

Concurrent Inhibition of NF- κ B, Cyclooxygenase-2, and Epidermal Growth Factor Receptor Leads to Greater Anti-Tumor Activity in Pancreatic Cancer

Shadan Ali,¹ Sanjeev Banerjee,² Jacqueline M. Schaffert,² Bassel F. El-Rayes,¹ Philip A. Philip,¹ and Fazlul H. Sarkar^{2*}

¹Division of Hematology/Oncology, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201

²Department of Pathology, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201

ABSTRACT

Inactivation of survival pathways such as NF- κ B, cyclooxygenase (COX-2), or epidermal growth factor receptor (EGFR) signaling individually may not be sufficient for the treatment of advanced pancreatic cancer (PC) as suggested by recent clinical trials. 3,3'-Diindolylmethane (B-DIM) is an inhibitor of NF- κ B and COX-2 and is a well-known chemopreventive agent. We hypothesized that the inhibition of NF- κ B and COX-2 by B-DIM concurrently with the inhibition of EGFR by erlotinib will potentiate the anti-tumor effects of cytotoxic drug gemcitabine, which has been tested both in vitro and in vivo. Inhibition of viable cells in seven PC cell lines treated with B-DIM, erlotinib, or gemcitabine alone or their combinations was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Significant inhibition in cell viability was observed in PC cells expressing high levels of COX-2, EGFR, and NF- κ B proteins. The observed inhibition was associated with an increase in apoptosis as assessed by ELISA. A significant down-regulation in the expression of COX-2, NF- κ B, and EGFR in BxPC-3, COLO-357, and HPAC cells was observed, suggesting that simultaneous targeting of EGFR, NF- κ B, and COX-2 is more effective than targeting either signaling pathway separately. Our in vitro results were further supported by in vivo studies showing that B-DIM in combination with erlotinib and gemcitabine was significantly more effective than individual agents. Based on our preclinical in vitro and in vivo results, we conclude that this multi-targeted combination could be developed for the treatment of PC patients whose tumors express high levels of COX-2, EGFR, and NF- κ B. *J. Cell. Biochem.* 110: 171–181, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: PANCREATIC CANCER; 3,3'-DIINDOLYLMETHANE; ERLOTINIB; GEMCITABINE; NF- κ B; COX-2

Pancreatic cancer (PC) is the fourth leading cause of cancer-related deaths in the United States with an annual mortality of approximately 34,000 and remains a drug resistant cancer [Jemal et al., 2009]. The prognosis of patients diagnosed with PC is very poor, which is mainly due to the difficulties in the early detection and presence of metastasis in the majority of patients at diagnosis. Although gemcitabine has at best modest activity in PC, it is considered the standard treatment for advanced disease for over a decade. Many attempts to improve the survival of patients with PC remain disappointing, suggesting newer treatment strategies must be developed.

In recent years, various approaches have been tested for targeting different survival and cell proliferation pathways such as epidermal growth factor receptor (EGFR) signaling that is known to be

associated with lower sensitivity to cytotoxic therapy [Dediu et al., 2007]. Activation of the EGFR signaling pathway also contributes to the chemo-resistance of PC cells. Randomized trials evaluating erlotinib a tyrosine kinase inhibitor of EGFR in patients with PC had disappointing results with only a modest survival advantage for the erlotinib/gemcitabine combination over gemcitabine alone [Moore et al., 2007]. The presence of multiple dysregulated signaling pathways could have contributed to the resistance to EGFR inhibitors; therefore, targeting multiple signaling pathways maybe required in order to make an impact on the killing of chemo-resistance cells for achieving better treatment outcome in patients diagnosed with PC.

In addition to the aberrant activation of EGFR signaling in PC, there are several other key genes that are known to be important in

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*Correspondence to: Prof. Fazlul H. Sarkar, PhD, Department of Pathology, Karmanos Cancer Institute, Wayne State University School of Medicine, 740 Hudson Webber Cancer Research Center, 4100 John R. Street, Detroit, MI 48201.

E-mail: fsarkar@med.wayne.edu

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drug-resistant PC cells. Among many of those genes, the nuclear factor kappa beta (NF- κ B) is a key regulator of genes that are involved in cell survival, proliferation, and inhibition of apoptosis and is commonly dysregulated in PC cells. In addition to EGFR-dependent activation of NF- κ B, constitutive activation of the NF- κ B has been reported in PC [Niu et al., 2004]. Over-expression of cyclooxygenase-2 (COX-2), a transcriptional downstream target of NF- κ B that has been linked with chronic inflammation and chemoresistance, further suggests that targeting NF- κ B could be a novel approach for the clinical management and treatment of human PC [Colby et al., 2008]. The COX-2 generated PGE₂ also plays important roles during pancreatic tumorigenesis and clearly suggests that enzymes involved in the PGE₂ production may be potential targets for the treatment of PC [Hasan et al., 2008]. In preclinical models, celecoxib, a COX-2 inhibitor, has been shown to sensitize PC cells to the pro-apoptotic effects of conventional chemotherapeutic agent such as gemcitabine [El-Rayes et al., 2004]. In addition to the role of the COX-2 and NF- κ B in chemo-resistance, preclinical and clinical studies suggest a role for these pathways in the toxicity observed with chemotherapeutic agents. For example, both NF- κ B and COX-2 showed significant increase in oral mucosal staining in patient with chemotherapy induced mucositis [Logan et al., 2007], suggesting that the chemotherapeutic agents mediated induction of these signaling pathways will not only cause adverse side effects but will also contribute to the reduction in drug sensitivity and acquired drug resistance. Therefore, collectively, EGFR, COX-2, and NF- κ B are considered important targets for developing novel therapies for PC because of their over-expression and/or activation in PC. Moreover, these pathways with their feedback loop maintains the persistent activation of NF- κ B and COX-2, leading to the production of PGE₂ that inhibits apoptosis [Pai et al., 2002; Ali et al., 2008], suggesting that combinatorial approach using novel agents targeting these pathways could be useful for the treatment of human PC.

Previous studies have shown that 3,3'-diindolylmethane (DIM) is a major acid condensation product of indole-3-carbinol (I3C) found in most cruciferous vegetable, which has been shown to possess anti-carcinogenic effects in experimental animals [Tiwari et al., 1994; Hong et al., 2002]. Recent published results further demonstrated that DIM could inhibit cell growth, invasion, and angiogenesis of PC cells both in vitro and in vivo [Ali et al., 2008]. The anti-proliferative effect of B-DIM, a formulated DIM, is in part mediated through the inhibition of the NF- κ B pathway [Rahman et al., 2007; Ali et al., 2008], suggesting that this non-toxic agent could be useful for the inactivation of NF- κ B signaling toward the treatment of human PC. Since the crosstalk between the EGFR, NF- κ B, and COX-2 pathways has been reported for PC, inhibition of these signaling pathways may require simultaneous targeting of EGFR, NF- κ B, and COX-2, which will constitute a novel approach for the successful treatment of PC. Therefore, we hypothesize that a combination of an inhibitor of NF- κ B (B-DIM), which in turn will inhibit COX-2, and EGFR inhibitor (erlotinib) would be more effective in sensitizing PC cells to the cytotoxic effects of gemcitabine than either agents alone in vitro, and will likely contribute to a greater anti-tumor activity in animal model in vivo.

MATERIALS AND METHODS

CELL CULTURE, DRUGS, AND REAGENTS

Seven human PC cell lines such as AsPC-1, BxPC-3, COLO-357, L3.6pl, HPAC, MIA PaCa-2, and PANC-1 were randomly chosen for this study. Cells were grown as a monolayer cell culture in DMEM containing 4.5 mg/ml D-glucose and L-glutamine supplemented with 10% fetal bovine serum. The cell lines have been tested and authenticated in core facility Applied Genomics Technology Center at Wayne State University on March 13, 2009. The method used for testing was short tandem repeat (STR) profiling using the PowerPlex[®] 16 System from Promega (Madison, WI). B-DIM and erlotinib were obtained through generous gifts from Dr. Michael Zeligs (BioResponse, LLC, Boulder, CO), and OSI Pharmaceuticals (Melville, NY), respectively. Gemcitabine (Eli Lilly, Indianapolis, IN) was purchased from our institutional pharmacy (Karmanos Cancer Institute, MI) and was dissolved in water to make a 100 μ M/L stock solution. The antibodies were purchased from following sources: p65 (Santa Cruz Biotechnology), EGFR (Neo Markers, Fremont, CA), pEGFR (Cell Signaling Technology, MA), COX-2 (Cayman Chemicals, Ann Arbor, MI), β -actin (Sigma Chemicals, St. Louis, MO).

CELL VIABILITY ASSAY

The effects of individual agents and double and triple combination on all seven cell lines were determined by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 72 h and reproducibility was confirmed in three independent experiments. To test the viability of cells treated with B-DIM, erlotinib, gemcitabine, or in combination cells were plated (3,000–5,000/well) in a 96-well plate and incubated overnight at 37°C. Based on our previous studies, we chose the concentration of B-DIM as 25 μ M, erlotinib as 2 μ M, and gemcitabine as 10 nM for all assays. The color intensity was measured by TECAN's microplate fluorometer (TECAN, Research Triangle Park, NC) at 595 nm. DMSO-treated cells were considered to be the untreated control and assigned a value of 100%. Combination index and Isobologram for combination treatment in BxPC-3, COLO-357, and HPAC cells were calculated and plotted using CalcuSyn software (Biosoft, Cambridge, United Kingdom). In addition to the above assay, we have also done clonogenic assay for assessing the effects of treatment as demonstrated below.

CLONOGENIC ASSAY

To test the survival of cells treated with B-DIM, erlotinib, gemcitabine, or in combinations, BxPC-3, COLO-357, and HPAC cells were plated (50,000–100,000/well) in a six-well plate and incubated overnight at 37°C. After 72 h exposure to 25 μ M of B-DIM, 2 μ M of erlotinib, 10 nM of gemcitabine, and the combinations, the cells were trypsinized, and the viable cells were counted (trypan blue exclusion) and plated in 100 mm Petri dishes in a range of 100–1,000 cells to determine the plating efficiency as well as assessing the effects of treatment on clonogenic survival. The cells were then incubated for about 10–12 days at 37°C in a 5% CO₂/5% O₂/90% N₂ incubator. The colonies were stained with 2% crystal violet.

QUANTIFICATION OF APOPTOSIS BY ELISA

Based on the results of MTT assay, three cell lines were chosen for apoptosis assays. The Cell Death Detection ELISA kit (Roche Applied Science, Indianapolis, IN) was used to detect apoptosis in untreated and treated BxPC-3, COLO-357, and HPAC cells. Cells were seeded in six-well plates and treated with B-DIM (25 μ M), erlotinib (2 μ M), gemcitabine (10 nM), or the double and triple combinations. The treated cells were trypsinized and approximately 10,000 cells were used for apoptosis assays as described earlier [El-Rayes et al., 2004]. TECAN's microplate fluorometer (TECAN) was used to measure the color intensity at 405 nm. The experiment was repeated three times.

PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

The expression of the COX-2, EGFR, and pEGFR proteins was determined in untreated AsPC-1, BxPC-3, COLO-357, L3.6pl, HPAC, MIAPaCa, and PANC-1 cells. Subsequently only three cell lines were selected BxPC-3, COLO-357, and HPAC for further study. The cells were treated with B-DIM (25 μ M), erlotinib (2 μ M), gemcitabine (10 nM), or the combination for 72 h to evaluate the effects of treatment on COX-2, EGFR, EGFR-p-Tyr (1173), NF- κ B, and β -actin expression levels. Cells were harvested as described previously [El-Rayes et al., 2004]. The samples were loaded on 7–10% SDS-PAGE for the separation of proteins and electrophoretically transferred to a nitrocellulose membrane. Each membrane was incubated with monoclonal antibodies against COX-2, EGFR, EGFR-p-Tyr-1173, NF- κ B, and β -actin and the blots were incubated with secondary antibodies conjugated with peroxidase. The signal intensity was then measured using chemiluminescent detection system (Pierce, Rockford, IL).

ANIMAL EXPERIMENTS

Female CB17 SCID mice (about 4 weeks old) were purchased from Taconic Farms (Germantown, NY) and fed Lab Diet 5021 (Purina Mills, Inc., Richmond, IN). We conducted the animal studies using paired isogenic human PC cell lines with differences in metastatic behavior (L3.6pl and COLO-357 cells). Tumors were developed in SCID mice bilaterally by injecting subcutaneously 50,000–100,000 cells/animal suspended in PBS. Mice were randomized into the following treatment groups ($n = 7$ per group): (1) untreated control; (2) B-DIM (3.5 mg/mice/day), intragastric once daily for 12 days; (3) erlotinib (1 mg/mice/day), daily intraperitoneally for 12 days; (4) gemcitabine (1 mg/mice/day), intravenous every third day for a total of three doses; (5) B-DIM and erlotinib; (6) B-DIM and gemcitabine; (7) erlotinib and gemcitabine; (8) B-DIM, erlotinib, and gemcitabine. Tumor measurements were performed at multiple time points during the course of treatment and observation periods. Tumor weights were calculated as described earlier [Mohammad et al., 2007]. Mice carrying L3.6pl tumors were euthanized after 3 days of the last dose of treatment and their body weights were determined. Tissue was rapidly frozen in liquid nitrogen and stored at -70°C to measure the DNA binding activity of NF- κ B by EMSA. For the second set of experiment containing isogenic paired COLO-357 tumors, two mice from each group were euthanized after last dose of treatment and their tumors were frozen for the analysis of DNA binding activity of NF- κ B by EMSA and COX-2 and EGFR protein by Western blot analysis. The remaining mice in each group

were euthanized as weight approached 1,500 mg, and the tumors were stored at -70°C for future analysis. The \log_{10} kill and the activity scores were calculated as described earlier [Mohammad et al., 2007].

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA) FOR ASSESSING THE DNA BINDING ACTIVITY OF NF- κ B

Nuclear extracts were prepared from all seven PC cell lines that were untreated and from tumors removed from mice 3 days after the last treatment. Cells and minced tumor tissue were homogenized using a Dounce homogenizer (Kontes Co., Vineland, NJ) in 400 μ l of ice-cold lysis buffer as described earlier [El-Rayes et al., 2004]. EMSA was performed using the Odyssey Infrared Imaging System with NF- κ B IRDye labeled oligonucleotide from LI-COR, Inc. (Lincoln, NE). The DNA binding reaction was set up using either 5 μ g (cells) or 10 μ g (tumor tissue) of the nuclear extract as described earlier [El-Rayes et al., 2004]. The samples were loaded and run at 30 mA for 1 h. The gel was scanned using Odyssey Infrared Imaging System (LI-COR, Inc.).

STATISTICAL METHODS

Comparisons of treatment outcome were tested for statistical difference by the paired *t*-test. Statistical significance was assumed at a *P*-value of <0.05 .

RESULTS

EFFECTS OF B-DIM, ERLOTINIB, AND GEMCITABINE ON THE VIABILITY OF PC CELLS

The viability of AsPC-1, BxPC-3, COLO-357, L3.6pl, HPAC, MIA PaCa-2, and PANC-1 PC cells treated with B-DIM (25 μ M), erlotinib (2 μ M), gemcitabine (10 nM) or the combinations were determined by the MTT assay (Fig. 1). In the BxPC-3, COLO-357, and HPAC cell lines, a significant potentiation in the inhibition of cell viability was observed by double or triple combinations compared to single agents (Fig. 1). A trend in favor of potentiation of growth inhibition of erlotinib by B-DIM and gemcitabine was observed in the L3.6pl cell lines. No potentiation was observed with double or triple combination in AsPC-1, MIA PaCa-2, or PANC-1 cell lines. Because of this variability in cell viability by B-DIM, erlotinib, and gemcitabine combination treatments in seven PC cell lines, we elected to determine the basal level of expression of COX-2, EGFR, and NF- κ B in all seven PC cell lines by Western blot analysis, and by assessing the DNA binding activity of NF- κ B.

EGFR AND COX-2 SIGNALING PATHWAYS IN HUMAN PANCREATIC CANCER CELLS

Baseline expression and activation levels of EGFR and COX-2 signaling proteins was determined in a panel of seven human PC cell lines that included AsPC-1, BxPC-3, COLO-357, L3.6pl, HPAC, MIAPaCa, and PANC-1. In BxPC-3, COLO-357, and HPAC cells, we found a significant reduction in cell viability when treated by the triple combination and in those cells we found higher expression of all three targets (COX-2, EGFR, and NF- κ B). Alternatively, the cells that had no potentiation to the triple combination lacked the expression of one or two proteins out of the three targets (Fig. 2A).

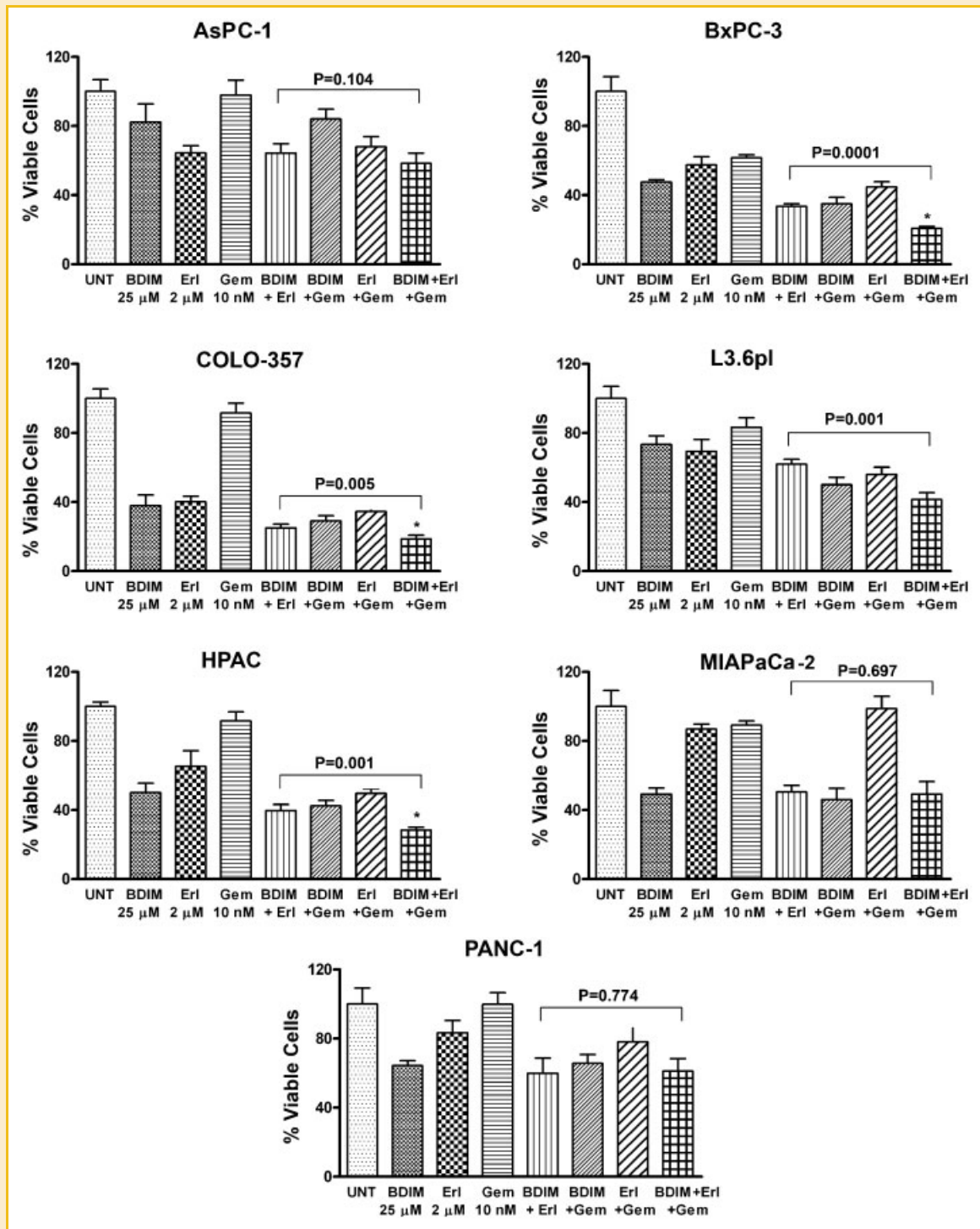


Fig. 1. Cell viability of human pancreatic cancer (PC) cell lines treated with B-DIM, erlotinib (Erl), gemcitabine (Gem), and the combination evaluated by the MTT assay. AsPC-1, BxPC-3, COLO-357, L3.6pl, HPAC, MIA PaCa-2, and PANC-1 cells were treated with B-DIM (25 μM), erlotinib (2 μM), gemcitabine (10 nM), and the combination. There was a significant reduction in cell growth in the BxPC-3, COLO-357, and HPAC cells treated with B-DIM in combination with either erlotinib or gemcitabine or triple combination compared to cells treated with single agents. A trend for potentiation of growth inhibition by B-DIM was observed in L3.6pl cells when combined with erlotinib or gemcitabine, but no such potentiation was observed in AsPC-1, MIA PaCa-2, and PANC-1 cells.

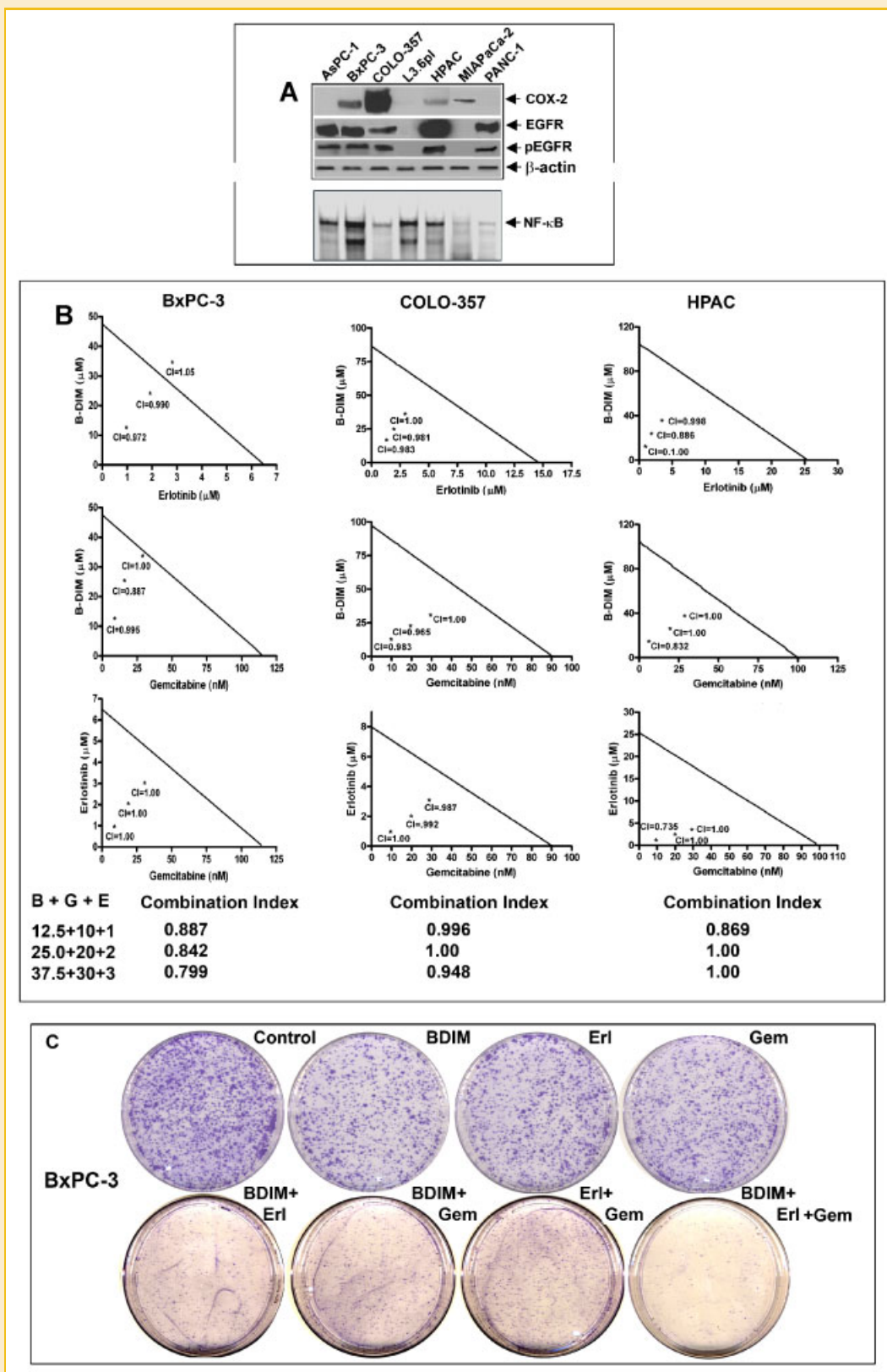


Fig. 2. The level of COX-2, EGFR, pEGFR, and NF- κ B activation was compared between a panel of seven pancreatic cancer cell lines (A). Expression of protein was assayed by Western blot analysis (upper panel), NF- κ B activation was evaluated by the EMSA (lower panel). Isobologram plots for combination treatments with B-DIM (12.5, 25, and 37.5 μ M), erlotinib (1, 2, and 3 μ M), and gemcitabine (10, 20, and 30 nM) in BxPC-3, COLO-357, and HPAC cell line was evaluated by the MTT assay. CI, combination index (B). Cell survival of BxPC-3, cells treated with B-DIM (25 μ M), erlotinib (2 μ M), gemcitabine (10 nM), and the combinations were evaluated by the clonogenic assay (C). Photo micrographic differences in colony formation in BxPC-3 cell line untreated and treated are shown. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Based on these results, the BxPC-3, COLO-357, and HPAC cells were further used to evaluate the mechanism of the observed growth inhibition.

ISOBOLOGRAM ANALYSIS

Analysis of individual drug and combination treatment of BxPC-3, COLO-357, and HPAC cells showed that the combination index for all the combination treatment was less than 1.00 (Fig. 2B), suggesting the synergistic effect of each combination treatment. In addition, we have also tested the effects of treatment on cell viability by clonogenic assay as shown below.

INHIBITION OF CELL GROWTH/SURVIVAL BY CLONOGENIC ASSAY

To determine the effect of B-DIM, erlotinib, and gemcitabine on cell growth, cells were treated with each of the single agents or their combination and assessed for cell viability by clonogenic assay. The combination of treatment resulted in a significant inhibition of colony formation in BxPC-3 cells when compared to either agent alone (Fig. 2C). Similar results were obtained with COLO-357 and HPAC (data not shown). Overall, the results from clonogenic assay were consistent with the MTT data as shown in Figure 1. The mechanisms of such differences were further investigated, and the results are presented in the following sections preceding our observations on the effects of B-DIM, erlotinib, gemcitabine, and the combination on apoptotic cell death.

INDUCTION OF APOPTOSIS BY B-DIM, ERLOTINIB, GEMCITABINE, AND IN COMBINATIONS

Apoptosis assays were performed using BxPC-3, COLO-357, and HPAC cell lines to determine the mechanism of the observed cell growth inhibition. The effects of B-DIM (25 μ M), erlotinib (2 μ M), and gemcitabine (10 nM) individually and in double and triple combinations were tested using the ELISA for assessing apoptosis. Exposure of BxPC-3, COLO-357, and HPAC cells to B-DIM, erlotinib, or gemcitabine for 72 h significantly enhanced apoptosis (Fig. 3). The addition of B-DIM to erlotinib and/or gemcitabine in a double and triple combination further increased apoptosis in all three cell lines. These results are consistent with cell viability MTT assay, suggesting that potentiation in overall cell growth inhibition by the combination could in part be due to the induction of apoptosis.

THE EFFECTS OF B-DIM, ERLOTINIB, GEMCITABINE, AND THE COMBINATION ON THE COX-2, EGFR, AND NF- κ B SIGNALING PATHWAYS

The effects of the drugs on COX-2 and NF- κ B in the context of EGFR inhibition were determined in the BxPC-3, COLO-357, and HPAC cells (Fig. 4). Down-regulation of EGFR and its phosphorylation by erlotinib together with inactivation of COX-2 and NF- κ B by B-DIM potentiated gemcitabine effects in all three cell lines tested (Fig. 4). We found that the combination treatment down-regulated COX-2 and EGFR proteins through inhibition of NF- κ B. This shows that NF- κ B is involved in the transcriptional regulation of the expression of COX-2 and EGFR in all the PC cell lines that have activated levels of COX-2, EGFR, and NF- κ B.

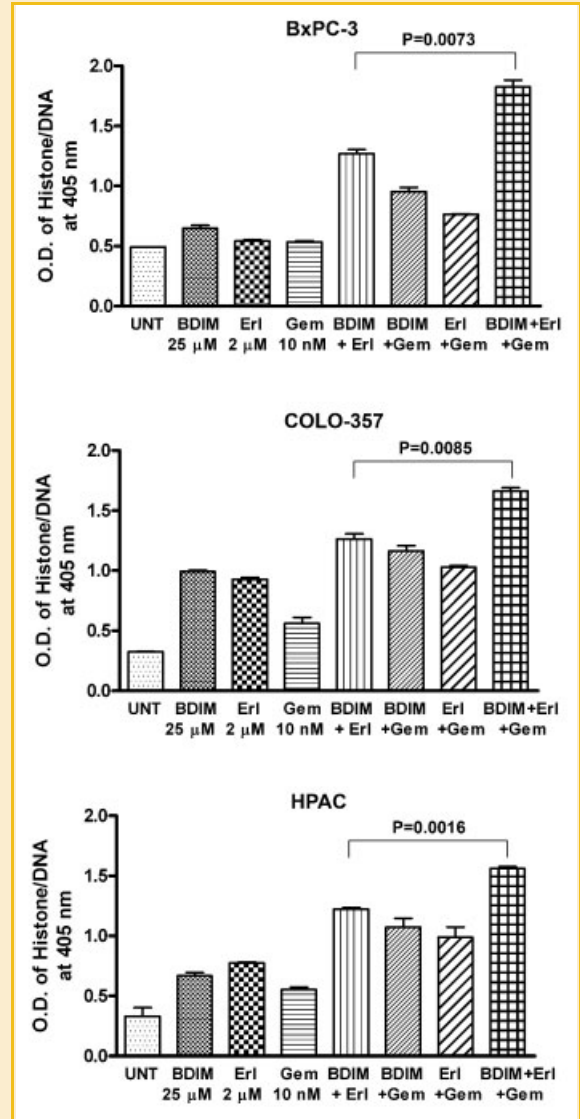


Fig. 3. Induction of apoptosis in PC cell lines treated with B-DIM, erlotinib (Erl), gemcitabine (Gem), and the combination, which was evaluated by the ELISA assay. Cells were treated with 25 μ M B-DIM, 2 μ M erlotinib, 10 nM gemcitabine, or the combination. There was a significant potentiation in the induction of apoptosis in BxPC-3, COLO-357, and HPAC cells treated with B-DIM in combination with erlotinib or gemcitabine and triple combination as compared to cells treated with either agent alone.

THE EFFECT OF B-DIM, ERLOTINIB, AND GEMCITABINE ON PANCREATIC TUMOR GROWTH IN VIVO

To determine whether B-DIM in combination with erlotinib and/or gemcitabine could result in greater inhibition of tumor growth in animals, a subcutaneous xenograft tumors induced by L3.6pl and COLO-357 human PC cells in CB17 SCID mice was developed. B-DIM treatment in combination with erlotinib and/or gemcitabine significantly inhibited tumor growth in L3.6pl (Fig. 5A) and to a greater extent in COLO-357 cells (Fig. 5B) compared with either untreated controls or those treated with a single drug. The mice did not show any weight loss during the treatment period (16–24 days) suggesting that no major adverse effects occurred. However, mice with L3.6pl tumor had to be

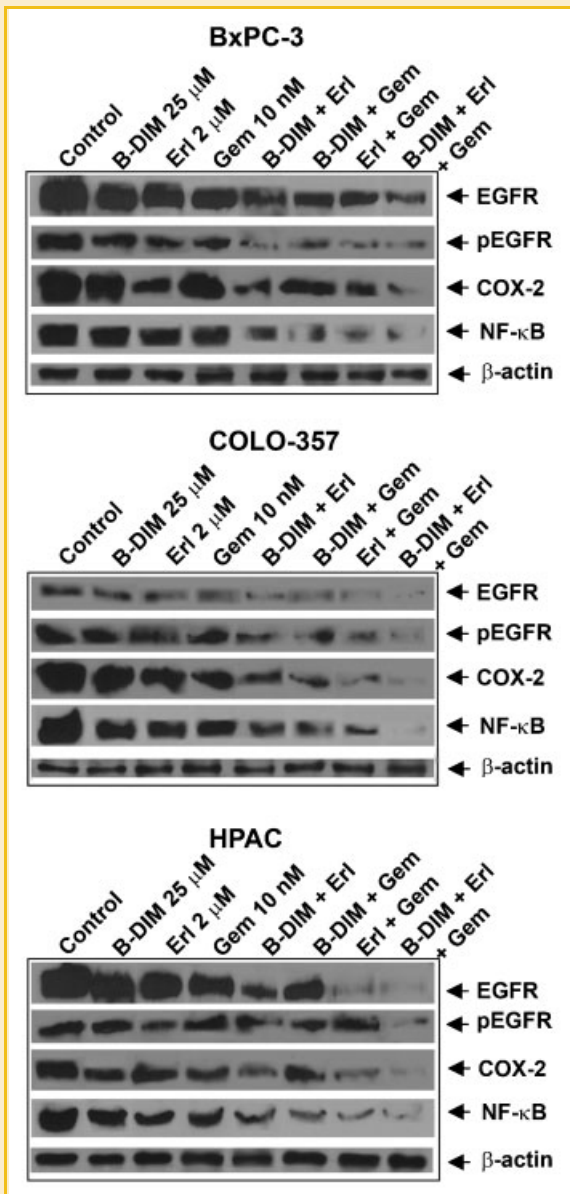


Fig. 4. The expression of EGFR, EGFR-p-tyrosine, COX-2, and NF-κB by Western blot analysis. BxPC-3, COLO-357, and HPAC human pancreatic cell lines were treated with B-DIM (25 μM), erlotinib (2 μM), gemcitabine (10 nM), or the combination. Significant down-regulation of all proteins was observed in cells treated with the double and triple combinations as compared to cells treated with either agent alone.

ethanized earlier than the mice with COLO-357 tumor because the tumor size reached the 1,500 mg target size. There was a significant decrease in tumor weight of L3.6pl tumors in drug combination treated groups containing gemcitabine but not in other combination groups (Fig. 5C). However, significant decrease in tumor weight was seen in COLO-357 bearing mice treated with any of the combinations (Fig. 5D).

THE EFFECTS OF B-DIM, ERLOTINIB, AND GEMCITABINE ON NF-κB ACTIVATION IN VIVO

NF-κB activation was determined in the B-DIM, erlotinib, and gemcitabine-treated tumor tissues derived from L3.6pl and

COLO-357 PC cells. The specificity of the band was confirmed by performing a supershift assay with anti-p65 antibody treatment (lane-1; Fig. 6A,B). B-DIM and erlotinib as single agent down-regulated NF-κB activation whereas gemcitabine activated NF-κB in both tumors. However, double and triple combination treatment in L3.6pl tissue (Fig. 6A; upper panel and the densitometric quantitation in lower panel) showed a slight decrease in NF-κB level and the decrease was more pronounced in COLO-357-derived tumor tissues (Fig. 6B; upper panel and the densitometric quantitation in lower panel). These *in vivo* results were similar to our *in vitro* results in COLO-357 cells, suggesting that the treatment with B-DIM and erlotinib abrogated the gemcitabine-induced NF-κB activation, further suggesting that the inactivation of NF-κB as one of the molecular mechanisms by which anti-tumor activity was observed in our animal model.

Since there are no detectable basal levels of COX-2 or EGFR in L3.6pl cells, we did not perform Western blot analysis (Fig. 2); however, in COLO-357 tumor tissues, the total protein was extracted, loaded on 7–10% gel, and COX-2 and EGFR expression was determined by Western blot analysis. A significant down-regulation in the expression of COX-2 and EGFR was observed in both the double and triple combination groups as compared to untreated and individual drug treatment groups (Fig. 6C).

DETERMINATION OF EFFICACY IN COLO-357 IN *sc* TUMOR MODEL

Anti-tumor activity of B-DIM, erlotinib, gemcitabine, and their combinations were also determined in COLO-357-derived tumors in mice as deduced by T/C, T-C, and log₁₀ as shown in Table I. Triple combination treatment group showed log₁₀ reduction of 4.1. Tumor response as determined by T/C values were consistent with *in vitro* findings showing that the double and triple combination of drug treatment tend to be more efficacious in tumors or cell lines that had modest levels of COX-2, EGFR, and NF-κB.

DISCUSSION

PC cells have multiple complex genetic alterations; hence targeting single signaling pathway produces only a modest clinical benefit as demonstrated by recent clinical trials with erlotinib. EGFR, NF-κB, and COX-2 are frequently over-expressed in various malignancies including PC [Ali et al., 2005; Fukata et al., 2007; Kourelis et al., 2008; Bergmann et al., 2009]. Activation of the EGFR can promote tumor growth, invasiveness, and chemo-resistance in PC [Woodburn, 1999], and has often been linked with poor survival in various malignancies including PC [Dong et al., 1998; Papouchado et al., 2005]. Moreover, over-expression and activation of the COX-2 and NF-κB pathways through EGFR-independent pathways is common in PC cells and contributes to the observed resistance of PC cells to chemotherapeutic agents. A rational approach for effective inhibition of cell growth would be to combine EGFR blockers with agents that inhibit the activation of NF-κB and COX-2. To that end, curcumin, a natural dietary chemopreventive agent with multi-targeted effects, when combined with gemcitabine showed increased inhibitory effects in cell lines that had high expression levels of COX-2 which was associated with the inhibition of Erk1/2 signaling

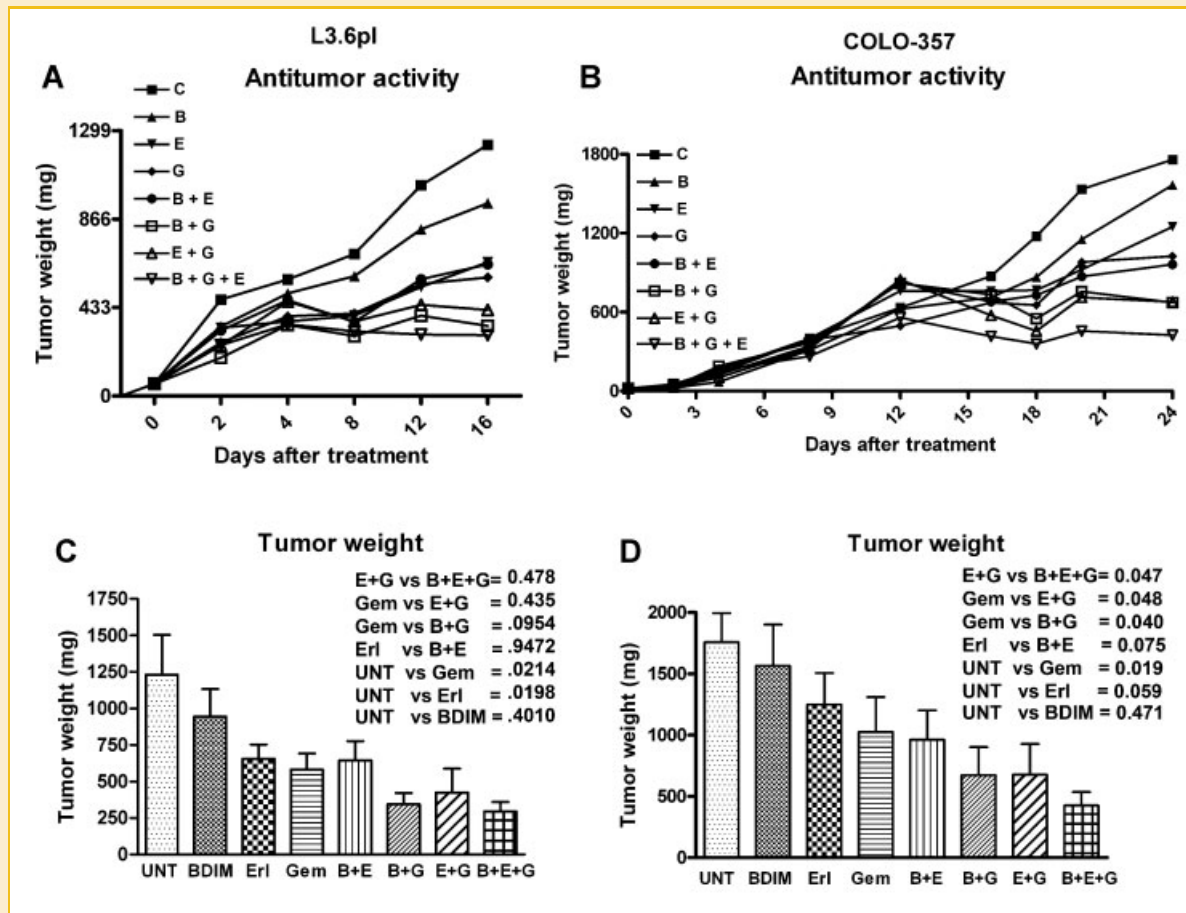


Fig. 5. Anti-tumor activity in L3.6pl (A) and COLO-357 (B) cells derived tumors. Changes in tumor weight showing efficacy of B-DIM, erlotinib, and gemcitabine combination treatment in L3.6pl (C) and COLO-357 (D) cells derived tumors, respectively.

in both pancreatic and lung adenocarcinomas [Lev-Ari et al., 2006, 2007]. Similarly, the growth inhibitory effect of B-DIM, another potent natural chemopreventive agent, was partly mediated through the inhibition of Akt and NF- κ B signaling [Ali et al., 2008]. Since simultaneous targeting of the EGFR, NF- κ B, and COX-2 may represent a more effective approach for sensitizing PC to the effects of gemcitabine, we evaluated the combination of B-DIM, erlotinib, and gemcitabine in PC cells in vitro and in animal model in vivo.

Since the molecular aberrations in PC are heterogeneous, our first aim was to determine the molecular profile of the PC cell lines that show potentiation of growth inhibition with the combination of B-DIM, erlotinib, and gemcitabine. Accordingly, we noticed only cell lines with over-activation/over-expression of COX-2, NF- κ B, and EGFR (BxPC-3, COLO-357, and HPAC) demonstrated potentiation of growth inhibition by the triple combination. In contrast, there was no such potentiation in AsPC-1, MIA PaCa-2, or PANC-1 cell lines. This novel observation suggests the role of all three signaling pathways in potentiating the combined effects of erlotinib and gemcitabine by B-DIM. Furthermore, our data provide support as a platform for future clinical trials evaluating B-DIM, erlotinib, and gemcitabine combination based on assessing the activation of these pathways in the tumor samples prior to the enrolment of

patients into such clinical trials, which would be a rational approach toward customize therapy.

Our observation on potentiation of growth inhibition was consistent with the induction of apoptosis as confirmed by ELISA. At the molecular level, the triple combination of B-DIM, erlotinib, and gemcitabine resulted in the down-regulation of EGFR, phospho-EGFR, NF- κ B, and COX-2 expression. Although erlotinib is known to inhibit EGFR phosphorylation; interestingly the inhibition of EGFR phosphorylation by erlotinib was further potentiated by the addition of B-DIM and gemcitabine. This observation maybe explained by the inhibition of the expression of EGFR protein by the triple combination, and thus the observed effect could be mechanistically linked with inactivation of NF- κ B. NF- κ B plays a central role in the transcriptional regulation of several genes including COX-2 and EGFR. Similarly, B-DIM is known to inhibit NF- κ B activation, which is consistent with our findings showing that the inhibition of NF- κ B by B-DIM results in the potentiation of the combined effect of erlotinib and gemcitabine. This observation may be related to the crosstalk between the EGFR and Akt/NF- κ B activation. These molecular findings lend support in favor of simultaneous targeting of all three pathways for the effective killing of PC cells compared to targeting each pathway separately. The inhibition of COX-2 expression mediated via the inhibition of EGFR

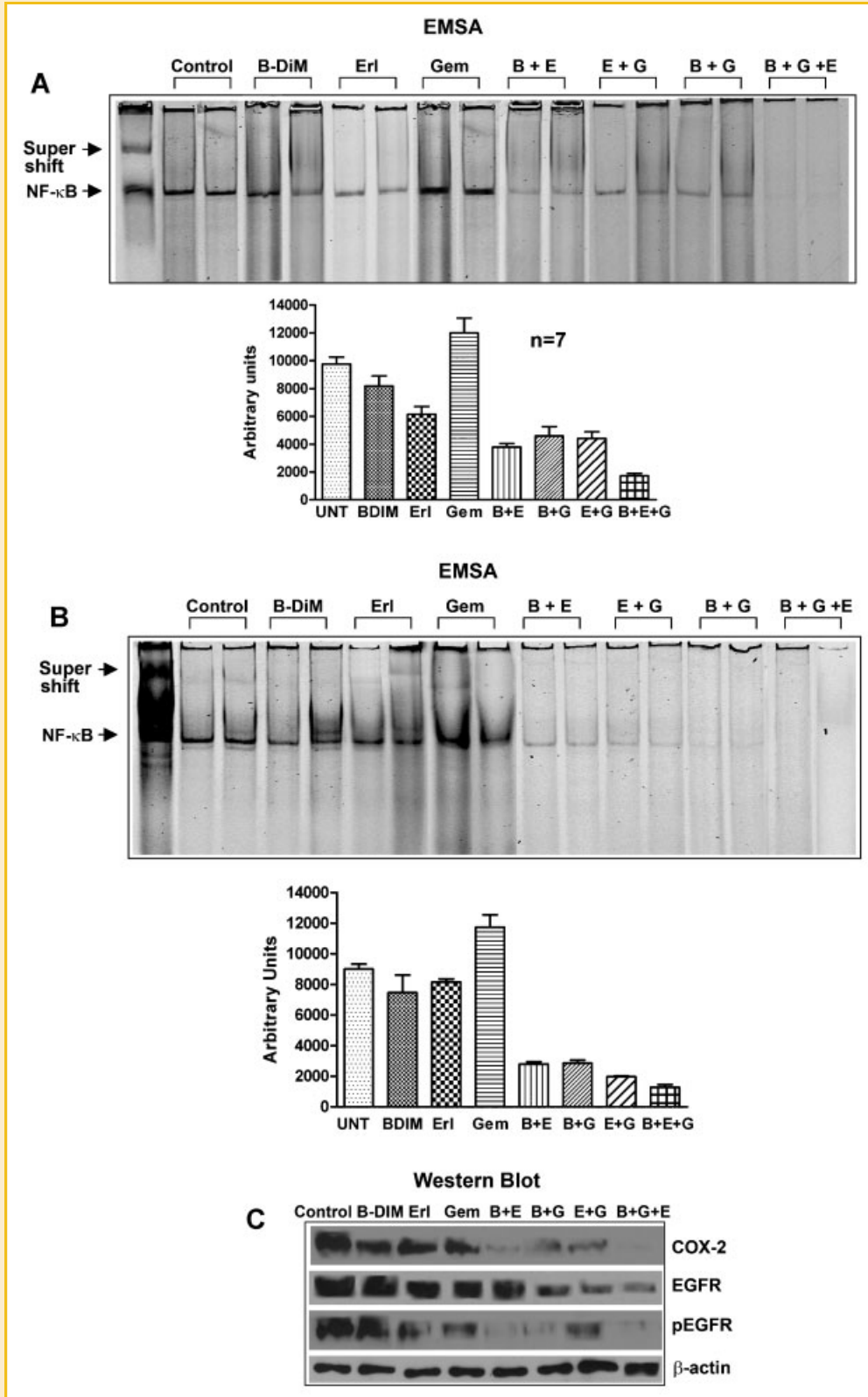


Fig. 6. NF-κB DNA binding activity in nuclear extracts of randomly selected tumor tissues by EMSA in L3.6pl derived tumors (A; upper panel); quantification of NF-κB DNA binding activity (A; lower panel; n = 7). NF-κB DNA binding activity in nuclear extracts of randomly selected tumor tissues by EMSA in COLO-357 derived tumors (B; upper panel), quantification of NF-κB DNA binding activity (B; lower panel), and the inhibition of protein expression of COX-2, EGFR, and pEGFR in COLO-357 derived tumors (C).

TABLE I. Anti-Tumor Activity of B-DIM, Erlotinib, Gemcitabine, and Their Combination in COLO-357-Bearing CB17 SCID Mice

Agent	No. of animals (bilateral)	Route	T/C (%)	T-C (days)	Log ₁₀ kill		Activity score
					Gross	Net	
Control	7	N/A	100	0	0.0	0.0	N/A
B-DIM	7	PO	88	2	0.37	N/A	-
Erlotinib	7	IP	89	3	0.55	N/A	-
Gemcitabine	7	IV	79	4	0.74	N/A	+
B-DIM + erlotinib	7	PO + IP	74	7	1.3	N/A	++
B-DIM + gemcitabine	7	PO + IV	76	9	1.7	N/A	++
Erlotinib + gemcitabine	7	IP + IV	59	14	2.6	0.37	+++
B-DIM + erl + gem	7	PO + IP + IV	47	22	4.1	1.86	++++

Doubling time was estimated at 1.62 days.

The drugs were administered for 12 consecutive days.

and NF-κB pathway is also mechanistically associated with the observed potentiation effects of erlotinib by B-DIM. Similar results were observed when the induction of COX-2 expression in prostate cancer cells by hydroxyflutamide, which targets androgen-androgen receptor signaling, was suppressed by the addition of COX-2 inhibitor NS398. This effect was mediated at the transcriptional level by the modulation of NF-κB signaling pathway [Cai et al., 2008]. Therefore, we believe that the inactivation of drug-induced activation of NF-κB and COX-2 is required prior to intervention using specific therapeutic agents for better therapeutic outcome.

To support our in vitro results, an in vivo tumor model was used to assess the anti-tumor activity of our triple combination. Our in vivo results are consistent with in vitro findings showing that the combined treatment is much more superior than single or double agents, and these results are consistent with inactivation of EGFR, COX-2, and NF-κB signaling in the tumor remnant, suggesting that B-DIM-induced inhibition of NF-κB results in the inhibition of both EGFR and COX-2, which leads to better killing of PC tumor by the combined effect of EGFR inhibitor (erlotinib) and gemcitabine. In summary, the inhibition of EGFR, NF-κB, and COX-2 could be useful for potentiating the anti-tumor activity of gemcitabine in vitro, which appears to be responsible for the observed better anti-tumor activity in vivo. However, only the PC cells that over-express COX-2, NF-κB, and EGFR demonstrates this potentiation, suggesting that targeting all three pathways (EGFR, COX-2, and NF-κB) by B-DIM could be a promising approach for designing tailored novel combination therapies for the treatment of human PC.

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