells were incubated in serum-free medium overnight and treated with DIM, paclitaxel, and the combination for an additional 24 h before stimulation with epidermal growth factor for 15 min. Cells were lysed and proteins extracted with cell lysis buffer (Cell Signaling). Proteins were separated with SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with %0.4 casein (I-Block, Tropix) in PBS-T and then incubated with primary antibodies overnight at 4°C. Blots were developed using a horseradish peroxidase-conjugated secondary antibody and proteins were visualized by enhanced chemiluminescence (ECL), scanned with a densitometer (Sharp JX-330), quantitated by Image Master 1D, and expressed as arbitrary units relative to the control (β-actin).

Statistical Analysis

Cell survival and apoptosis data were analyzed using simple linear regression (equivalent to two-way ANOVA) to compare the groups.

RESULTS

Effects of DIM on Cell Growth

DIM alone had both a time and concentration dependent effect on cell growth. After 72 h; 5 uM DIM decreased cell growth by 23% (SEM ±1.08%), 10 uM DIM decreased cell growth by 28% (+/- 1.31%), and 15 uM DIM decreased cell growth by 34% (±2.27%) in comparison to control ($P < 0.001$) (Fig. 1).

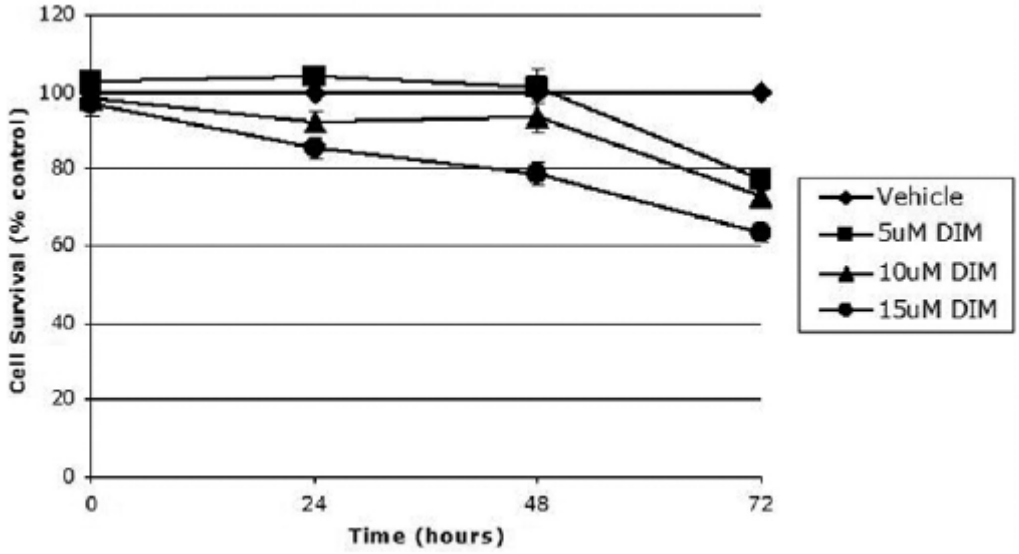

FIG. 1. Increasing concentrations of DIM inhibit cell growth in human breast carcinoma 435 eb1 cells (Her2/neu positive). Cells were plated in 96-well plates, incubated for 24 h and then treated with 0, 5, 10, and 15 uM DIM for 0, 24, 48, and 72 h. The number of viable cells was determined with ProCheck cell viability assay. Values represent mean ±SEM.

Effects of DIM and Paclitaxel on Cell Growth

DIM and paclitaxel together significantly decreased cell growth when compared to control or either treatment alone. After 72 h of incubation, cell growth was inhibited by 15 uM DIM, 10 nM paclitaxel and the combination of the two treatments by 42% (±3.26%), 62%(±1.12%), and 74% (±1.07%), respectively ($P < 0.001$) (Fig. 2).

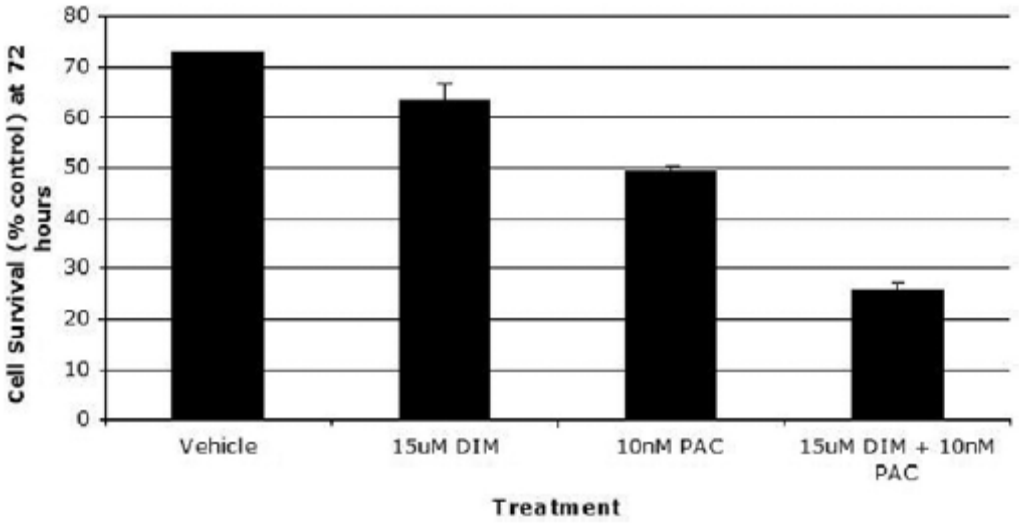

FIG. 2. DIM in combination with paclitaxel inhibits cell growth in human breast carcinoma 435 eb1 cells (Her2/neu positive). Cells were plated in 96-well plates, incubated for 24 h and then treated with vehicle, 10 uM DIM, 15 uM DIM, 10 nM paclitaxel, 10 uM DIM +10 nM paclitaxel, and 15 uM DIM +10 nM paclitaxel for 0, 24, 48, and 72 h. The number of viable cells was determined with ProCheck cell viability assay. Values represent mean ± SEM.

Effects of DIM and Paclitaxel on Cell Cycle

After treatment with 15 uM DIM and 10 nM paclitaxel in a starvation media (5% FBS), a small, but not significant increase was seen in the number of cells in the G2M phase of the cell cycle (Fig. 3).

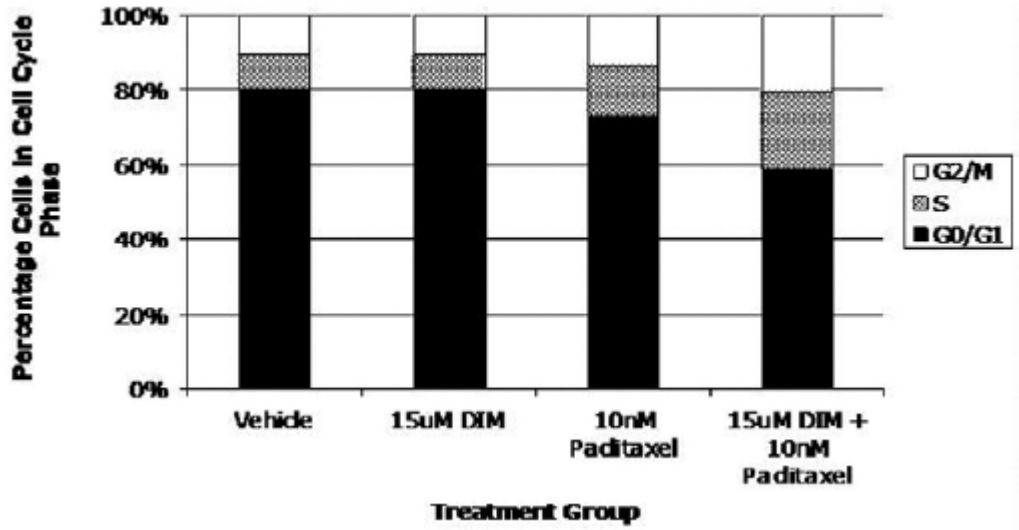

FIG. 3. DIM in combination with paclitaxel induces G2/M cell cycle phase arrest. Cells were plated in T-175 flasks, incubated for 24 h and then treated with vehicle, 15 uM DIM, 10 nM paclitaxel, and 15 uM DIM _10 nM paclitaxel and incubated for an additional 72 h. Cells were then harvested and underwent cell cycle analysis by flow cytometry.

Effects of DIM and Paclitaxel on Aapoptosis

The combination of DIM and paclitaxel synergistically increased apoptosis, detected by the TUNEL assay, a measure of DNA fragmentation. The combined treatment induced apoptosis in 34.8% of treated cells. The percentage of apoptotic cells was increased in 435 eb1 cells treated with DIM and paclitaxel compared with vehicle-treated cells (control 1.82%, DIM 6.59%, paclitaxel 7.39%) (Fig. 4).

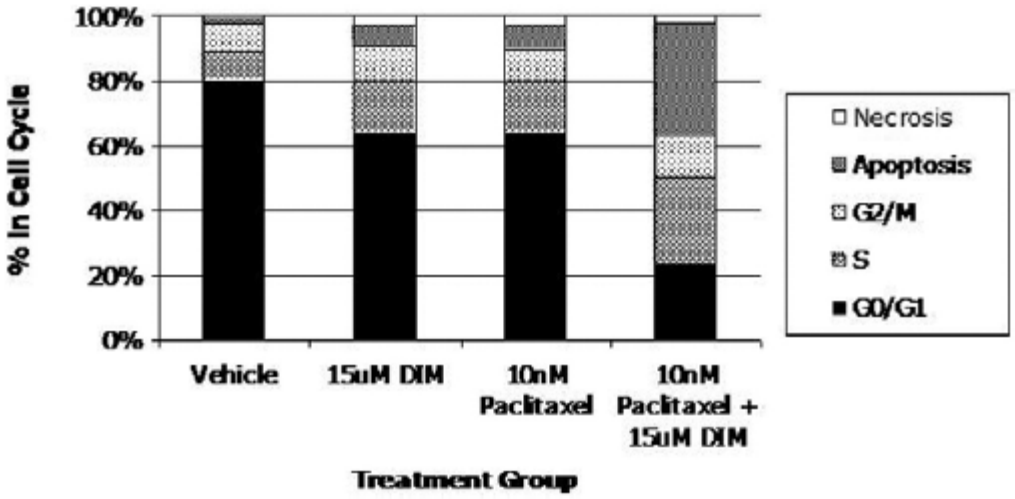

FIG. 4. DIM in combination with paclitaxel increases apoptosis. Cells were plated in T-175 flasks, incubated for 24 h and then treated with vehicle, 15 uM DIM, 10 nM paclitaxel, and 15 uM DIM +10 nM paclitaxel and incubated for an additional 72 h. Cells were then harvested and underwent TUNEL analysis.

Effects of DIM and Paclitaxel on Apoptosis-Related Protein Expression

Protein expression of cleaved poly (ADP-Ribose) polymerase (PARP), an early marker of apoptosis, was up regulated in cells treated with DIM and paclitaxel. Up-regulation was synergistically increased by the combination of the two compounds (Fig. 5A).

Expression of the protein, BCL-2, an inhibitor of apoptosis, was significantly reduced in samples treated with DIM and paclitaxel. Paclitaxel's effect on the proapoptotic protein, Bax, was not altered by the addition of DIM (Fig. 5B).

Effects of DIM and Paclitaxel on Phosphorylation of HER-2/Neu/EGFR and ERK1/2

DIM, paclitaxel, and the combined treatment decreased phosphorylation of the Her2/neu receptor (Fig. 6A). We have not measure whether HER3 and HER-4 are present in these cells. There does appear to be an additive/synergistic effect when the two agents are combined.

However, the protein expression of ERK1/2 and phosph-ERK1/2 decreased in cells treated with DIM and paclitaxel and paclitaxel alone. The combination super-additively inhibited the activation of ERK1/ERK2.

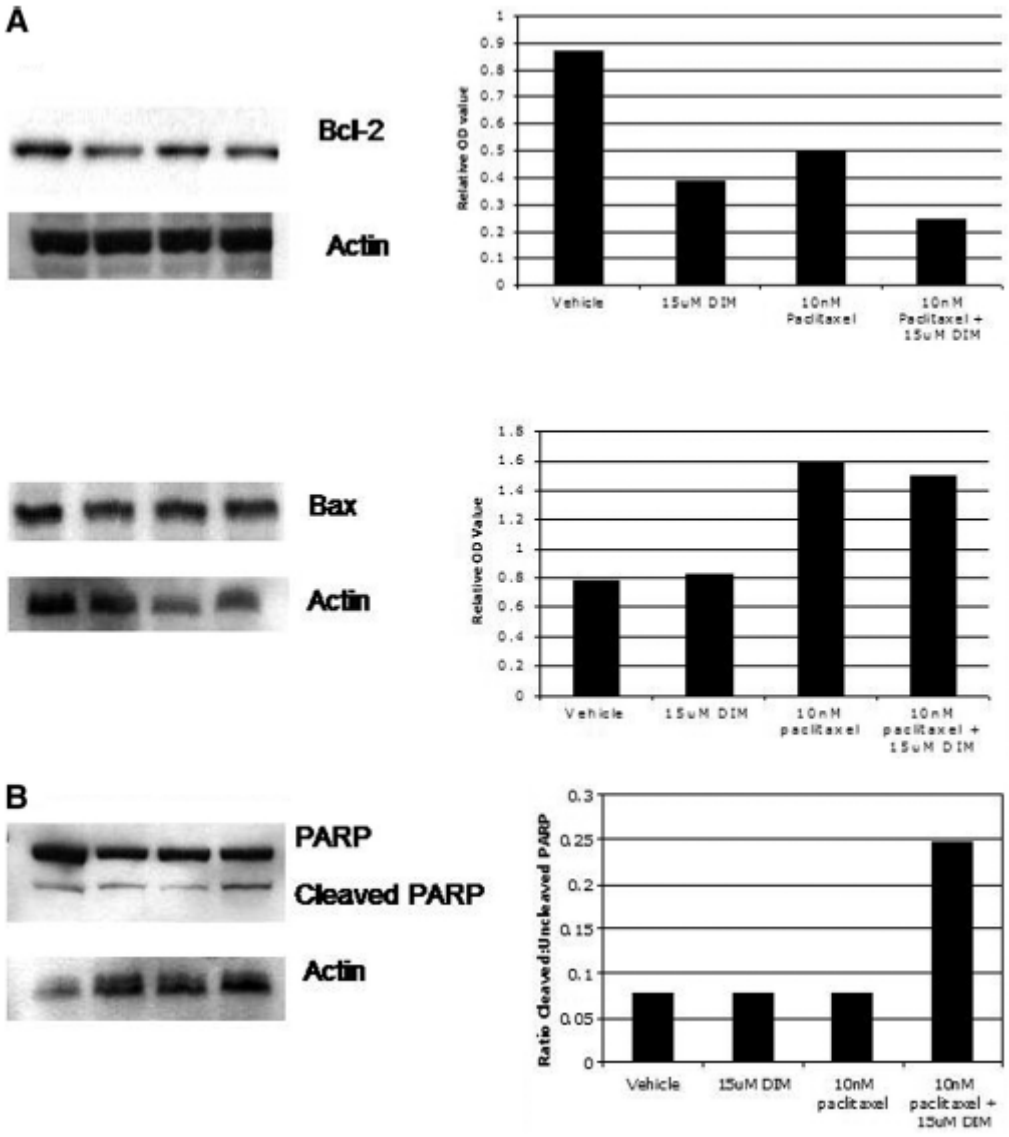

FIG. 5. DIM in combination with paclitaxel down-regulates BCL-2 expression. However, it appears to have little effect on Bax expression. PARP cleavage and activation is also increased by the treatments. Cells were plated in T-75 flasks, incubated for 24 h and then treated with vehicle, 15 uM DIM, 10 nM paclitaxel, and 15 uM DIM +10 nM paclitaxel and incubated for an additional 72 h. Cells were then lysed, the proteins were extracted, and Western blot analysis was performed.

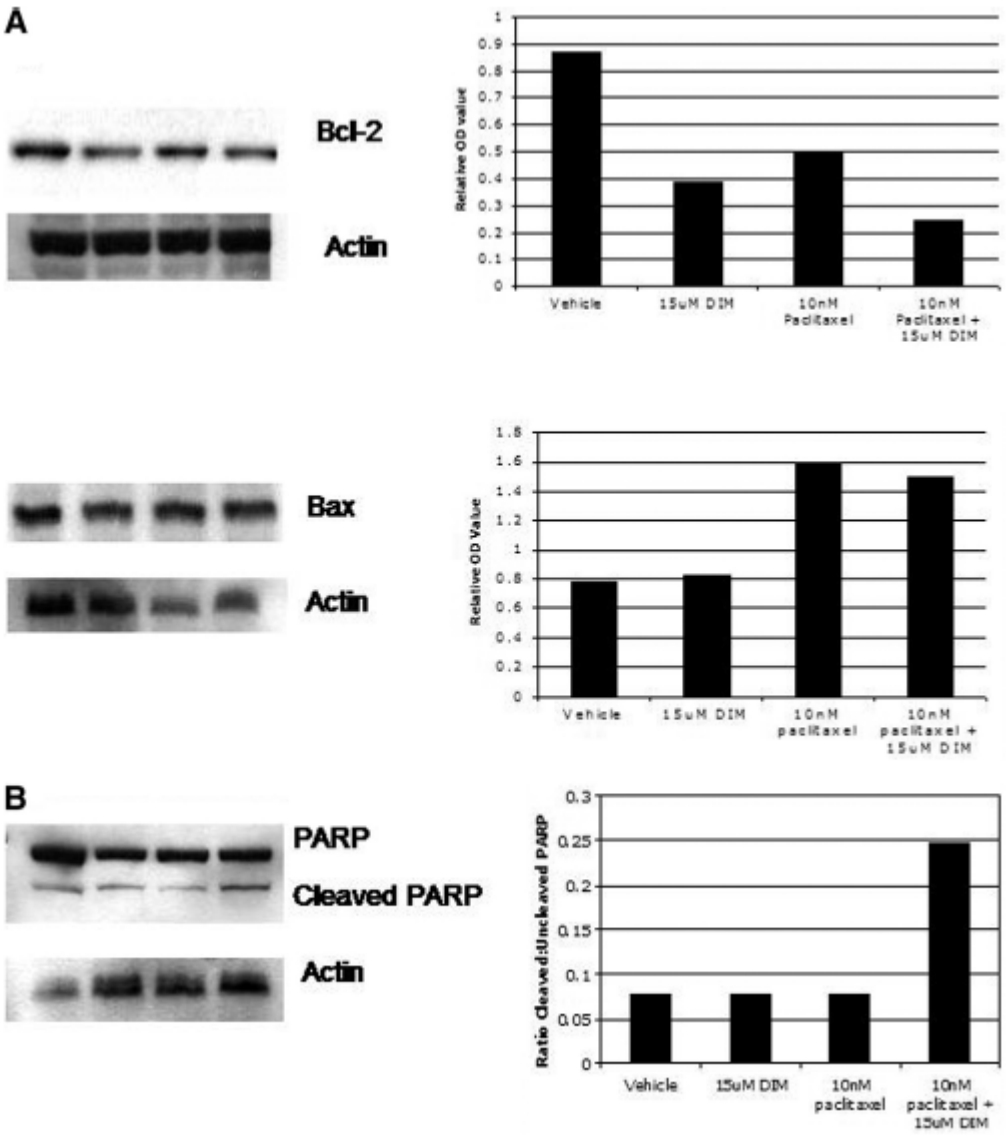

FIG. 6. DIM in combination with paclitaxel down-regulates Her2/neu and ERK1/ERK2 phosphorylation and activation. Cells were plated in T-75 flasks, incubated for 24 h in 5% (starving) media and then treated with vehicle, 15 uM DIM, 10 nM paclitaxel and 15 uM DIM +10 nM paclitaxel and incubated for an additional 24 h. Cells were then lysed, the proteins were extracted, and Western blot analysis was performed.

DISCUSSION

DIM and I3C have anticancer effects specifically through cell cycle arrest [7, 12, 14], apoptosis [17–19] as well as NF-κB, akt [18, 19], and Bax inhibition [17]. Paclitaxel's effects are mediated by inhibiting microtubule formation [3]. Each of these compounds are effective against breast cancer cells with clearly distinct mechanisms of action. Compounds similar to DIM, such as resveratrol have been shown to enhance the effects of paclitaxel on lung cancer cell lines [6]. In this study we demonstrate for the first time

that enhances the cytotoxicity of paclitaxel in a breast cancer cell line with overexpression of HER2/neu.

The combined treatments induced a greater apoptotic effect than either treatment alone. Several pathways leading to apoptosis converge at the mitochrondrial membrane; the breakdown of the mitochrondrial membrane is controlled, in part, by the balance of Bcl2 and Bax in cells. The protein expression of Bcl2 decreased in cells treated with DIM alone or the combination of DIM + paclitaxel, whereas there was no change in Bax protein expression. Thus, the ratio of Bax to Bcl2 was altered in favor of apoptosis. Studies using Her2/neu negative breast cancer cell lines have also shown an effect on the Bax/Bcl2 ratio by paclitaxel and DIM [25, 26]. Cleaved PARP, an early marker of apoptosis, is formed in significantly greater amounts with the combined treatment than with either treatment alone (Fig. 5A) [27]. Interestingly, we found no effect of DIM on Bax protein in these cells, an effect previously described in other cell lines [17]. However, the concentrations of DIM were as much as ten times higher than those used in our study.

Importantly, we observed that DIM decreased the phosphorylation of HER2. Because HER2/neu expression confers resistance to the taxanes, DIM may improve the response of the 435-eb cells to paclitaxel by down-regulating HER2. Studies have shown that combining paclitaxel with Herceptin improved the response of resistant HER2/neu overexpressing breast cancer cells to the taxanes [26, 27]. The observation that a nontoxic nutrient like DIM that is found in foods may block HER2 signaling may offer clinical advantages. Current therapeutic protocols use the taxanes in combination with Herceptin. However, Herceptin is associated with cardiotoxic effects [28] that may be minimized by use of DIM. Recent studies have demonstrated that DIM down-regulates the MAPK pathway [29]. In this study we demonstrated that ERK1/ERK2 is inhibited more by the combination therapy than with either treatment alone. This result suggests that paclitaxel may be acting on one of the upstream proteins in the ERK1/ERK2 pathway. In fact, DIM has been shown to down-regulate Ras signaling, which is upstream of MAPK [30]. It is often the case in cell culture experiments that high concentrations of the agent must be used to achieve cytoxic effects. In this study the concentration of DIM (15 _M) found to enhance the effectiveness of paclitaxel and alter signaling corresponds to the plasma level of humans taking dietary supplements [31].

In summary, DIM appears to significantly enhance the cytotoxicity of paclitaxel in the 435eb1 Her2/neu positive, ER/PR negative breast cancer cell line—a cell line known to be particularly resistant to taxane treatment both *in vitro* and *in vivo*. This effect is produced primarily through the up-regulation of apoptosis in the cells treated with a combination of DIM and paclitaxel. Further study into the mechanism of this effect is necessary to pinpoint the precise proteins regulated by these compounds and confirm the two distinct implicated in this study. *In vivo* studies will be necessary to confirm not only the effectiveness of this combination treatment, but also to evaluate any additional cytotoxicity against normal cells. These early studies do suggest that DIM may possibly be used clinically as a chemosensitizing agent with taxanes in treatment of traditionally taxane-resistant tumors [32,33].

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