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Research Article

3,3′-Diindolylmethane Enhances Chemosensitivity of Multiple Chemotherapeutic Agents in Pancreatic Cancer

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Abstract

Clinical management of pancreatic cancer is a major problem, which is in part due to both *de novo* and acquired resistance to conventional therapeutics. Here, we present *in vitro* and *in vivo* preclinical evidence in support of chemosensitization of pancreatic cancer cells by 3,3-diindolylmethane (DIM), a natural compound that can be easily obtained by consuming cruciferous vegetables. DIM pretreatment of pancreatic cancer cells led to a significantly increased apoptosis ($P < 0.01$) with suboptimal concentrations of chemotherapeutic agents (cisplatin, gemcitabine, and oxaliplatin) compared with monotherapy. It is known that resistance to chemotherapy in pancreatic cancer is associated with constitutively activated nuclear factor-κB (NF-κB), which becomes further activated by chemotherapeutic drugs. Our data provide mechanistic evidence for the first time showing that DIM potentiates the killing of pancreatic cancer cells by down-regulation of constitutive as well as drug-induced activation of NF-κB and its downstream genes (Bcl-xL, XIAP, cIAP, and survivin). Most importantly, using an orthotopic animal model, we found reduction in tumor size ($P < 0.001$) when DIM was given in combination with oxaliplatin compared with monotherapy. This was accompanied by loss of phospho-p65 and down-regulation of NF-κB activity and its downstream genes (Bcl-xL, survivin, and XIAP), which correlated with reduced cell proliferation (as assessed by Ki-67 immunostaining of tumor specimens) and evidence of apoptosis [as assessed by poly(ADP-ribose) polymease cleavage and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining]. These results provide strong *in vivo* evidence in support of our hypothesis that DIM could abrogate chemotherapeutic drug (cisplatin, gemcitabine, and/or oxaliplatin)–induced activation of NF-κB, resulting in the chemosensitization of pancreatic tumors to conventional therapeutics. [Cancer Res 2009;69(13):5592–600]

Introduction

Despite advances in multimodality treatment including targeted therapies, pancreatic cancer remains the fourth leading cause of cancer death in the United States (1), accounting for 37,680 estimated new cases diagnosed in the year 2008 with 34,290 deaths. These grim statistics are in part due to the indolent nature of this disease and lack of specific biomarkers for early detection. Clinical management of pancreatic cancer becomes further complicated due to both *de novo* chemoresistance and acquisition of chemoresistance during therapy by conventional cytotoxic agents (2–5). Moreover, primary treatment by surgery is most often palliative; thus, postoperative therapy including chemotherapy with and without chemoradiation therapy is necessary for the management of pancreatic cancer, although the therapeutic outcome of current strategies is dismal without any survival benefit. Similarly targeted therapies have been proven to be ineffective in this disease. For example, inactivation of epidermal growth factor receptor signaling pathway by the epidermal growth factor receptor-related tyrosine kinase inhibitor erlotinib have been tried in pancreatic cancer, showing only modest survival benefit in large phase III clinical trial when combined with gemcitabine (6), suggesting that novel approaches must be devised for improving the survival outcome for this deadly disease. Here, we report the results of one such novel strategy using "natural agents" that could be useful for the treatment of pancreatic cancer.

It is now well accepted that "natural agents" from vegetables of the family Cruciferae yield a bioactive phytochemical known as indole-3-carbinol (I3C; refs. 7, 8), which is chemically unstable in aqueous and gastric acidic environment; thus, it is rapidly converted to numerous condensation products, among which 3,3′-diindolylmethane (DIM) showed biological activities (9). In a study reported by Reed and colleagues, I3C was not detectable in the plasma of women ingesting I3C but DIM was the only I3C-derived compound detected in the plasma (10), suggesting that DIM is the predominant bioactive compound. Emerging preclinical evidence also suggest that I3C and its dimeric product DIM possess anticarcinogenic effects in experimental animals and also inhibits the growth and induce apoptosis in prostate, breast, colon, cervix, and pancreas cancer cells (11–20), which is mediated by alterations in multiple signaling pathways (9, 14, 18, 21, 22). Recently, a study reported from our laboratory concluded that inhibition of cell proliferation by DIM (a formulated DIM with enhanced pharmacokinetics) is mediated through the regulation of Akt/FOXO3a/GSK-3β/β-catenin signaling and induction of Par-4 (20, 22). Additional clinically relevant study reported by our laboratory and others confirmed that DIM inhibits human primary endothelial cell migration in culture and decreased blood vessel formation in xenograft tumors of human breast and prostate (21, 23). Furthermore, we and others have shown that DIM and I3C reduce the activity of nuclear factor-κB (NF-κB) in prostate, breast, and other cancer cells (15, 24). Collectively, these scientific findings led to a significant interest in the past few years to explore the potential chemopreventive and therapeutic activity of DIM against multiple cancers. It is, however, important to note that DIM but not I3C is safer in humans and that administration of DIM to human volunteers results in adequate serum levels that could be biologically important (10, 25, 26).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Previously, we have conceptualized that natural dietary substances (natural agents such as DIM) may have therapeutic benefit in addition to their role as chemopreventive agent by virtue of their pleiotropic activity on cancer cells including inactivation of survival signaling pathways (epidermal growth factor receptor/Akt/NF-κB) and simultaneous activation of multiple death pathways (27). Moreover, we have shown that the apoptosis-inducing effect of erlotinib could be potentiated by DIM in pancreatic cancer cells *in vitro* (11); however, such studies have not been reported using conventional chemotherapeutic agents such as gemcitabine or oxaliplatin especially because platinum-containing chemotherapeutic agents including cisplatin and oxaliplatin are used as an alternate treatment option for pancreatic cancer (28–31). Because chemoresistant phenotype is a major impediment in delivering effective cytotoxic therapy to cancer cells using conventional therapeutics, here, we report for the first time the effect of DIM in sensitizing pancreatic cancer cells *in vitro* and pancreatic cancer tumors *in vivo* to lower concentrations of the conventional cytotoxic chemotherapeutic drugs.

Materials and Methods

Cell culture. The human pancreatic carcinoma cell lines PANC-1 were obtained from the American Type Culture Collection. Human pancreatic ductal epithelial cells Colo-357 and Panc-28 were obtained from University of Texas M. D. Anderson Cancer Center. The cell lines were maintained in continuous exponential growth by twice a week passaging in DMEM or keratinocyte serum-free medium for human pancreatic ductal epithelial cells (Life Technologies) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 10 mg/mL streptomycin.

Antibodies were obtained from the following commercial sources: caspase-3, caspase-9, phospho-Akt, cytochrome *c*, and cytochrome *c* oxidase (Cell Signaling); anti-mouse Bcl-xL, Bax, Mcl-1, and anti-retinoblastoma antibody (Santa Cruz Biotechnology); and anti-poly(ADP-ribose) polymease (PARP) antibody (Biomol Research). Anti-XIAP, cIAP (pan), and survivin were from R&D Systems. Anti-β-actin antibody was from Sigma. DIM (LKT Laboratories) was dissolved in DMSO to make 20 mmol/L stock solution. Cisplatin, oxaliplatin, and gemcitabine was obtained from the Barbara Ann Karmanos Cancer Institute pharmacy.

Cell viability inhibition by MTT assay. Cells were seeded at a density of 2×10^3 to 3×10^3 per well in 96-well microtiter culture plates. After overnight incubation, fresh medium containing different concentrations of DIM (0-60 μmol/L; 0.1% final concentration of DMSO; similar approach was used for BITC and the final concentration of 10 μmol/L BITC was used) was added to each well and incubated for 72 h and then subjected to MTT assay as described earlier (32) with or without cytotoxic agents.

Cell viability inhibition by cytotoxic agents. PANC-1, Panc-28, and Colo-357 cells were plated as described above and allowed to attach overnight. The culture medium was replaced with fresh medium containing 30 μmol/L DIM for 24 h and then exposed to cytotoxic agents for an additional 72 h. Thus, for single agent, cells were exposed to DIM for 96 h and to cisplatin, gemcitabine, or oxaliplatin for 72 h. The effect of pretreatment on cell viability was examined by MTT assay as described earlier (32) and synergism was calculated using CalcuSyn software (Biosoft).

Quantification of apoptosis. The Cell Apoptosis ELISA Detection Kit (Roche) was used to detect apoptosis as described earlier (33).

Protein extraction and Western blot analysis. The pancreatic cancer cells PANC-1 were plated and allowed to attach for 36 h. DIM was directly added to cell cultures at the indicated concentrations and incubated for 72 h. Cell lysates were prepared by suspending the cells in radioimmunoprecipitation assay lysis buffer and subjected to routine Western blot analysis as described earlier (33).

Cytochrome *c* release assay. Cells were plated at a density of 5×10^6 in 100 mm dish and allowed to attach overnight. DIM was added to freshly replaced medium at indicated concentrations (0-45 μmol/L). Following termination of incubation period, cells were collected, washed with ice-cold PBS, lysed and processed to obtain cytosolic and mitochondria fraction for cytochrome *c* immunoblotting as described earlier (33).

Caspase-3 activity assay. Caspase-3 activity were measured in whole-cell lysates using commercially available assay kit (R&D Systems) according to the manufacturer's instruction.

DNA cell cycle analysis. PANC-1 cells were seeded and treated with DIM (0-45 μmol/L) for 72 h. After treatments, the cells were collected by trypsinization, washed with cold PBS, fixed with 70% ethanol, and stained with propidium iodide for 30 min. Flow cytometric analysis was carried out using FACScan.

Electrophoretic mobility shift assay. Nuclear extracts were prepared from treated samples and electrophoretic mobility shift assay was done by incubating 10 μg nuclear extract with IRDye-700-labeled NF-κB oligonucleotide as described earlier (32). The DNA-protein complex formed was visualized by Odyssey Infrared Imaging System using Odyssey Software Release 1.1.

Experimental animals. Female ICR-SCID mice were purchased from Taconic Farms. The mice were housed and maintained under sterile conditions and used in accordance with Animal Care and Use Guidelines of Wayne State University. Mice received Lab Diet 5021 (Purina Mills).

Orthotopic implantation of tumor cells. PANC-1 cells were harvested from subconfluent cultures washed once in serum-free medium and resuspended in PBS. Cells (1×10^6) in 15 μL PBS were injected into the parenchyma of pancreas with a 27-gauge hypodermic needle as described earlier (33).

Experimental protocol. Mice were randomized into the following treatment groups ($n = 7$): (*a*) untreated control; (*b*) BR-DIM 5.0 mg/mice daily orally by gavage for 25 days; (*c*) oxaliplatin 15 mg/kg body weight intravenously given once as a bolus; and (*d*) BR-DIM and oxaliplatin following the schedule as for individual treatments. All mice were killed on day 25 since the initiation of BR-DIM treatment. Body weight of mice from all the groups was recorded every fifth day after cell implantation. For imaging, 2 to 3 mice per group were injected with EGF-IRDye-800CW (epidermal growth factor receptor antibody) via tail vein (1 nmol/L per mice) 72 h before euthanizing the animals. Imaging of live animals was done using Odyssey Infrared Imaging system under anesthesia. Upon autopsy, the pancreas was excised neatly, weighed, and subsequently processed for H&E, immunohistochemical staining, and preparation of nuclear protein extracts for electrophoretic mobility shift assay and Western immunoblotting.

Histologic sections and immunohistochemistry. Formalin-fixed tissue sections were evaluated for tumor cell cytology, mitotic rate, growth pattern, necrosis, cystic change, and associated inflammatory cellular response. Immunohistochemical studies were done after staining with specific primary antibodies against phospho-p65 and Ki-67 followed by 3,3′-diaminobenzidine staining. Apoptotic cells were identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling using Chemicon Apotag *In situ* Apoptosis Detection kit (Chemicon) and visualized under an Olympus microscope (Olympus).

Statistical analysis. Data are represented as mean ± SD for the absolute values or percent of controls as indicated in the vertical axis legend of Figs. 1 to 5. The statistical significance of differential findings between experimental groups and control was determined by Student's *t* test. P values < 0.05 were considered statistically significant.

Results

Effect of DIM on cell viability and apoptosis induction and cell cycle arrest. As shown in Fig. 1*A*, in almost all pancreatic cancer cell lines, DIM suppressed viability in a dose-dependent manner and had minimal effect on human pancreatic ductal epithelial cells, suggesting the relatively nontoxic nature of this compound on normal cells. These results were also consistent with induction of apoptosis induced by DIM treatment (Fig. 1*B* and *C*). Moreover, the sub-G_1 DNA content analysis showed increased accumulation of cells in the sub-G_1 phase in a dose-dependent

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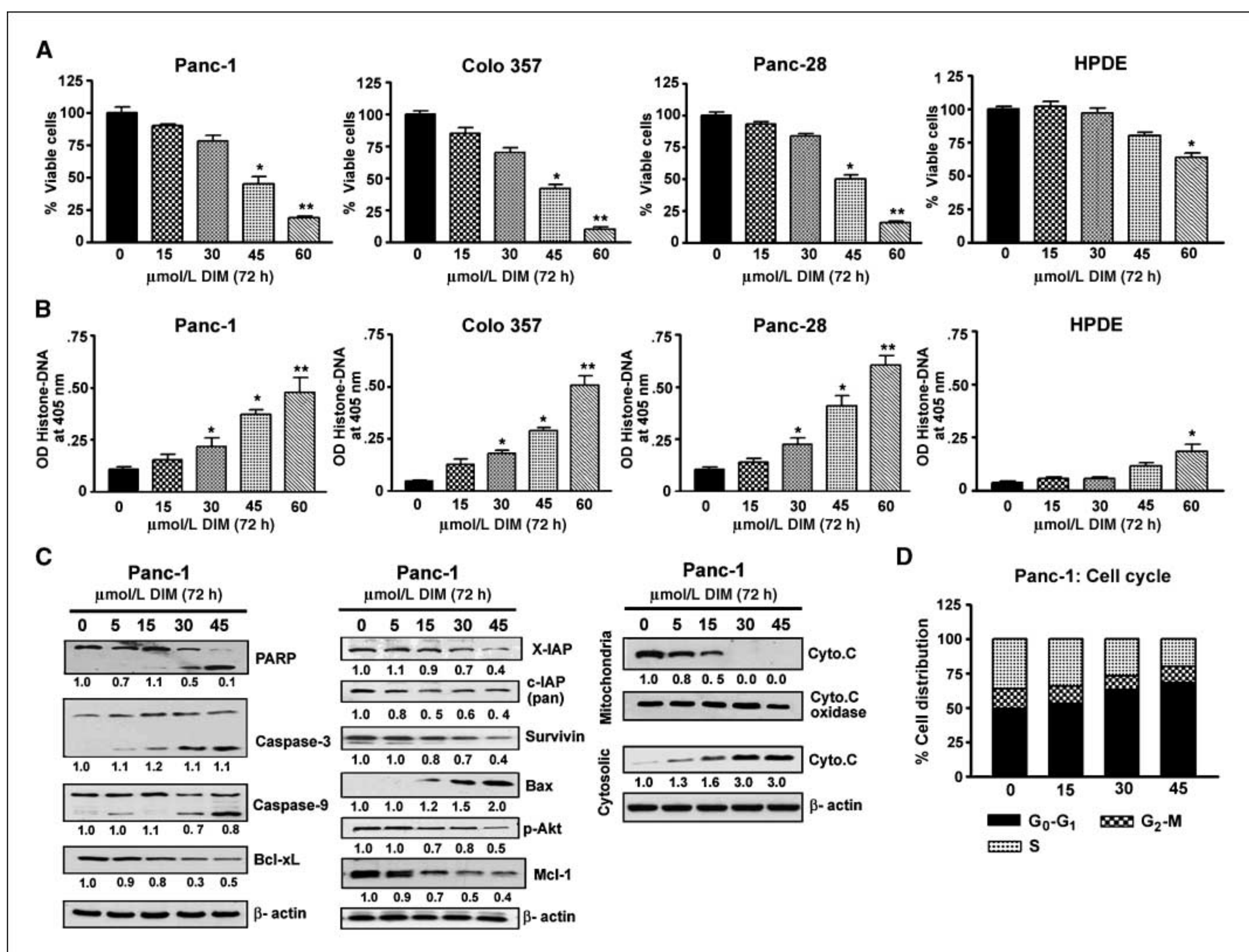

Figure 1. Evaluation of cell viability and apoptosis induced by DIM treatment (72 h) to PANC-1, Panc-28, and Colo-357 and human pancreatic ductal epithelial (*HDPE*) cells by MTT (*A*) or histone DNA ELISA for apoptosis (*B*). *, $P < 0.01$; **, $P < 0.001$, relative to control. *C,* Western blot depicting alterations in the expression of apoptosis-related proteins in whole-cell lysates and the release of cytochrome *c* (*Cyto.C*) prepared from PANC-1 cells after treatment with different concentrations of DIM (0-45 μmol/L) for 72 h. β-Actin protein was used as loading control and the signal was quantified as presented. *D,* cell cycle analysis by flow cytometry and % distribution of cells in G_0-G_1, G_2-M, or S phase of the cell cycle.

manner (data not shown). Further analysis showed that DIM treatment resulted in a significant increase of cell population in the G_0-G_1 phase of the cell cycle (49% versus 71% cells at 0 and 45 μmol/L concentrations of DIM, respectively; Fig. 1*D*). The increase in cell population in the G_0-G_1 phase was found to be associated with a concomitant decrease in cell population in the S phase, whereas the population of cells in G_2-M phase did not change significantly compared with the corresponding controls (Fig. 1*D*). Overall, these results support the notion that the observed decline in cell viability by DIM was in part due to cell cycle arrest and induction of apoptosis. We then investigated the status of cell survival and apoptosis-related molecules in DIM-treated cells using PANC-1 as representative cell type, because this cell line is moderately resistant to chemotherapeutic drugs and have molecular signature similar to human pancreatic tumors harboring K-ras and p53 mutations.

DIM inhibits apoptotic molecules in PANC-1 cells. As shown in Fig. 1*C*, Bcl-xL protein level was inhibited, whereas proapoptotic Bax protein level was markedly induced in response to DIM treatment, indicating that the apoptotic effects of DIM are partly due to increased Bax/Bcl-xL protein ratio. Relative to control, Mcl-1, survivin, XIAP, cIAP (pan), and phospho-Akt expression was down-regulated in cells exposed to DIM for 72 h. These results provide convincing mechanistic evidence in support of DIM-induced apoptosis in pancreatic cancer cells, which is consistent with increase levels of active caspase-3, caspase-9, and 85 kDa cleaved intermediate of PARP in PANC-1 cells (Fig. 1*C*), suggesting that DIM-induced apoptosis is mediated, at least in part, by the mitochondrial pathway. Therefore, to confirm the involvement of mitochondrial pathway and the release of cytochrome *c*, we separated cytosolic and mitochondrial fractions from PANC-1 cells and showed that DIM was able to induce the release of cytochrome *c* from mitochondria into cytosol in a dose-dependent manner (Fig. 1*C*). We next assessed the effects of conventional therapeutics alone and in combination with DIM.

DIM sensitizes pancreatic cancer cells to multiple cytotoxic agents by reducing cell viability and promoting apoptosis. We assessed the effect of cisplatin, gemcitabine, and oxaliplatin alone

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on the viability of different pancreatic cancer cells by MTT assay and found a concentration-dependent inhibition of pancreatic cancer cell viability (data not shown). We noted differential sensitivity of cells toward gemcitabine and oxaliplatin, with Colo-357 being highly sensitive to low concentrations of gemcitabine as well as oxaliplatin compared with other pancreatic cancer cells (data not shown). In our subsequent studies, we found that treatment of cells with DIM or cisplatin, gemcitabine, or oxaliplatin alone (for 72 h) caused >25% to 50% (P < 0.01) loss of pancreatic cancer cell viability (Fig. 2*A*); however, pretreatment of cells with DIM for 24 h followed by treatment with the cytotoxic chemotherapeutic agents (cisplatin, gemcitabine, and oxaliplatin) for 72 h resulted in a significant loss of cell viability (<65-80%; P < 0.001) in all the cell lines tested. Morphologic feature characteristics of apoptosis were observed as depicted by the microphotographs (Fig. 2*B*). In control culture, the cells were seen attached and reaching near confluent, whereas cells tend to show slightly less proficiency in growth and viability following single-regimen treatments. However, the changes tend to become more pronounced and prominent in the combination group, wherein the cells appears to round off, detach, and were seen floating, all of which are typical characteristics of apoptotic cell death. The attached cells appeared pleiomorphic and elongated.

Next, we determined the combination index values for all three combination treatment groups, where combination index < 1 indicates synergism, combination index > 1 indicates antagonism, and combination index = 1 indicates additive effect. Our results (Fig. 2*C*) clearly showed that PANC-1 cells pretreated with DIM showed synergistic loss of the cell viability when combined with cisplatin, gemcitabine, and/or oxaliplatin (combination indices =

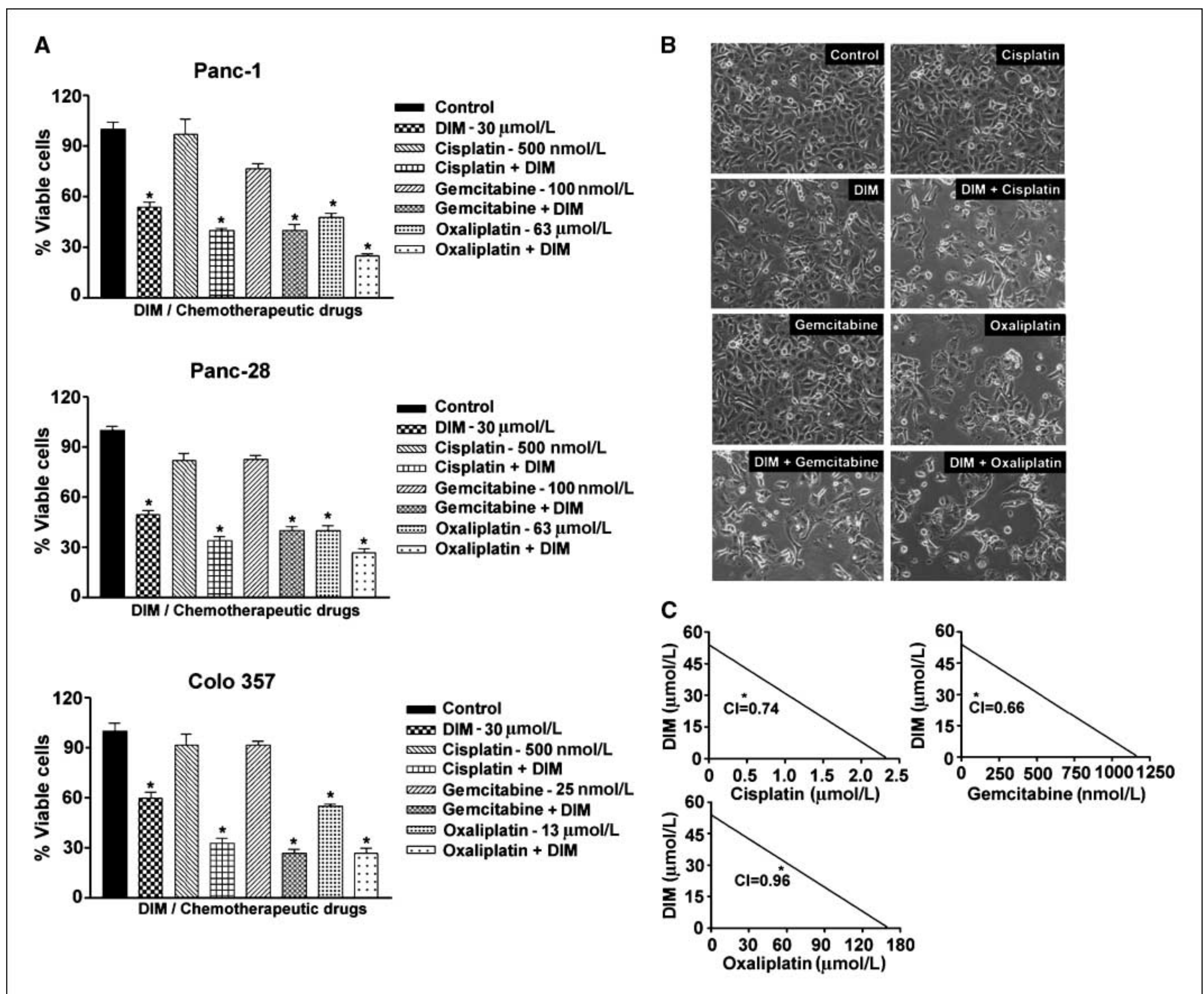

Figure 2. *A,* chemosensitization by DIM preexposure (30 μmol/L DIM for 24 h) of pancreatic cancer cells (PANC-1, Panc-28, and Colo-357 cells) followed by coincubation with cisplatin, gemcitabine, or oxaliplatin for additional 72 h. Viable cells were evaluated by MTT. *, P < 0.01; **, P < 0.001, relative to control. *B,* photomicrograph of PANC-1 cells morphology following exposure to only DIM or combination treatment. *C,* isobologram depicting synergy between combinations of DIM-cisplatin, DIM-gemcitabine, and DIM-oxaliplatin in PANC-1 cells. Concentrations of cisplatin, gemcitabine, oxaliplatin, and DIM are reflected on X and Y axes, respectively.

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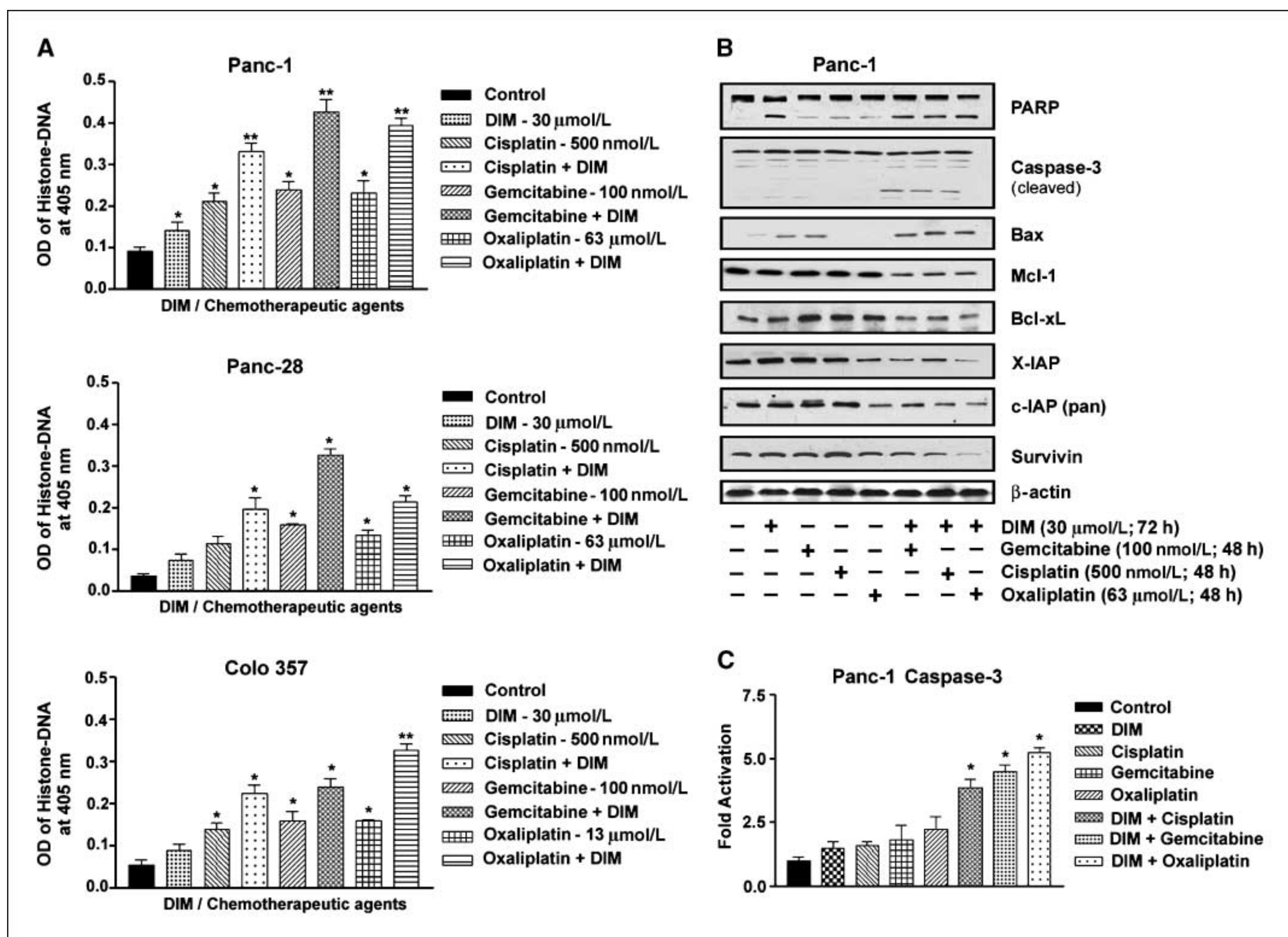

Figure 3. *A,* sensitization of pancreatic cancer cells (PANC-1, Panc-28, and Colo-357 cells) to apoptosis as determined by histone-DNA ELISA. Increased apoptotic response was evident in the combination group relative to untreated control or individual treatment groups. *, $P < 0.01$; **, $P < 0.001$, relative to control. *B,* Western blot analysis of antiapoptotic and prosurvival molecules in whole-cell lysates of PANC-1 pancreatic cancer cells exposed to either only DIM or cytotoxic chemotherapeutic drugs either single or in combination. *C,* caspase-3 activity in cell lysates derived from PANC-1 pancreatic cancer cells under the conditions of preexposure to DIM as described above. A significant increase in caspase-3 activity over that of control and relative to individual drugs are evident. *, $P < 0.001$.

0.74, 0.66, and 0.96, respectively).These results are of paramount interest clinically in minimizing toxic side effects of chemotherapeutic agents on normal cells. These results were also seen consistent with synergistic induction of apoptosis (~50% more; $P < 0.01$; Fig. 3*A*). It is important to note that we did not find synergistic effects using BITC (Supplementary Fig. S1), suggesting that DIM is superior to BITC in our experimental system. Further molecular mechanistic investigations by Western immunoblotting revealed in PANC-1 cells pretreatment with DIM augmented PARP cleavage and the appearance of active cleaved caspase-3 (Fig. 3*B*), which was consistent with spectrophotometric assay results showing significant increase in caspase-3 activity (Fig. 3*C*). In agreement with our results with PARP and caspase-3 activity, we found significant up-regulation of Bax and down-regulation of Bcl-xL, Mcl-1, survivin, cIAP, and XIAP proteins in the combination treatment group (Fig. 3*B*), indicating that DIM indeed sensitizes pancreatic cancer cells to the cytotoxic effect of cisplatin, gemcitabine, and oxaliplatin. Because many of the antiapoptotic proteins are regulated by NF-κB, we assessed the role of NF-κB in our experimental system.

DIM inhibits activation of NF-κB and down-regulates NF-κB activation stimulated by oxaliplatin and gemcitabine. As shown in Fig. 4*A*, DIM resulted in a concentration-dependent decrease in the DNA-binding activity of NF-κB in PANC-1 and Panc-28 pancreatic cancer cells, which is consistent with the down-regulation of the transcriptional target genes of NF-κB such as Bcl-2 family of antiapoptotic proteins survivin and XIAP. To further assess the role of NF-κB, experiments were done to determine optimal treatment schedule and dose of individual chemotherapeutic agents in stimulating basal level of NF-κB in PANC-1 cells. For this study, we exposed PANC-1 cells to 30 μmol/L DIM for 48 h followed by 3 h of either gemcitabine (100 nmol/L), cisplatin (500 nmol/L), or oxaliplatin (63 μmol/L), prepared nuclear extracts, and subjected to NF-κB DNA-binding assay by electrophoretic mobility shift assay. Consistent with previously published data from our laboratory (32), we found that gemcitabine and oxaliplatin treatment alone for 3 h induced NF-κB DNA-binding activity (Fig. 4*B*). Interestingly, we also found that pretreatment of cells with 30 μmol/L DIM significantly reduced chemotherapeutic agents-induced activation of NF-κB DNA-binding activity (Fig. 4*B*).

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These results show that DIM not only down-regulates the preexisting basal levels of NF-κB DNA-binding activity in unstimulated pancreatic cancer cells but could also inhibit gemcitabine, cisplatin, and/or oxaliplatin-induced NF-κB activation, which is consistent with our hypothesis.

Moreover, using pancreatic cancer cell line expressing phosphorylation-defective IκBα (S32 and 36A) cells (generous gift from Dr, Paul Chiao, University of Texas M. D. Anderson Cancer Center), we confirmed the absence of NF-κB and its target genes in these cells (Fig. 4*C*). We treated these cells with DIM and oxaliplatin using our protocol employing pretreatment with DIM followed by oxaliplatin treatment. As anticipated, inhibition of NF-κB signaling in these cells leads not only to a significant decrease in the expression of several of the NF-κB downstream targets (Fig. 4*C*) but also exhibited loss of cell viability (>50% loss) compared with Panc-28 (containing constitutively active NF-κB) cells in the combination treatment group (Fig. 4*D*). These *in vitro* studies prompted us to conduct *in vivo* testing of our hypothesis, and the results are presented below.

DIM enhances *in vivo* therapeutic effect of oxaliplatin on primary tumor. To be consistent with clinical relevance of our results, we used an absorption-enhanced formulation of DIM, henceforth called BR-DIM (obtained from Bio-Response) for our *in vivo* studies. Under our experimental conditions (as depicted in Fig. 5*A*), administration of BR-DIM by gavage caused only a minimal (20% reduction) effect on tumor weight. Additionally, relative to control group, oxaliplatin treatment alone caused 50% reduction in tumor weight (Fig. 5*B*). However, under identical experimental conditions, the combination of BR-DIM and oxaliplatin showed significant decrease ($P < 0.001$) in tumor weight relative to untreated control group. These results were consistent with imaging results (Fig. 5*C*). Of interest, on autopsy, 86% of mice from the control group showed evidence of nodal metastasis. In contrast, a progressive decline in the percentage of mice harboring nodal metastasis, as well as metastatic tumor size, was prominently evident in the BR-DIM and oxaliplatin combination groups as represented in Fig. 5*D*. Moreover, no evidence of any toxicity as inferred from body weight loss criteria or signs of aversion to food intake or diarrhea were evident within therapeutic window.

NF-κB DNA-binding activity and PARP cleavage *in vivo*. Similar to our *in vitro* findings, NF-κB in the nuclear extracts of tumor samples was moderately down-regulated by BR-DIM alone, but, unlike *in vitro* situation, oxaliplatin did not reveal any overtly induced NF-κB DNA-binding activity relative to control specimens. Interestingly, constitutively expressed NF-κB was seen abrogated in tumor samples obtained from mice treated with BR-DIM and oxaliplatin (Fig. 6*A*). Whole-tissue lysates from harvested tumors revealed down-regulation of a few important NF-κB regulated antiapoptotic molecules such as Bcl-xL, survivin, and XIAP proteins *in vivo*, which is consistent with our *in vitro* results.

Tumor histology and immunohistochemistry. H&E evaluation of the tumors from all four groups showed high-grade carcinoma associated with tumor apoptosis and necrosis (Fig. 6*C*). In the control group, the tumor was largely viable and consisted entirely of neoplastic cells with minimal intratumoral stroma. In contrast, in the group receiving combined treatment, there was severe tumor destruction throughout the entire tumor and it was associated with increased stromal fibrosis. Similar but only milder changes were also seen in the tumors of the group treated with BR-DIM or oxaliplatin alone. The expression of phospho-p65 was significantly decreased in the combination group compared with the control (Fig. 6*C*) and milder effects were seen BR-DIM or oxaliplatin alone group, which is consistent with our results on the DNA-binding activity of NF-κB. Likewise, significant apoptosis was

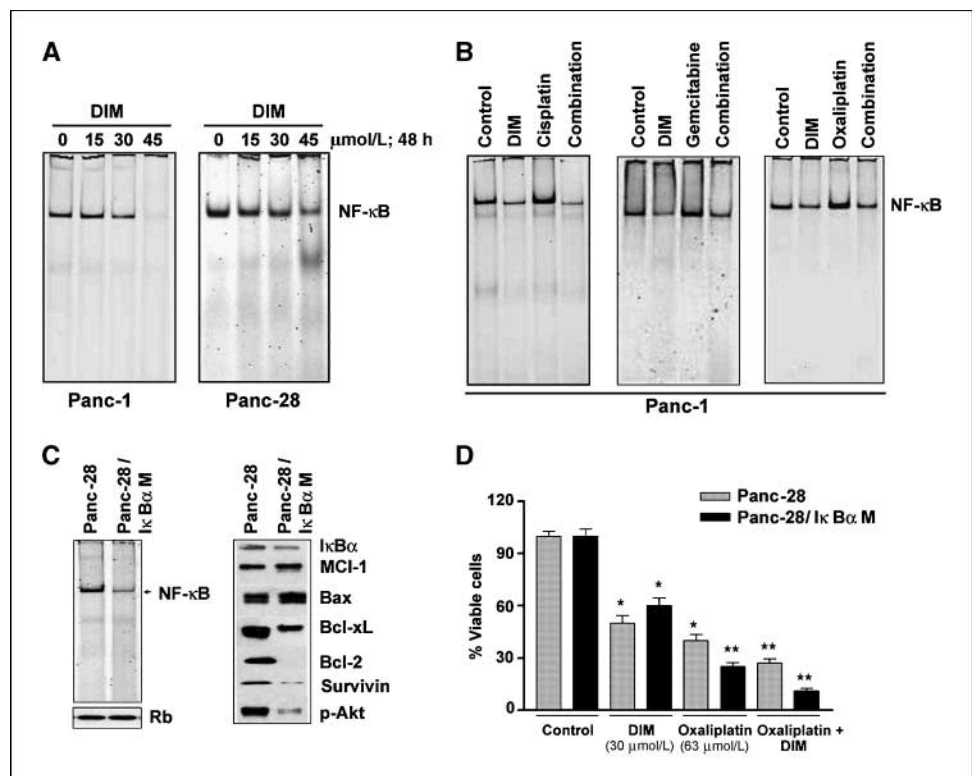

Figure 4. *A,* gel shift assay showing DIM-induced down-regulation of NF-κB DNA-binding activity in the nuclear extract of PANC-1 and Panc-28 cells treated with increasing concentrations of DIM (0-45 μmol/L) for 48 h. *B,* NF-κB DNA-binding activity in the nuclear extract of PANC-1 cells in the presence and/or absence of DIM, gemcitabine, oxaliplatin, and the combinations as detailed in Materials and Methods. *C,* comparative NF-κB DNA-binding activity in nuclear extracts from Panc-28 and Panc-28IκBαM cells and Western blot showing down-regulation of antiapoptotic proteins. *D,* comparative chemosensitization effect of DIM and oxaliplatin in Panc-28 and Panc-28IκBαM cells showing greater loss of cell viability in Panc-28IκBαM cells lacking activated NF-κB.

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evident in the combination group with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling-positive apoptotic cells seen randomly distributed in tumor parenchyma along with reduced staining for Ki-67 (Fig. 6*C*). Together, these results provide convincing evidence in support of the superior antitumor activity of the combination of BR-DIM with oxaliplatin, and these *in vivo* results are consistent with our *in vitro* findings.

Discussion

Here, we report for the first time that DIM could be therapeutically exploited for the treatment of pancreatic cancer in combination with conventional therapeutics. We found that DIM was effective as a general inducer of apoptosis in pancreatic cancer cells by down-regulating several antiapoptotic proteins but had no effect on normal human pancreatic ductal epithelial cells. Moreover, DIM was effective in down-regulating Bcl-2 family proteins as well as IAPs and survivin in pancreatic cancer cells, abrogating treatment resistance via inhibition of caspase cascade (34, 35). Evidence from our laboratory and others have shown that the transcription factor, NF-κB, is constitutively active in human pancreatic tumor specimens as well as in pancreatic cancer cell lines and that NF-κB is intimately involved with *de novo* and acquired chemoresistant phenotype of pancreatic cancer cells (5, 36–38). Moreover, during chemotherapy, NF-κB is transiently activated leading to chemoresistance phenotype and the inactivation of NF-κB before treatment with conventional therapeutics leads to sensitization (better cell killing) of cancer cells to conventional therapeutics as shown by our laboratory and others (5, 27, 32, 33, 39). Zhang and colleagues recently reported that IKK inhibitor could confer sensitivity to pancreatic cancer cells and xenograft tumors by blocking activation of IKK/NF-κB pathway and downstream genes, which leads to enhanced effect of tumor necrosis factor-α on the growth of tumor cells through activation of apoptosis (40). We hypothesized that DIM at the molecular level can function as a "double-edged sword" by abrogating the constitutively active DNA-binding activity of NF-κB and also by attenuating the chemotherapeutic drugs-induced activation of NF-κB. Our findings using isogenic Panc-28 and Panc-28 IκBαM PaCa cell line [with mutation in the two serine residues 32 and 36 of inhibitory IκBα, which blocks phosphorylation and degradation of IκBα protein and results in superrepressor form of IκBα, preventing nuclear translocation of NF-κB and its binding to regulatory sequences ref. 41] convincingly showed that NF-κB activation plays a critical role in protecting the cells against apoptosis induced by cytotoxic agents, which provide direct evidence in support of our hypothesis and other published data (42).

It is known that Bcl-xL, XIAP, cIAP, and survivin (members of IAP proteins) are regulated by NF-κB at the transcriptional level

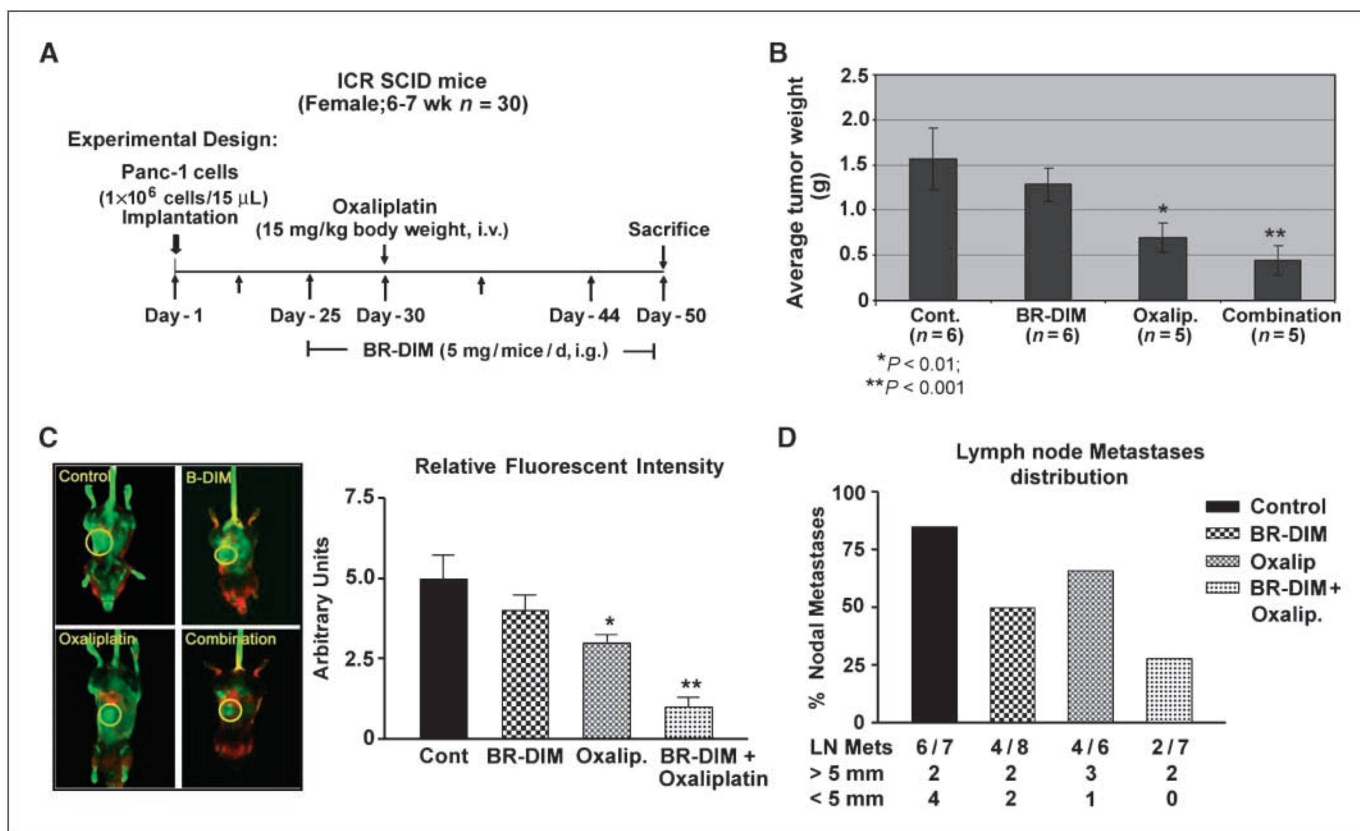

Figure 5. *A,* schematic representation of *in vivo* experimental design. *B,* isolated pancreatic tumor weight showing greater *in vivo* therapeutic efficacy between BR-DIM and oxaliplatin treatment based on tumor weight relative to untreated control group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *C,* comparative IR fluorescence imaging of orthotopic tumors (*left*) in mice showing therapeutic benefit of BR-DIM pretreatment and oxaliplatin. Less fluorescent intensity of the IRDye-800CW EGF-targeting agent in the BR-DIM pretreated group was found, which parallels with reduced tumor size seen after sacrificing the animals. *Right,* quantification of the imaging data using Odyssey software. *, $P < 0.05$; **, $P < 0.01$. *D,* comparative metastatic pancreatic tumor frequency and tumor size distribution between different groups of mice treated with either BR-DIM, oxaliplatin alone, or their combinations.

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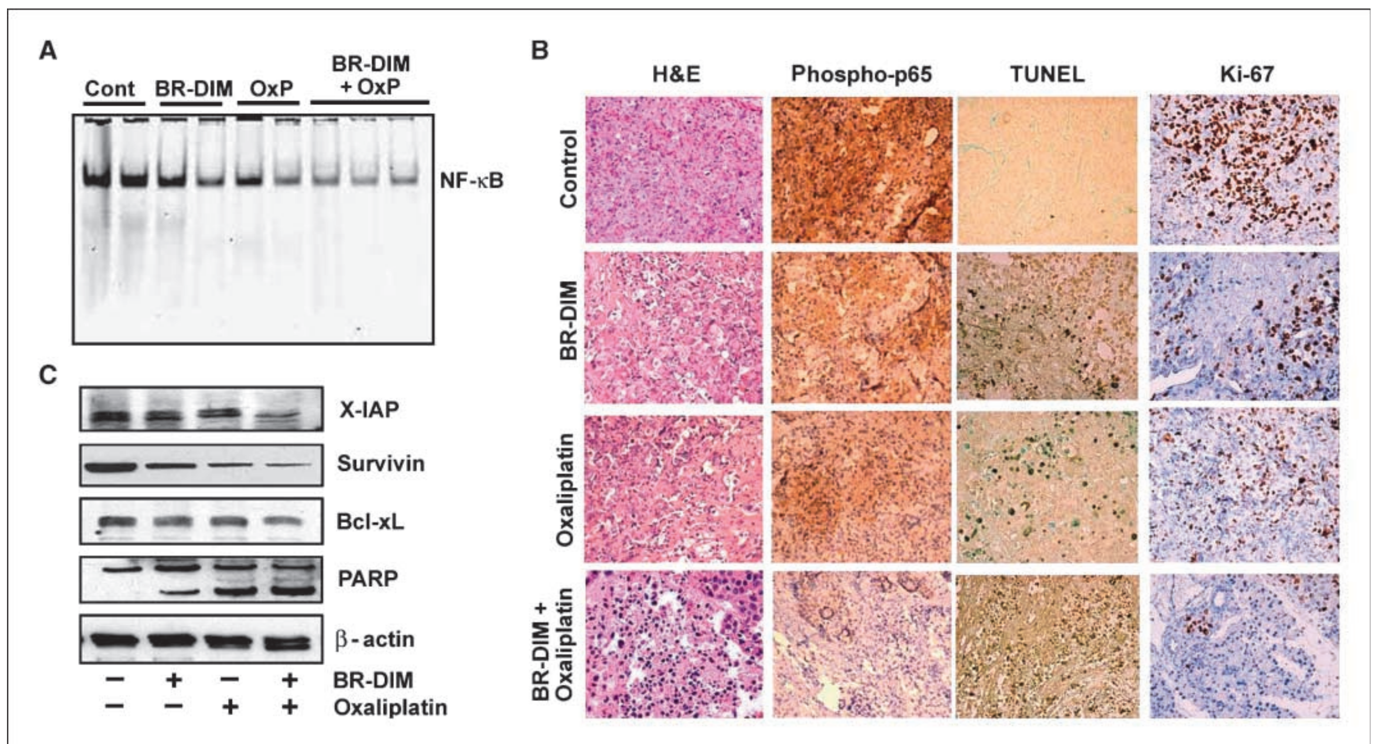

Figure 6. *A,* gel shift assay for NF-κB on two to three randomly selected primary pancreatic tumor tissues from each experimental group showing loss of NF-κB in combination treatment group. *B,* Western blots analysis for survivin, Bcl-xL, and XIAP and PARP in primary pancreatic tumors harvested from mice of different treatment groups showing loss of NF-κB-related antiapoptotic proteins and induced PARP cleavage. *C,* H&E and immunohistochemical demonstration of phospho-p65, apoptosis (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling), and Ki-67 protein in tissues harvested from tumor-bearing mouse.

and also contributes to pancreatic cancer chemoresistance, which can be suppressed by NF-κB inhibition (34, 35, 43–45). Thus, by suppressing NF-κB, DIM induces cell growth inhibition and apoptosis, which is in part due to inactivation of NF-κB and its downstream genes contributing to the reversal of chemoresistance. This phenomenon could be universal among various tumors because high expression of these proteins has also been shown to be associated with resistance to chemotherapy and poor prognosis in carcinomas of the lung, breast, ovary, and esophagus (46, 47). Survivin has been validated as a therapeutic target because of its dual function in inhibiting apoptosis and regulation of mitosis in concert with different cell cycle regulators (48). Recently, small interfering RNA directed against survivin and NF-κB p65/relA leading to enhanced chemosensitivity of pancreatic cancer cells to gemcitabine has been reported (38, 49) and these results are consistent with our current findings.

Interestingly, our *in vitro* results were recapitulated *in vivo* using oxaliplatin as a test agent and we believe that similar phenomenon may exist with many other conventional therapeutics. Our data clearly show that the down-regulation of NF-κB and its downstream targets such as Bcl-xL, survivin, and XIAP is responsible for the enhanced antitumor activity of the combination treatment in our orthotopic pancreatic tumor model, which support our *in vivo* imaging results and complement pancreatic tumor weight at autopsy. Immunohistochemistry of tumor samples showed significantly reduced phospho-p65 immunostaining in the combination group and increased apoptosis as documented by increased terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining and less immunoreactivity toward Ki-67, indicative of reduced proliferation of cells in tumors treated with BR-DIM and oxaliplatin. These *in vivo* results were also consistent with our molecular studies *in vitro*, which clearly provide strong support in favor of our hypothesis that NF-κB is an important target in overcoming *de novo* and acquired chemoresistance in pancreatic cancer, which could be easily achieved by our nontoxic strategy by using BR-DIM.

In conclusion, we have presented evidence showing that pancreatic cancer cells with *de novo* and acquired resistance to chemotherapeutic drugs such as gemcitabine, cisplatin, and oxaliplatin could be reversed by DIM pretreatment and that this beneficial effect is in part due to inactivation of NF-κB and its downstream genes. Our *in vitro* findings together with our *in vivo* results provide confidence in support of further development of DIM (a nontoxic natural agent) as an adjunct to conventional therapeutics in future clinical trial for improving the treatment outcome of patients diagnosed with pancreatic cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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3,3′-Diindolylmethane Enhances Chemosensitivity of Multiple Chemotherapeutic Agents in Pancreatic Cancer

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