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# 3,3′-Diindolylmethane (DIM) and its derivatives induce apoptosis in pancreatic cancer cells through endoplasmic reticulum stress-dependent upregulation of DR5

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**3,3′-Diindolylmethane (DIM), ring-substituted DIMs and 1,1-bis(3′-indolyl)-1-(*p*-substitutedphenyl)methanes (C-DIMs) inhibit growth of Panc-1 and Panc-28 pancreatic cancer cells. Although DIMs (diarylmethanes) and selected C-DIMs (triarylmethanes), such as the *p-t*-butyl derivative (DIM-C-pPhtBu), activate the aryl hydrocarbon receptor and peroxisome proliferator-activated receptor γ, respectively, this study shows that both DIM and DIM-C-pPhtBu induce common receptor-independent pathways. Both DIM and DIM-C-pPhtBu increased endoplasmic reticulum (ER) staining and ER calcium release in Panc-1 cells, and this was accompanied by increased expression of glucose related protein 78 and C/EBP homologous transcription factor (CHOP/GADD153) proteins. Similar results were observed after treatment with thapsigargin (Tg), a prototypical inducer of ER stress. The subsequent downstream effects of DIM/DIM-C-pPhtBu- and Tg-induced ER stress included CHOP-dependent induction of death receptor DR5 and subsequent cleavage of caspase 8, caspase 3, Bid and PARP. Activation of both receptor-dependent and receptor-independent (ER stress) pathways by DIM and DIM-C-pPhtBu in pancreatic cancer cells enhances the efficacy and potential clinical importance of these compounds for cancer chemotherapeutic applications.**

## Introduction

The endoplasmic reticulum (ER) plays a critical role in post-translational modification of newly synthesized proteins, and the unfolded protein response (UPR) is activated under conditions of physiological ER stress (1–6). The UPR relieves the condition of ER stress by modulating expression of genes required for protein processing and allows the cell to revert to homeostasis. ER-dependent folding and other modifications of proteins are critical for cell function, and genetic defects in these responses can lead to Alzheimer's, Parkinson's and Huntington diseases; tissue ischemia; atherosclerotic lesions; viral infection and diabetes (7–17). The failure of cells to counteract ER stress initiates activation of multiple pathways that lead to apoptosis (18).

Activation of cell death pathways is an important function for many anticancer drugs and other pharmacologically active compounds, and for some agents this may involve activation of ER stress (12–21). For example, non-steroidal anti-inflammatory drugs induce gastric lesions and their anti-tumorigenic activity has been linked to apoptosis through activation of ER stress responses (20). Moreover, the anticancer activities of the plant alkaloid ellipticine are also due, in part, to activation of ER stress in breast cancer cells (21). Research in this laboratory has been focused on the characterization and mechanism of action of the anticancer activities of 1,1-*bis*(3′-indolyl)methane (DIM), several symmetrical ring-substituted DIMs and 1,1-*bis*(3′-indolyl)-1-(*p*-substitutedphenyl)methanes (methylene substituted DIMs) (22–28). DIM and ring-substituted DIMs activate the aryl hydrocarbon receptor (AhR) in breast cancer cells and their activity in breast cancer may be due, in part, to inhibitory AhR–estrogen receptor crosstalk (23–25). However, it is clear that DIM induces multiple growth inhibitory/cell death pathways in breast and other cancer cell lines and these responses are AhR-independent (29–39). Recent studies in this laboratory have identified selected methylene-substituted DIMs (C-DIMs) that activate peroxisome proliferator-activated receptor γ (PPARγ). Similar to other structure classes of PPARγ agonists, PPARγ-active C-DIMs inhibit growth of cancer cells and induce apoptosis through receptor-dependent and -independent pathways (26–28).

Preliminary studies with PPARγ-active C-DIMs in Panc-1 pancreatic cancer cells which express mutated p53 showed that these compounds induced PPARγ-independent growth inhibition/cell death. This study now demonstrates that DIM, 5,5′-dibromoDIM and the PPARγ-active 1,1-*bis*(3′-indolyl)-1-(*p-t*-butylphenyl)methane (DIM-C-pPhtBu) inhibit Panc-1 cell growth and this response correlates with the induction of multiple ER stress pathways in Panc-1 and Panc-28 cancer cell lines. These DIM-derived compounds induce several characteristic markers of ER stress including glucose-related protein 78 (GRP78) and C/EBP homologous transcription factor (CHOP/GADD153). These stress-related responses are accompanied by upregulation of the death receptor DR5 and activation of extrinsic and intrinsic pathways of apoptosis. The results demonstrate that the potent anticancer activities of diarylmethane (DIM) and triarylmethane (C-DIM) compounds that contain bis-indolyl substituents are due, in part, to activation of ER stress pathways.

**Abbreviations:** AhR, aryl hydrocarbon receptor; ChIP, chromatin immunoprecipitation; DIM, 3,3′-diindolylmethane; ER, endoplasmic reticulum; GRP78, glucose-related protein 78; PPARγ, peroxisome proliferator-activated receptor γ.

 

## Materials and methods

*Cell lines, chemicals, biochemical constructs and oligonucleotides*

Panc-1 and Panc-28 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). DME/F12 with and without phenol red, 100× antibiotic/antimycotic solution, thapsigargin (Tg) and tunicamycin (Tm) were purchased from Sigma–Aldrich (St Louis, MO). Fetal bovine serum (FBS) was purchased from Intergen (Purchase, NY). The GRP78 promoter-luciferase construct contains 374 bp from the promoter and was provided by Dr K.Park, Center for Molecular Medicine, Sungkyunkwan University (Seoul, Korea) (40). Human CHOP promoter constructs were provided by Dr Pierre Fafournoux (Saint Genes, Champarelle, France) (41), and the DR5 constructs were from Dr H.G.Wang, Moffitt Cancer Center (Tampa, FL) (42). [$\gamma$-$^{32}$P]ATP (300 Ci/mmol) was obtained from Perkin Elmer Life Sciences (Boston, MA). Poly(dI–dC) and T4 polynucleotide kinase were purchased from Roche Molecular Biochemicals (Indianapolis, IN). ER-Tracker Blue-White DPX and Fluo-3 calcium indicator were purchased from Molecular Probes (Eugene, OR). Z-VAD-FMK (general caspase inhibitor) and Z-IETD-FMk (caspase 8 inhibitor) were obtained from BD Bioscience (San Diego, CA). Antibodies for GRP78/Bip, GADD153/CHOP, caspase 8 PARP, rabbit IgG and $\beta$-tubulin proteins were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for ATF6 and DR5 were obtained from Imgenex (San Diego, CA). Antibodies for caspase 3 and BID were obtained from Cell Signaling Technology (Beverly, MA). Lysis buffer and luciferase reagent were obtained from Promega (Madison, WI).

*Cell proliferation assay*

Panc-1 and Panc-28 cells were seeded in DMEM:F-12 media with 5% FBS and treated on the next day with either vehicle ($Me_2SO$) (0.1% of volume) or with the indicated compounds and concentrations. Cells were counted at the indicated times using a Coulter Z1 cell counter. Each experiment was carried out in triplicate, and results are expressed as mean ± SD for each determination.

*Transfection of pancreatic cancer cells and preparation of nuclear extracts*

Cells were cultured in 6-well plates in 2 ml of DME/F12 medium supplemented with 5% FBS. After 16–20 h, when cells were 50–60% confluent, reporter gene constructs were transfected using Lipofectamine Reagent (Invitrogen, Carlsbad, CA). The effects of different treatments on transactivation were investigated in Panc-1 and Panc-28 cells. Cells were transfected with (500 ng) GRP78, GADD153 or DR5 constructs for 16 h, and then treated with the indicated concentrations of $Me_2SO$ (control), DIM, C-DIMs, Tg or tunicamycin (Tm) for 18 h. Cells were harvested, and luciferase activity of lysates (compared with $\beta$-galactosidase activity) was determined. For the EMSA assay, nuclear extracts from the cells were isolated as described previously and aliquots were stored at −80°C until the analysis was carried out (43,44).

*Western immunoblot analysis*

Cells were washed once with phosphate-buffered saline (PBS) and collected by scraping in 200 μl of lysis buffer [50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 10% (v/v) glycerol, 1% Triton X-100 and 5 μl/ml of Protease Inhibitor Cocktail (Sigma)]. Lysates from cells were incubated on ice for 1 h with intermittent vortexing followed by centrifugation at 40 000 *g* for 10 min at 4°C. Equal amounts of protein from each treatment group were diluted with loading buffer, boiled, and loaded onto 10% SDS–polyacrylamide gel. Samples were electrophoresed and proteins were detected by incubation with the primary antibodies ATF6, GRP78 (H-129), GADD153 (R-20), caspase 8 (H-134), caspase 3 (3G2), BID (7A3), DR5 or $\beta$-tubulin (H-235) followed by blotting with appropriate horseradish peroxidase-conjugated secondary antibody as described previously (44). After autoradiography, band intensities were determined by a scanning laser densitometer (Sharp Electronics Corporation, Mahwah, NJ) using this Zero-D Scanalytics software (Scanalytics Corporation, Billerica, MA).

*Electrophoretic mobility shift assay (EMSA)*

Oligonucleotides were synthesized and annealed, and 5-pmol aliquots were 5′ end-labeled using T4 kinase and [$\gamma$-$^{32}$P]ATP. A 30 μl EMSA reaction mixture contained ~100 mM potassium chloride, 3 μg of crude nuclear protein or 1–2 band forming units of human recombinant Sp proteins, 1 μg poly(dI–dC), with or without unlabeled competitor oligonucleotide, and 10 fmol of radiolabeled probe. After incubation for 20 min on ice, antibodies against selected proteins were added and incubated another 20 min on ice. Protein–DNA complexes were resolved by 5% PAGE as described previously (43,44). Specific DNA–protein and antibody-supershifted complexes were observed as retarded bands in the gel. GRP78 ERSE, GADD153 ERSE, DR5 CHOP-like, and Sp1 oligonucleotide sequences used in gel shift analysis are as follows: Human GRP78 −94 ERSE, GGG CCA ATG AAC GGC CTC CAA CGA; Human GADD153 −103 ERSE, GGG GCC AAT GCC GGC GTG CCA CTT TCT; Human DR5 −276 CHOP site, TTG CGG AGG ATT GCG TTG ACG A; and Human DR5 −198 Sp1-2, CAT TCG GGG CGG GGC GAA TCA.

*Measurement of apoptosis*

Annexin V-FLUOS Staining Kit (Roche Applied Science, Penzberg, Germany) was used to detect apoptotic activity in Panc-1 cells after different treatments. Annexin V positivity of cells were determined by Flow Cytometric Analysis; briefly, cells were treated with $Me_2SO$ (control) or with the indicated concentrations of DIM, C-DIMs or Tg for 24 h. Cells were then harvested by typsinization and washed twice in Annexin binding buffer (5 mM $CaCl_2$, 140 mM NaCl and 1 mM HEPES, pH 7.4). Cells were then resuspended in 100 μl of Annexin V binding buffer containing 1:30 dilution of Annexin V–FITC conjugate and 1 μg/ml propidium iodide and incubated in the dark for 15 min. After washing three times with Annexin binding buffer, samples were analyzed by flow cytometry in a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) using a 488 nm argon excitation laser.

*Fluorescent microscopy and staining*

Panc1 cells were seeded in Lab-Tek Chamber slides (Nalge Nunc International, Naperville, IL) at 100 000 cells/well in DME/F12 medium supplemented with 5% FBS. Cells were then treated with 20 μM DIM or 10 μM 5,5′-dibromoDIM, DIM-C-pPhtBu or 0.5 μM Tg for 18 h, and were washed were washed in Dulbecco's PBS. For ER staining, ER-Tracker Blue-White DPX probe was diluted 1:1000 in the regular medium. Then, the prewarmed (37°C) probe-containing medium was added to the cells and incubated for ~30 min under the same growth conditions. The loading solution was removed and cells were then washed one more time with PBS before adding the fresh medium without stain. For $Ca^{2+}$ staining, cells were treated and stained as described for the ER staining experiment and incubated with Fluo-3 probe (1:1000 dilution) as recommended by the manufacturer. For both staining experiments, cells were analyzed using a fluorescence microscope and a confocal laser scanning microscope (Fluoview/FV500; Olympus America, Melville, NY) with appropriate filter settings.

*Chromatin immunoprecipitation assay*

Panc-1 cells ($2 \times 10^7$ cells) were treated with $Me_2SO$, 10 μM DIM-C-pPhtBu, or 0.5 μM Tg for 3, 6 or 12 h, respectively. Formaldehyde (1.5%) was added for 5 min, and the cross-linking reaction was terminated by the addition of 0.125 M glycine for 5 min. Cells were washed with PBS, scraped, pelleted and hypotonically lysed (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% CA-630 plus protease inhibitors). Nuclei were collected by centrifugation, dissolved in sonication buffer (1% SDS, 10 mM EDTA and 50 mM Tris–HCl, pH 8.0) and sonicated to desired chromatin length (500 pb to 1 kb). Chromatin was precleared by addition of protein A-conjugated beads (PIERCE) and incubated at 4°C for 1 h with shaking. The beads were pelleted, and the precleared chromatin supernatant was immunoprecipitated with antibodies [1–2 μg per chromatin immunoprecipitation (ChIP)] specific to IgG or CHOP (Santa Cruz Biotechnology) at 4°C overnight. Protein–antibody complexes were collected by addition of 5 μl protein A-conjugated beads at room temperature for 1 h; beads were washed with a low salt buffer (0.1% SDS, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA and 20 mM Tris–HCl, pH 8.0), high salt buffer (500 nM NaCl instead), LiCl buffer (1% CA-630, 1% sodium deoxycholate, 250 mM or 500 mM LiCl, 1 mM EDTA and 100 mM Tris–HCl, pH 8.0) and TE buffer (0.1% Tween 20, 0.1% SDS, 2 mM EDTA and 50 mM Tris–HCl, pH 8.0). Protein–DNA cross-links were eluted (1% SDS, 50 mM $NaHCO_3$ and 1.5 μg/ml of salmon sperm DNA) and reversed (5 μl of 5 N NaCl, 2 μl of 10 μg/μl RNase for 00 μl eluent) at 65°C for 5–6 h. DNA was purified by Qiaquick Spin Columns (Qiagen) followed by PCR amplification. The DR5 primers are 5′-AGGTTAGTTCCGGTCCCTTC-3′ (forward) and 5′-CAACTGCAAATTCCACCACA-3′ (reverse); they amplify a 111 bp region of the human DR5 promoter, which contains a CHOP binding site: GAGGATTGCGTTG. The positive control primers are 5′-TACTAGCGGTTTTACGGGCG-3′ (forward) and 5′-TCGAACAGGAGGAGCAGAGAGCGA-3′ (reverse), which amplify a 167 bp region of the human glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene. The negative control primers are 5′-ATGGTTGCCACTGGGGATCT-3′ (forward) and 5′-TGCCAAAGCCTAGGGGAAGA-3′ (reverse), which amplify a 174 bp region of genomic DNA between the GAPDH gene and the CNAP1 gene. PCR products were resolved on a 2% agarose gel in the presence of 1:10 000 SYBR gold (Molecular Probes).

*Statistical analysis*

Statistical significance was determined by the analysis of variance and Scheffe's test, and the levels of probability were noted. The results are expressed as mean ± SD for at least three separate (replicate) experiments for each treatment.

## Results

*Decreased cell survival and increased ER stress*

DIM and the ring-substituted 5,5′-dibromoDIM and DIM-C-pPhtBu were used in this study as representative di- and triarylmethanes containing two indolyl or ring-substituted indolyl groups. Results of concentration-dependent Panc-1 and Panc-28 cell proliferation curves (Fig. 1) show that the order of growth inhibitory potencies for these compounds was DIM-C-pPhtBu ≈ 5,5′-dibromoDIM > DIM, and we have observed similar results in other pancreatic, prostate and breast cancer cell lines (data not shown). Initial light microscopic analysis of Panc-1 cells treated with these compounds suggested some alteration in the ER, and results in Figure 2 summarize the staining of Panc-1 cells with ER-Tracker Blue-White DPX dye after treatment with 20 μM DIM, 10 μM 5,5′-dibromoDIM, 10 μM DIM-C-pPhtBu and 0.5 μM Tg, a well-characterized inducer of ER stress. This dye specifically stains the ER blue in live cells, and the results show that Panc-1 cells from all treatment groups exhibited significantly increased blue staining compared with the solvent ($Me_2SO$) control, suggesting the induction of ER stress. ER stress induced by Tg is accompanied by release of $Ca^{2+}$ which can be detected using Fluo-3 calcium indicators, and this is typified by the increased green staining in Panc-1 cells after treatment with Tg for 18 h (Fig. 3). Using the same staining protocol, similar results were obtained in Panc-1 cells after treatment with 20 μM DIM, 10 μM 5,5′-dibromoDIM and 10 μM DIM-C-pPhtBu for 18 h. These results suggest that like Tg the DIM compounds also induce release of $Ca^{2+}$ from the ER and activate ER stress in Panc-1 cells.

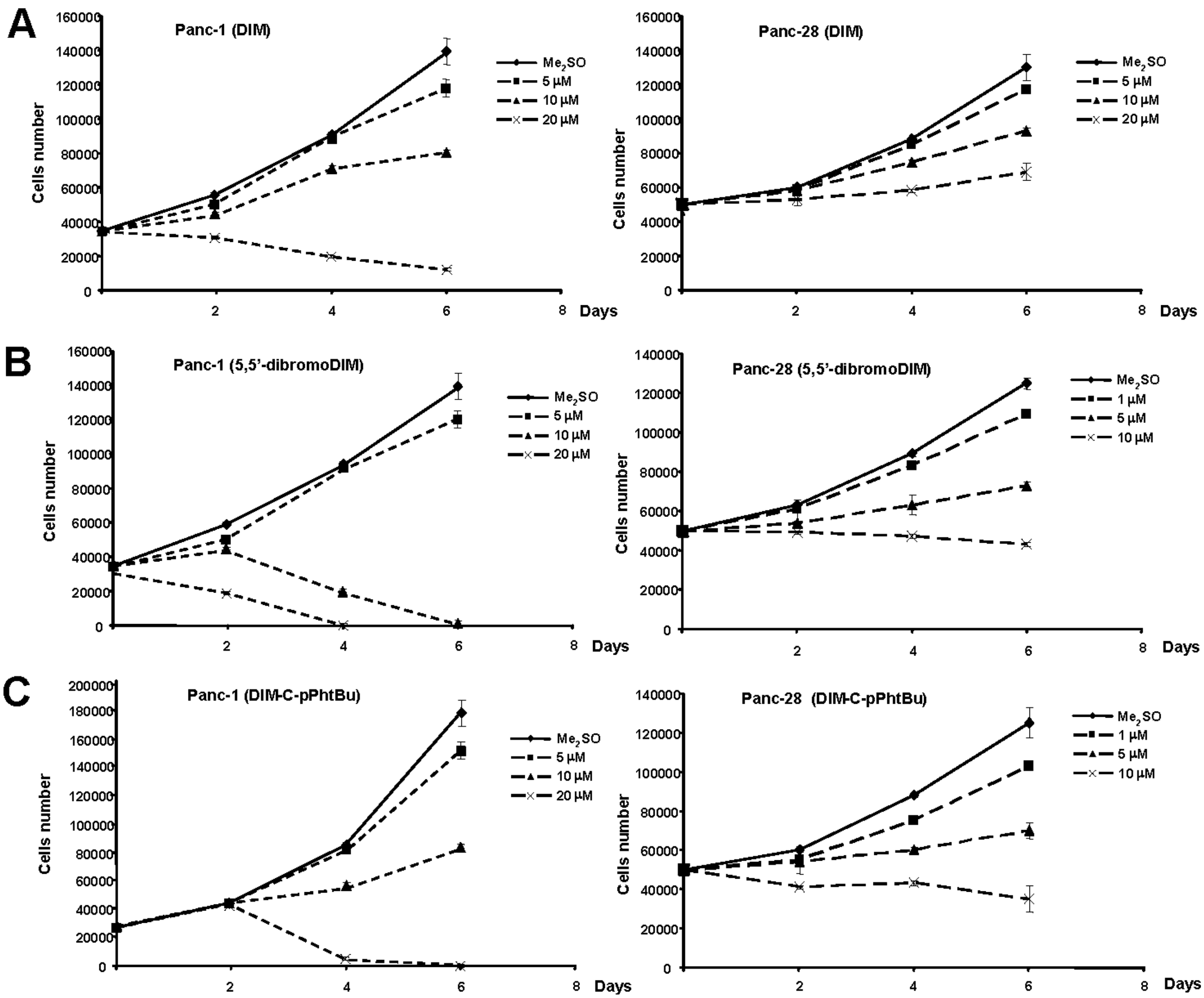


**Fig. 1.** Effects of DIM, 5,5′-dibromoDIM and DIM-c-pPhtBu on Panc-1 and Panc-28 cell proliferation. Cells were treated with $Me_2SO$ (solvent control) and different concentrations of DIM (**A**), 5,5′-dibromoDIM (**B**) or DIM-C-pPhtBu (**C**), and the number of cells in the $Me_2SO$ and chemical-treated groups were determined as described in Materials and methods. Between 1 and 5 μM DIM-C-pPhtBu, 5,5′-dibromoDIM or 5–10 μM DIM significantly ($P < 0.05$) decreased Panc-1 and Panc-28 cell proliferation. Doses of 5–10 μM for 5,5′-dibromoDIM and DIM-C-pPhtBu and 20 μM DIM decreased the number of Panc-1 and Panc-28 cells below the number of cells initially seeded.

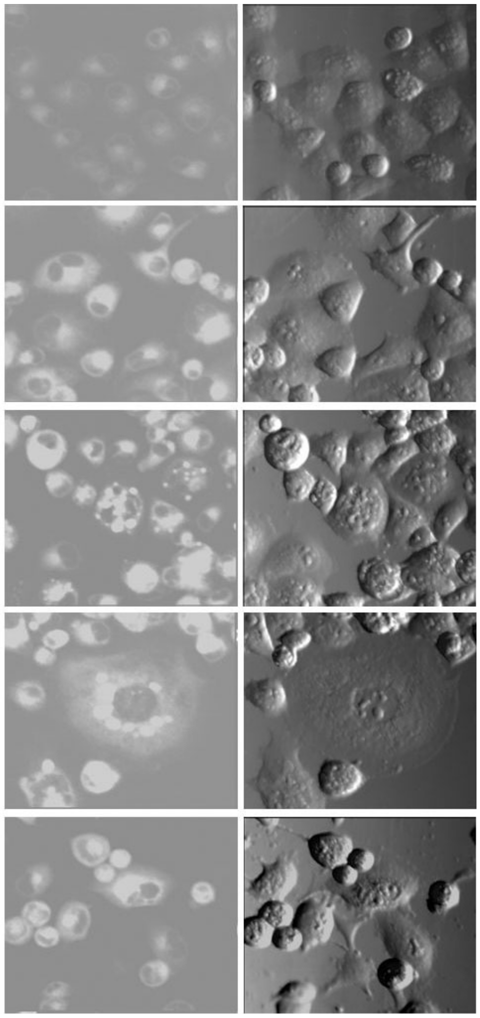


**Fig. 2.** Enhanced ER staining induced by DIM compounds and Tg. Panc-1 cells were treated with $Me_2SO$, 20 μM DIM, 10 μM 5,5′-dibromoDIM, 10 μM DIM-C-pPhtBu or 0.5 μM Tg for 18 h, and were then treated with ER Tracker Blue-White DPX for ER staining as described in Materials and methods.

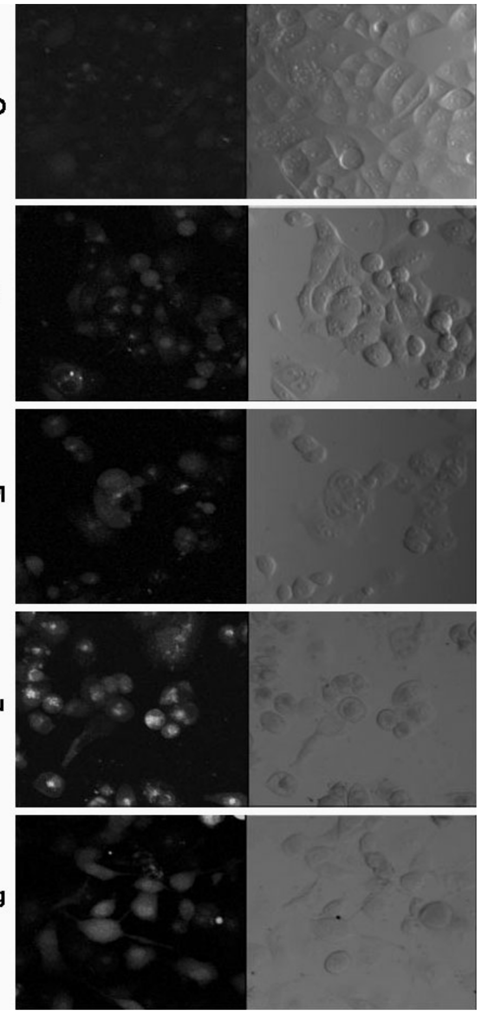


**Fig. 3.** ER calcium ion release. Panc-1 cells were treated with $Me_2SO$ (solvent), 20 μM DIM, 10 μM 5,5′-dibromoDIM, 10 μM DIM-C-pPhtBu and 0.5 μM Tg for 18 h, and were treated with fluo-3 for $Ca^{2+}$ staining as described in Materials and methods.

*Induction of GRP78 and CHOP by DIM Compounds*

One of the hallmarks of the UPR involves cleavage of ATF6 and the subsequent induction of GRP78 and CHOP. Results of initial studies in Panc-28 cells showed that after treatment with Tg, DIM-C-pPhtBu or DIM for 18 h there was significant cleavage of p90ATF6 to give p50ATF6 (Fig. 4A), and 5,5′-dibromoDIM treatment also induced cleavage of ATF6 (data not shown). Western blot analysis of whole cell lysates from Panc-1 and Panc-28 cells treated with Tg, Tm, DIM, 5,5′-dibromoDIM or DIM-C-pPhtBu for 18 h showed that GRP78 and CHOP protein levels were induced by these compounds (Fig. 4B and C). Quantification of GRP78 protein levels from three separate determinations shows that significant induction was observed for the five compounds in both cell lines. The results in Figure 4D and E show that the DIM compounds also activated transcription in Panc-1 cells transfected with pGRP78 and pCHOP which contain ER stress-responsive promoter inserts from the human GRP78 and CHOP genes linked to firefly luciferase. Similar results were obtained after transfection of pGRP78 and pCHOP in Panc-28 cells, respectively (data not shown). The GRP78 and CHOP promoters contain two or more ER stress elements (ERSEs), and gel mobility shift assays were used to investigate increased binding of nuclear extracts from Panc-28 cells treated with Tg, DIM and DIM-C-pPhtBu to $^{32}$P-radiolabeled ERSEs from the GRP78 (Fig. 5A) and CHOP (Fig. 5B) promoters. The results show that DIM, DIM-C-pPhtBu and Tg induce the ERSF retarded band complex compared with $Me_2SO$ (lane 2) using the $^{32}$P-labeled −94GRP78-ERSE and −103CHOP-ERSE (lanes 3–5) (45). Coincubation with excess unlabeled GRP78 or CHOP ERSEs decreased the ERSF retarded band as well as NFY–DNA and YY1–DNA complexes (lane 6). The increased intensity of the ERSF–ERSE complex using extracts from cells treated with DIM, Tg or DIM-pPhtBu (lanes 3–5) was due, in part, to cleavage and translocation of p50ATF6, which bind to this complex (45). These results, combined with the effects of the DIM compounds on $Ca^{2+}$ release (Fig. 3) indicate that ER stress pathways are activated by the di- and triarylmethane compounds containing two 3′-indolyl substituents.

*Induction of DR5 by Tg and DIM compounds*

A recent report showed that Tg-induced ER stress in HCT-116 colon and other cancer cell lines was accompanied by

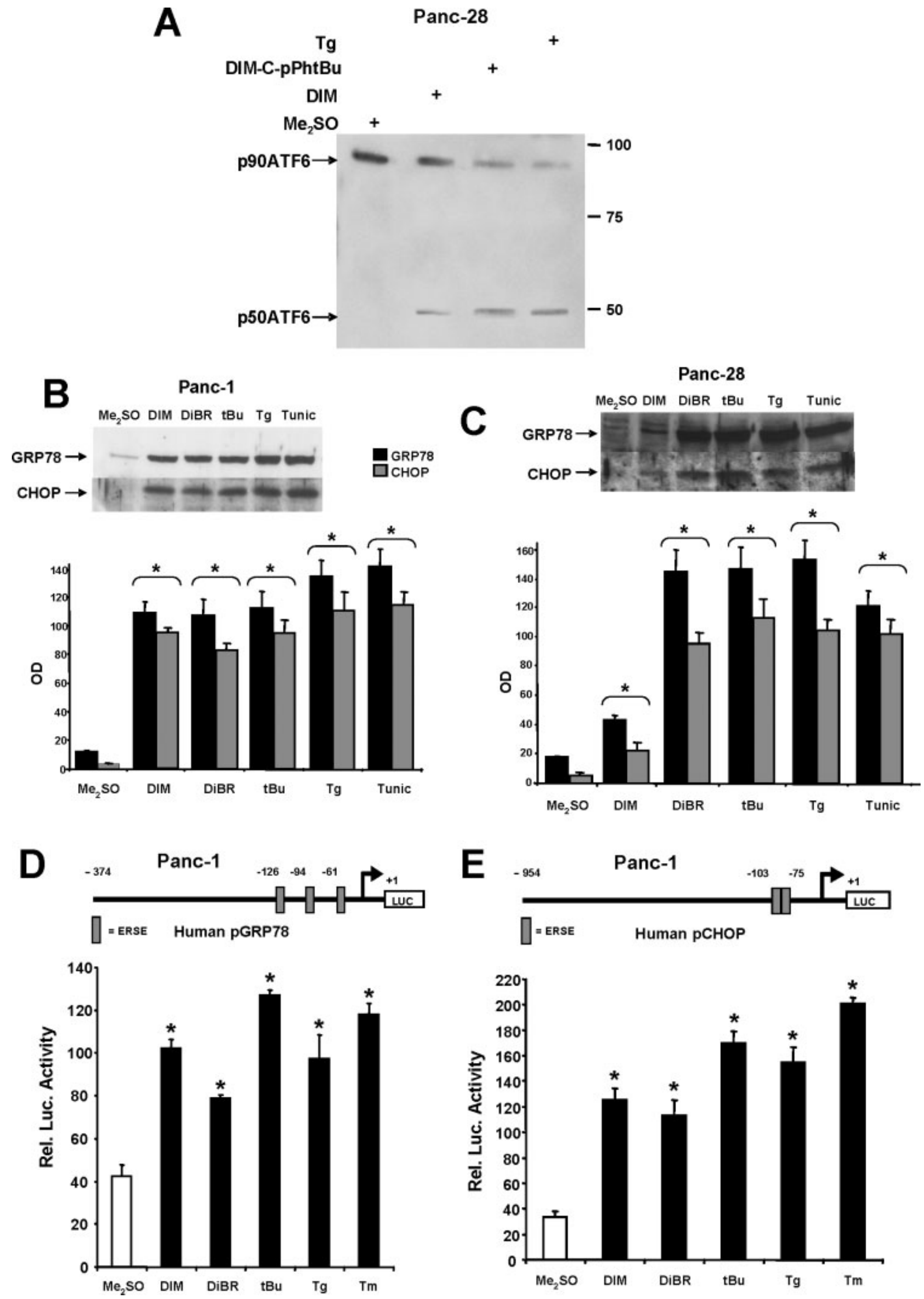


**Fig. 4.** Activation of markers of ER stress in pancreatic cancer cells by DIM compounds. (**A**) Cleavage of p90ATF6. Panc-28 cells were treated with $Me_2SO$, 10 μM DIM-C-pPhtBu, 20 μM DIM and 0.5 μM Tg for 18 h, and whole cell lysates were analyzed for p90ATF6/p50ATF6 by western blot analysis as described in Materials and methods. Induction of CHOP and GRP78 in Panc-1 (**B**) and Panc-28 (**C**) cells. Cells were treated with $Me_2SO$, 10 μM 5,5′-dibromoDIM, 10 μM DIM, 10 μM DIM-C-pPhtBu, 0.5 μM Tg or 0.5 μg/ml Tm for 18 h, and whole cell lysates were analyzed for GRP78 and CHOP proteins by western blot analysis as described in Materials and methods. The analysis was carried out three times for each treatment group, and levels of GRP78 protein were quantified and are presented as mean ± SE in the bar graphs. Significant ($P < 0.05$) induction by the compounds compared with $Me_2SO$ is indicated by an asterisk. GRP78 band intensities were quantified relative to a non-specific (NS) band for normalizing protein loading. Induction of transactivation in Panc-1 cells transfected with pGRP78 (**D**) and pCHOP (**E**) constructs, respectively. Cells were transfected with pGRP78 or pCHOP, treated with $Me_2SO$, 10 μM 5,5′-dibromoDIM, 20 μM DIM, 10 μM DIM-C-pPhtBu, 0.5 μM Tg and 0.5 μg/ml Tm, and luciferase activity was determined as described in Materials and methods. Results are expressed as mean ± SE for three separate determinations for each treatment group and significant ($P < 0.05$) induction is indicated by an asterisk.

increased transcription and translation of the death receptor DR5 and this was directly linked to the activation of CHOP, which is a critical *trans*-acting factor in the upregulation of DR5 (46). The results in Figure 6A illustrate the induction of DR5 protein by western blot analysis of whole cell lysates from Panc-28 cells treated with DIM, Tg, Tm and DIM-C-pPhtBu for 18 h. All compounds significantly induced levels of the CHOP protein. The DR5 promoter contains a *cis*-acting CHOP-like binding sequence at −276 (GAGGATTGCGTTG) (46), and this sequence was used to

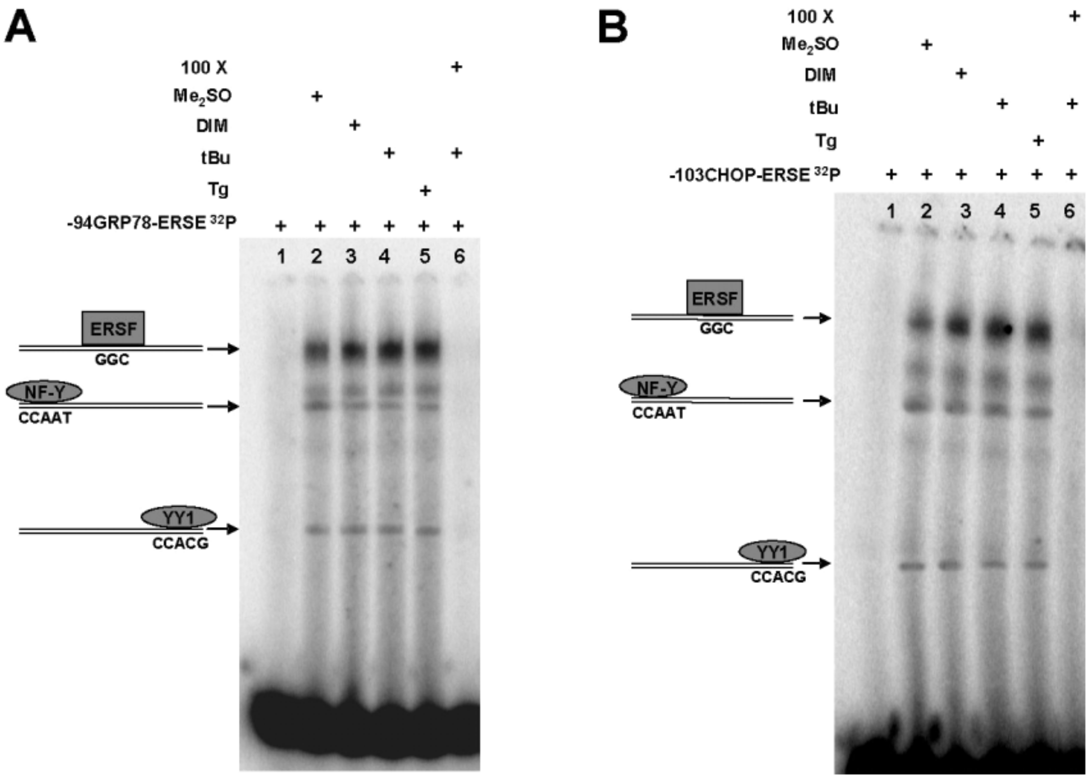


**Fig. 5.** Binding of nuclear extracts to $^{32}$P-labeled −94GRP78/ERSE (**A**) and −103CHOP/ERSE (**B**). Panc-28 cells were treated with $Me_2SO$, 10 μM DIM-C-pPhtBu, 20 μM DIM and 0.5 μM Tg for 18 h, and nuclear extracts ± antibodies or unlabeled oligonucleotides were incubated with radiolabeled ERSEs and analyzed by gel mobility shift assays as described in Materials and methods.

determine chemical-induced DNA–CHOP interactions in a gel mobility shift assay (Fig. 6B). There was constitutive binding of nuclear extracts from solvent ($Me_2SO$)-treated Panc-28 cells to the CHOP element (lane 2); however, the identities of protein–DNA moieties in this complex have not been identified (46). Extracts from cells treated with DIM (lane 3) or DIM-C-pPhtBu (lane 4) gave an increased retarded band complex. Coincubation with CHOP antibody (lane 5), but not IgG (lane 6), decreased intensity of the least retarded portion (upper part) of the retarded band formed with extracts from DIM-C-pPhtBu-treated cells; however, a supershifted ternary complex was not detected. Nuclear extracts from cells treated with Tm and Tg also gave an enhanced retarded band (lanes 7 and 8), and coincubation of nuclear extracts from Panc-28-treated cells with 100-fold excess of the unlabeled −276DR5 eliminated the radiolabeled retarded band. Results in Figure 6C used increasing amounts of CHOP antibody to further demonstrate CHOP immunodepletion and decreased intensity of the CHOP–DNA retarded band. These results demonstrate that elevated ER stress in Panc-28 cells after treatment with DIM, DIM-C-pPhtBu, Tg or Tm induced CHOP protein (Fig. 6A) and enhanced binding to the CHOP element in the DR5 promoter (Fig. 6B and C). Previous reports on sodium butyrate and bile acid activation of DR5 suggested that upregulation of this receptor depended on enhanced Sp1-DNA binding linked to GC-rich sites in the DR5 promoter (46,47). Analyses of nuclear extracts from cells treated with $Me_2SO$, DIM, DIM-C-pPhtBu and Tg (lanes 2–5) (Fig. 6D), binding to the $^{32}$P-labeled −198DR5-GC oligonucleotide (contains the proximal GC-rich motif in the DR5 promoter), showed that retarded band intensities were similar in all treatment groups. Unlabeled oligonucleotide (lane 6) decreased and antibodies for Sp1 (lane 7) and Sp3 (lane 8) supershifted the retarded bands. Thus, induction of ER stress was not accompanied by altered Sp1-DNA binding. We also investigated the effects of Tg and DIM-C-pPhtBu on the induction of CHOP binding to the DR5 promoter (Fig. 7). As a positive control for this experiment, we showed that TFIIB binds the glyderaldehyde-3-phosphate dehydrogenase (GAPDH) promoter but not exon 1 of the CNAP1 gene (Fig. 7A) (27,48). Both Tg and DIM-C-pPhtBu induce binding of CHOP to the DR5 promoter after 3 and 6 h, respectively (Fig. 7B), using primers that target the CHOP element in the DR5 promoter (Fig. 7C).

Results summarized in Figure 8 confirm transcriptional activation of DR5 in Panc-1 and Panc-28 cells transfected with pDR5a, pDR5b and pDR5c, which contain the −991 to −7, −552 to −7, and −216 to −7 DR5 promoter inserts, respectively (46). In Panc-1 cells transfected with pDR5a and pDR5b, DIM, DIM-C-pPhtBu and Tg significantly induced transactivation (Fig. 8A and B), whereas these same compounds did not induce transactivation in Panc-1 cells transfected with pDR5c, which does not contain a CHOP element but does contain the proximal GC-rich motif that binds the Sp1 protein (Fig. 6C). In a similar experiment, in Panc-28 cells transfected with pDR5b or pDR5c, induced luciferase activity by DIM, DIM-C-pPhtBu and Tg was only observed in cells transfected with pDR5b. These results confirm that chemical-induced ER stress in at least two pancreatic cancer cell lines is accompanied by the induction of DR5 which is also dependent on activation of CHOP but is independent of activation of the proximal GC-rich motif at −198.

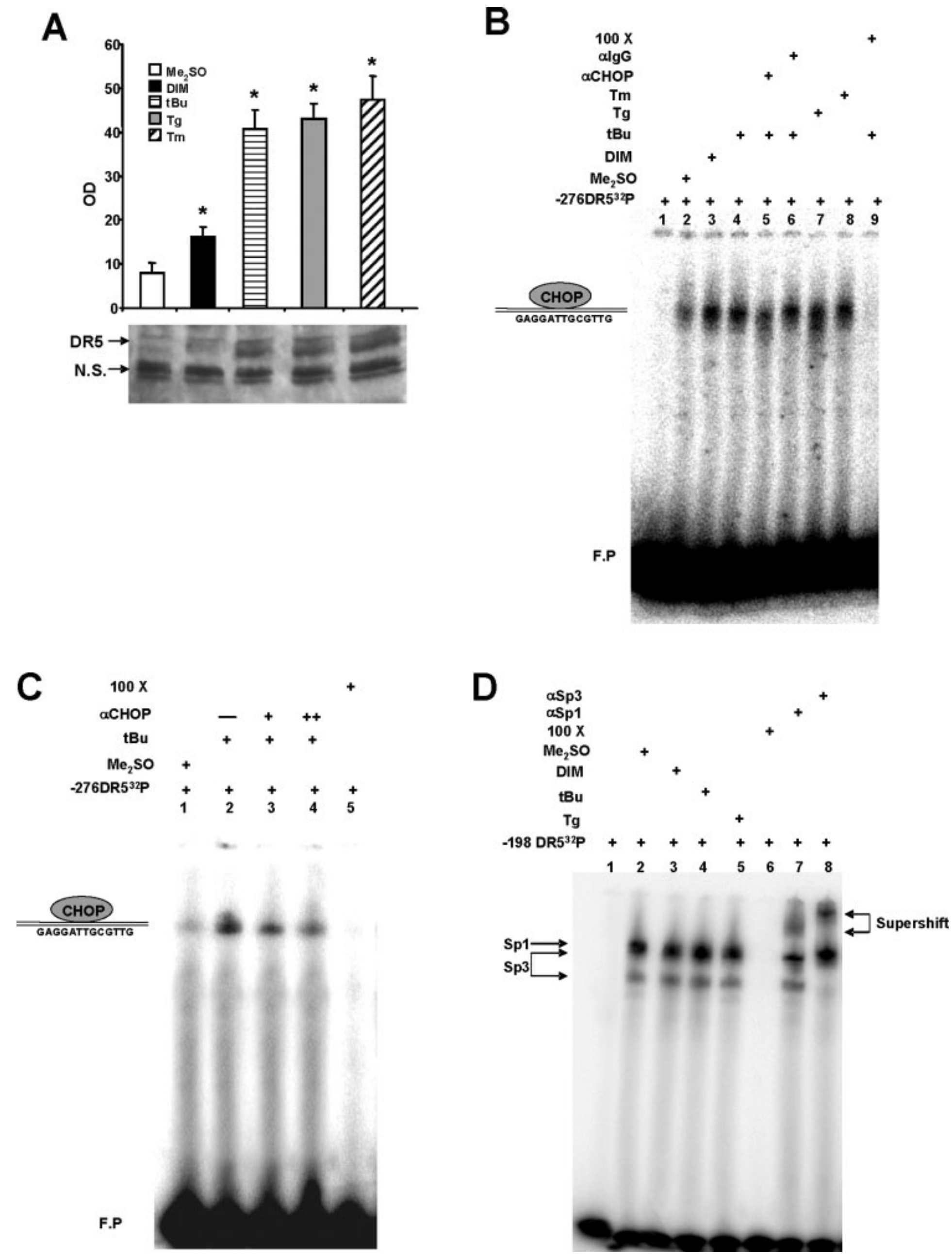


**Fig. 6.** Induction of DR5 protein and the analysis of binding to DR5 promoter sequences. (**A**) Induction of DR5 protein. Panc-28 cells were treated with $Me_2SO$, 20 μM DIM, 10 μM DIM-C-pPhtBu, 0.5 μM Tg and 0.5 μg/ml Tm, and whole cell lysates were analyzed by western blot analysis as described in Materials and methods. Results are presented as mean ± SE for three separate determinations for each treatment group, and significant ($P < 0.05$) induction (compared with $Me_2SO$) is indicated by an asterisk. DR5 band intensities were quantified relative to a non-specific (NS) loading control. Binding of nuclear extracts to $^{32}$P-labeled −276DR5 (**B**) and −198DR5 (**C** and **D**). Nuclear extracts from Panc-28 cells treated with 20 μM DIM, 10 μM DIM-C-pPhtBu, 0.5 μM Tg or 0.5 μg/ml Tm were incubated with radiolabeled oligonucleotides, antibodies or unlabeled oligonucleotides, and were analyzed in gel-mobility shift assays. Specifically bound retarded bands and supershifted complexes are indicated by arrows.

*Activation of the extrinsic apoptotic pathway by DIM compounds*

Tg-induced CHOP and upregulation of DR5 activates the extrinsic apoptotic pathway in HCT116 cells (42), and we therefore investigated the comparative effects of DIM, DIM-C-pPhtBu, Tg and Tm on events downstream from DR5 in Panc-28 cells. Treatment of these cells with DIM, DIM-C-pPhtBu, Tg and Tm decreased procaspase 8 and increased caspase 8 protein levels (Fig. 9A). In parallel studies, the caspase 8-dependent substrate BiD was cleaved (Fig. 9B) and procaspase 3 was also degraded (Fig. 9C). Activation of these pathways by Tg, DIM-C-pPhtBu and DIM was accompanied by induction of PARP cleavage (Fig. 9D), which was inhibited by the caspase 8 (Z-IETD-FMK) and pan-caspase (Z-VAD-FMK) inhibitors. In addition, these compounds also induced apoptosis as determined by increased Annexin V staining (Fig. 9E). These results are consistent with the DIM compound-dependent activation of ER stress pathways in pancreatic cancer cells, and this response induces apoptosis through upregulation of CHOP, which, in turn, activates DR5.

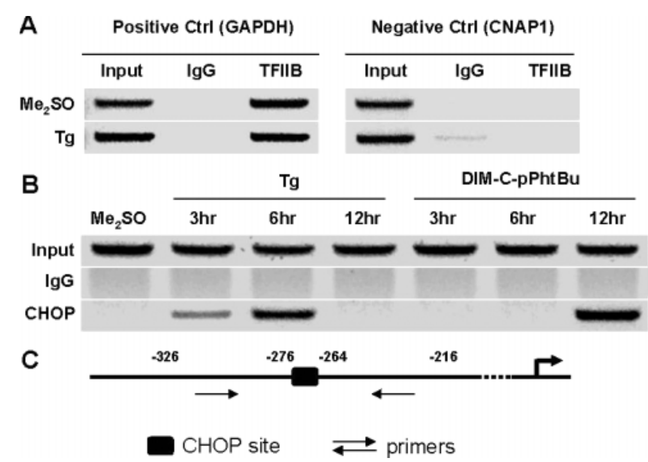


**Fig. 7.** CHOP interactions with the DR5 promoter-ChIP assay. (**A**) TFIIB binding to GAPDH. Cells were treated with $Me_2SO$ or Tg, and interactions of TFIIB with the GAPDH promoter or CNAP1 exon (negative control) were determined in a ChIP assay as described in Materials and methods. Binding of CHOP (**B**) to the proximal region of the DR5 promoter (**C**). Panc-1 cells were treated with $Me_2SO$, 0.5 μM Tg or 10 μM DIM-C-pPhtBu for 3, 6 or 12 h, and interactions of CHOP with the DR5 promoter were determined in a ChIP assay as described in Materials and methods. IgG served as a non-specific control for the immunoprecipitation experiments, and primers designed for determining ChIP-DR5 promoter interactions are summarized in Materials and methods.

## Discussion

DIM, ring-substituted DIMs and C-DIMs inhibit growth of carcinogen-induced mammary tumors at doses of 1–5 mg/kg/48 h (22–25). DIM and ring-substituted DIMs bind the AhR (22–24) and selected C-DIMs activate PPARγ (25), and their inhibition of mammary tumor growth may be related, in part, to receptor-mediated responses (26–39). Pancreatic cancer cells express the AhR and PPARγ, and previous studies indicate that DIM and other AhR agonists and PPARγ-active C-DIMs inhibit growth of pancreatic cancer cells (27,49). However, it is apparent from *in vitro* studies that many of the anticarcinogenic activities of DIM compounds are receptor-independent. For example, DIM inhibits growth of both Ah-responsive and Ah-non-responsive breast cancer cells, and growth inhibitory responses of PPARγ-active C-DIMs in MCF-7 cells were also PPARγ-independent (31). The growth inhibitory and apoptotic responses observed in cancer cells treated with DIM have been linked to the modulation of a number of genes/proteins. One study reported that DIM decreased bcl-2/bax ratios in MCF-7 cells (36), whereas another report indicated that DIM activated ER stress-dependent GRP78/CHOP genes/proteins in the same cell line. Rahman and Sarkar (35) recently reported that DIM induced apoptosis in an MCF-7 derived tumorigenic cell line by



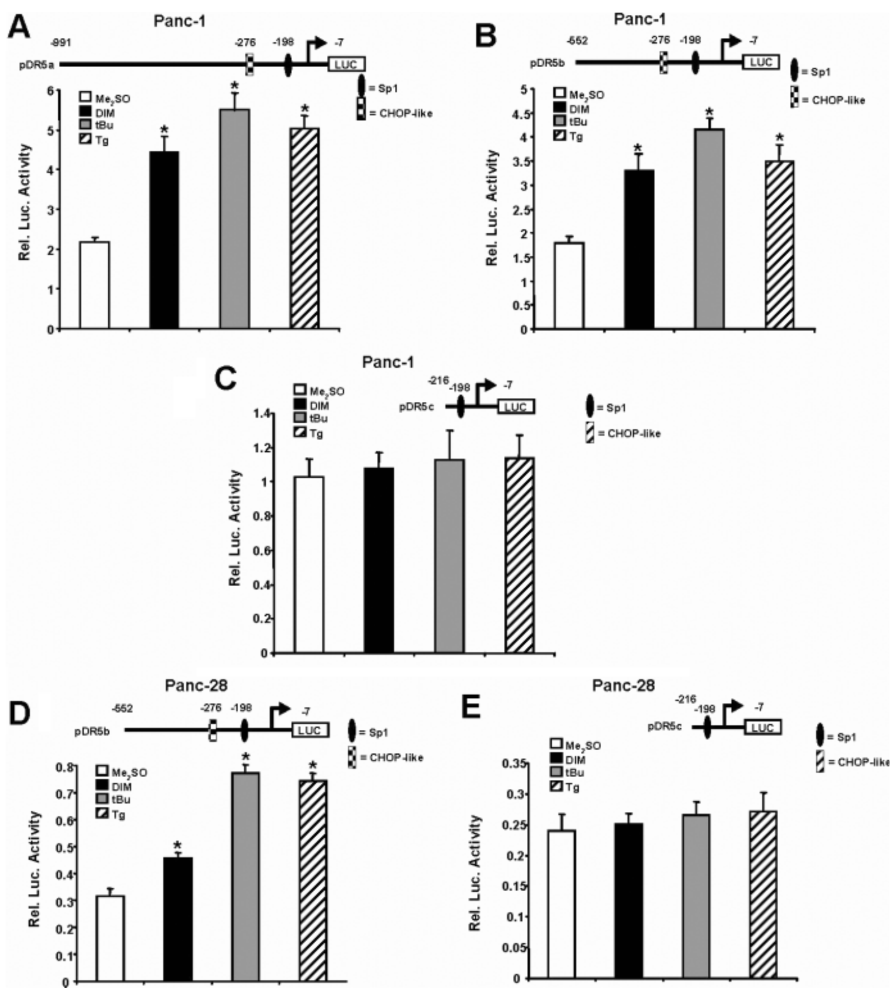


**Fig. 8.** Activation of DR5 promoter constructs. Panc-1 (**A–C**) and Panc-28 cells (**D** and **E**) were transfected with pDR5a, pDR5b or pDR5c constructs, treated with $Me_2SO$, 20 μM DIM, 10 μM DIM-C-pPhtBu or 0.5 μM Tg, and luciferase activity determined as describe in Materials and methods. Results are expressed as mean ± SE for three separate determinations for each treatment group, and significant ($P < 0.05$) induction is indicated by an asterisk.

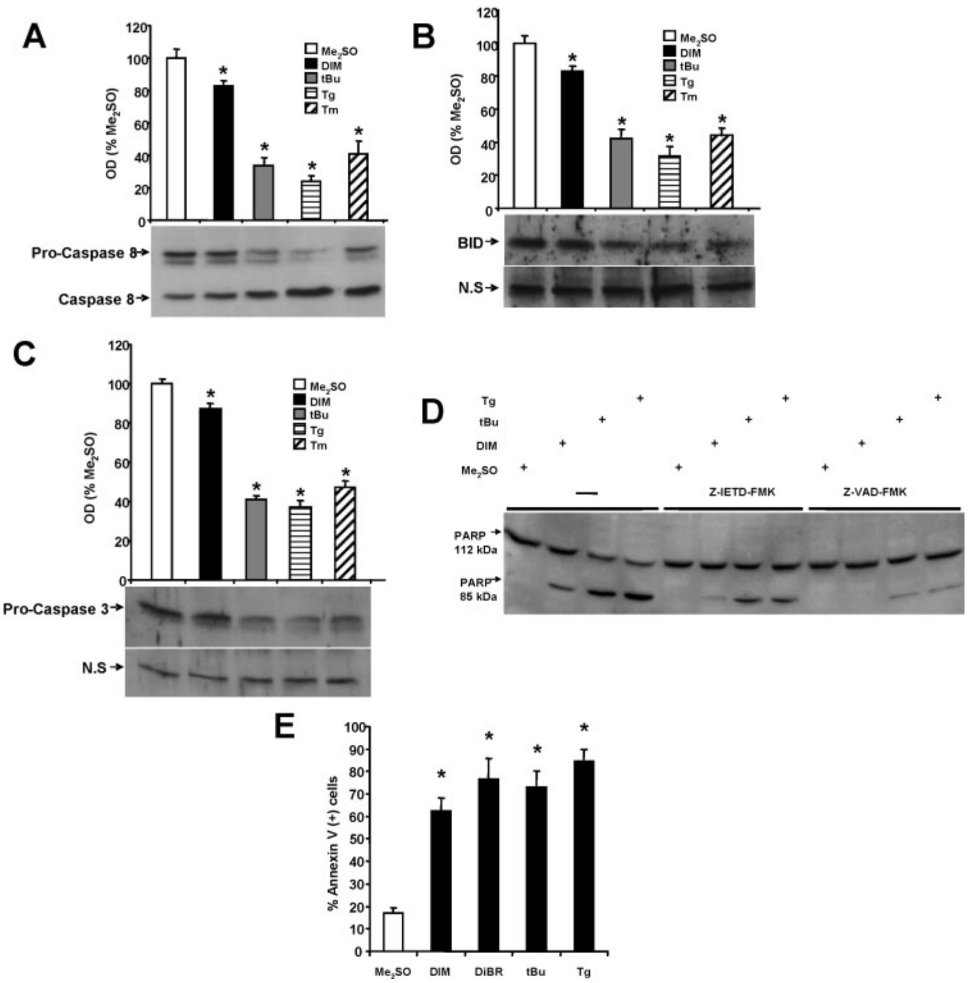


**Fig. 9.** Activation of apoptosis by DIM compounds. Caspase 8 and procaspase 8 (**A**), Bid (**B**) and procaspase 3 (**C**) cleavage proteins were determined in Panc-28 cells treated with $Me_2SO$, 20 μM DIM, 10 μM DIM-C-pPhtBu, 0.5 μM Tg or 0.5 μg/ml Tm for 18 h. Whole cells lysates from three separate determination processes for each treatment group were analyzed by western blot analysis as described in Materials and methods, and significant ($P < 0.05$) induction is indicated by an asterisk. Protein band intensities in the treated versus the $Me_2SO$ control were measured, and results are presented as mean ± SE. Protein loading in each treatment group was standardized using a non-specific (NS) loading control band. (**D**) Induction of PARP cleavage. Panc-1 cells were treated as described above or preincubated for 1 h with 20 μM Z-IETD-FMK or 20 μM Z-VAD-FMK, and whole cell lysates were analyzed by western blot analysis as described in Materials and methods. (**E**) Induction of Annexin V staining. Cells were treated with 20 μM DIM, 10 μM 5,5′-dibromoDIM, 10 μM DIM-C-pPhtBu and 0.5 μM Tg for 24 h, and Annexin V staining was determined as described in Materials and methods. Significant ($P < 0.05$) apoptosis is indicated by an asterisk and results are expressed as mean ± SE for three replicate determinations for each treatment group.

inactivating NFκB activity through inhibition of p65 nuclear translocation.

In this study, we have hypothesized that receptor-independent growth inhibition/apoptosis induced by DIMs and C-DIMs may be due to the activation of common pathways in pancreatic cancer cells. A recent report showed that the PPARγ-active C-DIMs induced p21 and growth inhibition in Panc-28 cancer cells (27); however, PPARγ antagonists such as GW9662 only partially reversed the growth inhibitory responses induced by PPARγ-active C-DIMs in Panc-28 cells and had no effect in Panc-1 cells treated with the same compounds (data not shown). We, therefore, used Panc-1 and Panc-28 cells as models for investigating the effects of DIM, 5,5′-dibromoDIM and DIM-C-pPhtBu on growth/apoptosis. These compounds decreased Panc-1 and Panc-28 cell proliferation (Fig. 1) and this was accompanied by increased staining of the ER (Fig. 2) and ER calcium release (Fig. 3), suggesting that the DIM compounds induce ER stress in Panc-1 cells. This was confirmed by western blot analysis of whole cell lysates from Panc-1 and Panc-28 cells treated with Tg and DIMs, showing induction of ER stress-dependent GRP78 and CHOP proteins, cleavage of p90ATF6 and transactivation in cells transfected with pCHOP/pGRP78 constructs (Fig. 4). DIM and DIM-C-pPhtBu and Tg also induced the intensity of the ERSF retarded band in gel-mobility shift assays using ERSEs from the GRP78 and CHOP gene promoters (Fig. 5), confirming that the DIM compounds induced at least two major pathways associated with ER stress, namely the release of ER calcium stores (Fig. 3) and the induction of GRP78 and CHOP. The latter responses are comparable to those previously observed in breast, prostate and cervical cancer cells treated with DIM (37).

Several studies report that death receptor DR5 is induced by diverse chemicals, such as sodium butyrate, Tg, the synthetic triterpenoid methyl 2-cyano-3,12-dioxooleana-1,9-dien-28-oate, bile acids, histone-deacetylase inhibitors and indole-3-carbinol (46,47,50–53). Relatively high concentrations (90 μM) of indole-3-carbinol were required to induce DR5 in LNCaP prostate cancer cells. However, this was not accompanied by enhanced PARP cleavage or activation of caspase 3

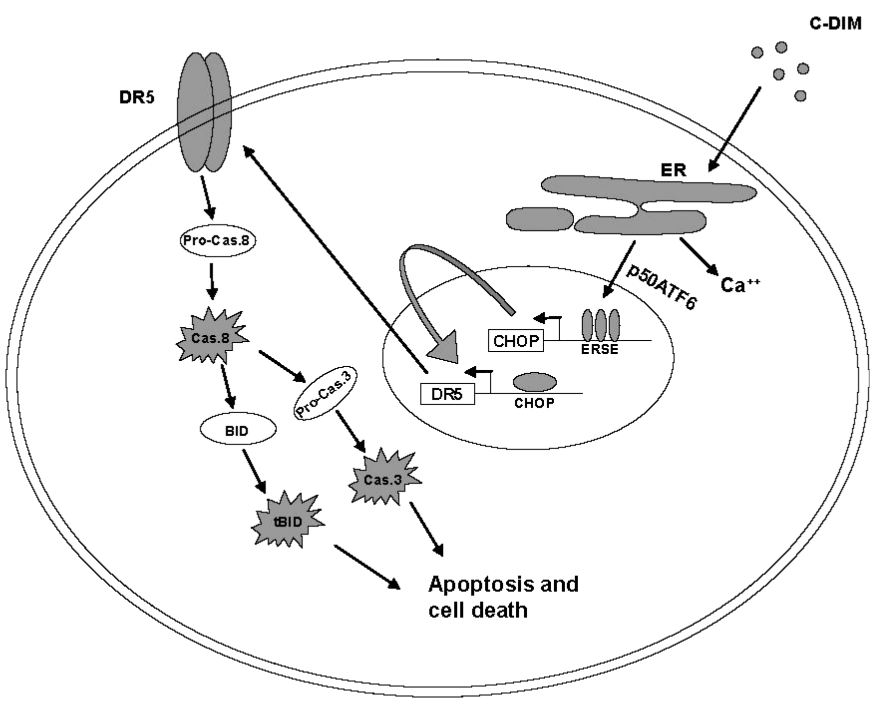


**Fig. 10.** Proposed model for ER-stress-induced apoptosis activated by DIM or DIM-C-pPhtBu in pancreatic cancer cells.



(53), although other studies report that DIM induces apoptosis in prostate cancer cells (38,39). Upregulation of DR5 by both bile acids and sodium butyrate has been linked to a single proximal GC-rich site in the DR5 promoter at −195 (46,47), in which both compounds appear to enhance Sp1-DNA binding to this site, and sodium butyrate also induced apoptosis (34). In contrast, it was reported that CHOP is involved in ER stress-induced DR5 upregulation in HCT116 colon cancer cells by Tg, and this resulted in activation of the extrinsic apoptotic pathway (42). DIM, DIM-C-pPhtBu, Tg and Tm also induced DR5 in Panc-28 cells, and this was accompanied by increased binding of CHOP to a GAGGATTGCGTTG motif (Fig. 6) previously identified in the DR5 promoter (42), and CHOP interactions with the DR5 promoter were confirmed in a chromatin immunoprecipitation assay (Fig. 7). In contrast, there was no evidence for induced binding of Sp1 to the −195 GC-rich DR5 site (Fig. 6D). Moreover, transient transfection studies in Panc-1 and Panc-28 cells (Fig. 8) showed that the constructs containing the CHOP response element and not the −195 GC-rich motif are required for activation of DR5 promoter constructs. Activation of DR5 by DIM and DIM-C-pPhtBu also induced PARP cleavage, a hallmark of apoptosis, and this was accompanied by induction of Annexin V staining, caspase 8, caspase 3 and Bid cleavage (Fig. 9). These results, coupled with the inhibitory effects of the caspase 8 and pan-caspase inhibitors, are consistent with the schematic summary (Fig. 10), which represents the ER stress pathways induced by DIM compounds in Panc-1 and Panc-28 cells.

These results demonstrate that ER stress induced by DIM and DIM-C-pPhtBu is similar to that observed for Tg/Tm and leads to activation of the extrinsic pathway of apoptosis in pancreatic cancer cells. Induction of DR5 through activation of CHOP plays a major role in DIM-/DIM-C-pPhtBu-induced apoptosis and thereby represents an important receptor-independent pathway activated by these compounds in pancreatic cancer cells and a potential mechanism for the anticarcinogenic activity of these compounds. Current studies are focused on the induction of ER stress pathways by DIMs/C-DIMs in other cancer cell lines and the identification of subcellular targets for these compounds (including the ER) that are required for these responses. The simultaneous activation of PPAR$\gamma$-dependent and PPAR$\gamma$-independent pathways by DIM-C-pPhtBu and related compounds in pancreatic and other cancer cell lines (25–28) adds to their potential efficacy for clinical applications in cancer chemotherapy.

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