Activation of Checkpoint Kinase 2 by 3,3'-Diindolylmethane Is Required for Causing G₂/M Cell Cycle Arrest in Human Ovarian Cancer Cells^[S]

Prabodh K. Kandala and Sanjay K. Srivastava

Department of Biomedical Sciences and Cancer Biology Center, Texas Tech University Health Sciences Center, Amarillo, Texas

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ABSTRACT

We evaluated the effect of 3,3'-diindolylmethane (DIM) in ovarian cancer cells. DIM treatment inhibited the growth of SKOV-3, TOV-21G, and OVCAR-3 ovarian cancer cells in both a dose- and time-dependent manner with effective concentrations ranging from 40 to 100 μ M. Growth-inhibitory effects of DIM were mediated by cell cycle arrest in G₂/M phase in all the three cell lines. G₂/M arrest was associated with DNA damage as indicated by phosphorylation of H₂A.X at Ser139 and activation of checkpoint kinase 2 (Chk2) in all the three cell lines. Other G₂/M regulatory molecules such as Cdc25C, Cdk1, cyclin B1 were down-regulated by DIM. Cycloheximide or Chk2 inhibitor pretreatment abrogated not only activation of Chk2 but also G₂/M arrest and apoptosis mediated by DIM. To further establish the involvement of Chk2 in DIM-mediated G₂/M arrest, cells

were transfected with dominant-negative Chk2 (DN-Chk2). Blocking Chk2 activation by DN-Chk2 completely protected cells from DIM-mediated G₂/M arrest. These results were further confirmed in Chk2 knockout DT40 lymphoma cells, in which DIM failed to cause cell cycle arrest. These results clearly indicate the requirement of Chk2 activation to cause G₂/M arrest by DIM in ovarian cancer cells. Moreover, blocking Chk2 activation also abrogates the apoptosis-inducing effects of DIM. Furthermore, our results show that DIM treatment cause ROS generation. Blocking ROS generation by *N*-acetyl cysteine protects the cells from DIM-mediated G₂/M arrest and apoptosis. Our results establish Chk2 as a potent molecular target of DIM in ovarian cancer cells and provide the rationale for further clinical investigation of DIM.

Ovarian cancer is a major cause of deaths among the female population in United States and European countries (Jemal et al., 2008). Ninety percent of cases of ovarian cancer are of epithelial origin. Activation of oncogenes such as B-Raf, PTEN, β -catenin, and K-Ras (Shih and Kurman, 2004) and inactivation or mutations in tumor suppressor genes such as *BRCA-1* or *BRCA-2* and *P53* are associated with ovarian cancer (Goodheart et al., 2002; McPherson et al., 2004). Studies suggest that inactivation of Chk2 similar to p53 results in increased survival and proliferation of cancer cells, indicating it as a tumor suppressor gene (McPherson et

al., 2004). Given its role in DNA repair, Chk2 is considered to be an important molecular target in ovarian cancer (Wang et al., 2007). There are no sufficiently accurate screening tests to diagnose this malignancy at its dormancy. Hence, it is usually identified in late stages with poor prognosis. Chemotherapy and radiotherapy are currently in use in patients with ovarian cancer (Pickel et al., 1999). However, these are associated with resistance or damage to normal cells. Hence, novel approaches targeting ovarian cancer at the molecular level are needed.

Epidemiological studies continue to indicate an inverse relationship between the consumption of cruciferous vegetables and risk of cancer of ovary, breast, lung, and pancreas (Bosetti et al., 2001; Zhang et al., 2002; Pan et al., 2004). 3,3'-Diindolylmethane (DIM) is an active metabolite of indole-3-carbinol present in cruciferous vegetables such as cabbage, broccoli, and kale (Grose and Bjeldanes, 1992). Previous studies have indicated that DIM has antiproliferative

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ABBREVIATIONS: Chk2, checkpoint kinase 2; DIM, 3,3'-diindolylmethane; DN-Chk2, dominant-negative checkpoint kinase 2; ROS, reactive oxygen species; NAC, *N*-acetyl cysteine; FBS, fetal bovine serum; DCFDA, 2'-7'-dichlorofluorescein diacetate; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; dbs, double-strand breaks; ATCC, American Type Culture Collection; CHX, cycloheximide; KO, knock out; Cdc25C, cell division cycle 25C; CDK, cyclin-dependent kinase; ATM, ataxia telangiectasia mutated; MG132, *N*-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal.

activity against various cancers (Hong et al., 2002a; Chen et al., 2006; Rahman et al., 2006; Kong et al., 2007; Bhatnagar et al., 2009). DIM has been shown to suppress cancer growth by inhibiting oncogenic molecules such as nuclear factor- κ B, Akt, and β -catenin in breast and prostate cancers (Chen et al., 2006; Rahman et al., 2006; Kong et al., 2007). Anti-apoptotic molecules such as Bcl-2 and proapoptotic proteins like Bax were also regulated by DIM (Hong et al., 2002a). Inhibition of H^+ -ATP synthase by DIM leads to the induction of p21 in breast cancer cells (Gong et al., 2006). DIM potentiates the effect of erlotinib, an epidermal growth factor receptor inhibitor, to suppress the growth of pancreatic cancer cells in vivo in orthotopic model (Ali et al., 2008). However, the exact mechanism by which DIM causes its antiproliferative effects is not clear, and the effect of DIM on ovarian cancer is not known. Clinical trials to evaluate the efficacy of DIM against prostate and cervical cancer are currently underway.

In the present study, we demonstrate the antiproliferative effects of DIM in human ovarian cancer cells. The growth-suppressive effects of DIM were associated with G_2/M cell cycle arrest. Our studies established that the cell cycle arrest by DIM was due to the activation of Chk2. Blocking the activation of Chk2 by Chk2 inhibitor, DN-Chk2, or by using Chk2 KO cells abrogated DIM-mediated G_2/M cell cycle arrest and protected the cells from apoptosis, indicating Chk2 as a potent molecular target of DIM in ovarian cancer cells.

Materials and Methods

Chemicals. BR-DIM was a kind gift from Dr. Michael Zeligs (Bio Response, Boulder, CO). We refer to BR-DIM as DIM in our studies. Sulforhodamine B, RNase A, propidium iodide, ampicillin, Luria broth, actin antibody, *N*-acetyl cysteine (NAC), trichloroacetic acid, medium 199, and MCDB 105 were obtained from Sigma-Aldrich (St. Louis, MO). Cycloheximide was obtained from Thermo Fisher Scientific (Waltham, MA), MG132 and Chk2 inhibitor 2-[4-(4-chlorophenoxy)phenyl]-*H*-benzoyl-imidazole-5-carboxylic acid amide was purchased from Calbiochem (San Diego, CA). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA). Antibodies against checkpoint kinase 2 (Chk2), phospho-Chk2 (Thr68), cell division cycle 25C (Cdc25C), phospho-Cdc25C (Ser216), phospho- $H_2A.X$ (Ser139), cyclin B1, Cdk1, p21, Cdk2, cyclin D1, and DNA polymerase β 1 were from Cell Signaling Technology (Danvers, MA). RPMI 1640 medium, McCoy 5A medium, F12K medium, trypsin, heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin antibiotic mixture were from Mediatech Inc. (Manassas, VA). Dulbecco's modified Eagle's medium was from the American Type Culture Collection (ATCC; Manassas, VA). Lipofectamine, Opti-MEM, and DCFDA were obtained from Invitrogen (Carlsbad, CA). Plasmid Midi kit to extract DNA was from QIAGEN (Valencia, CA). The DeadEnd Fluorometric TUNEL System kit was purchased from Promega (Madison, WI). Agarose A beads were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Apoptosis kit was purchased from EMD Biosciences (San Diego, CA).

Cell Culture. SKOV-3, OVCAR-3, and TOV-21G cell lines were obtained from ATCC. All the three cell lines were well differentiated epithelial ovarian adenocarcinoma's obtained from Caucasian female subjects. SKOV-3 cells were maintained in McCoy's 5A medium supplemented with 10% FBS. OVCAR-3 was maintained in RPMI medium supplemented with 20% FBS, 1 mM sodium pyruvate, 10 mM HEPES, 10 mg/l bovine insulin, and 4.5g/l glucose. TOV-21G cells were maintained in 1:1 mixture of MCDB 105 and Medium 199 supplemented with 15% FBS. 1% penicillin-streptomycin-neomycin antibiotic mixture was added to all of the above media. Chk2 knock-

out DT40 lymphoma cells, a kind gift from Dr. Mark Walker (University of Glasgow, Glasgow, UK), were maintained in Dulbecco's modified Eagle's medium with 10% FBS, 1% chick serum, 10 μ M β -mercaptoethanol, 1% penicillin-streptomycin-neomycin, and 2 mM L-glutamine. In addition, MDA-MB-231 and PC-3 cells were also obtained from ATCC and cultured in RPMI and F12K media, respectively. All of the cell lines were maintained in a humidified incubator with 5% $CO_2/95\%$ air. A 100 mM stock solution of DIM in a 1:4 H_2O /dimethyl sulfoxide mixture was prepared freshly before the experiment.

Sulforhodamine Cell Survival Assay. Approximately 5000 cells in 0.1 ml of medium were plated in 96-well plates and allowed to attach overnight. Desired concentrations of DIM were added to the cells and incubated at 37°C for 24, 48, and 72 h. The cells were then processed and stained with 0.4% sulforhodamine B solution, and the absorbance was read at 570 nm using a plate reader (BioTek Instruments, Winooski, VT) as described previously (Zhang et al., 2008; Sahu and Srivastava, 2009).

Flow Cytometric Analysis. The effect of DIM on cell cycle distribution was assessed by flow cytometry after staining the cells with propidium iodide. Stained cells were analyzed by using a FACSCaliber flow cytometer (BD Biosciences, San Jose, CA) as described previously (Sahu et al., 2009). Cell cycle data were reanalyzed using CellQuest Pro software (BD Biosciences). Approximately 2×10^4 cells were evaluated for each sample. In all determination, cell debris and clumps were excluded from the analysis.

Western Blot Analysis. SKOV-3, OVCAR-3, TOV-21G, MDA-MB-231, and PC-3 cells were exposed to varying concentrations of DIM for the indicated time periods. Cells were washed twice with ice-cold phosphate-buffered saline and lysed as described by us previously (Sahu and Srivastava, 2009). Protein content was determined using Bradford reagent, and lysate containing 20 to 80 μ g of protein was subjected to SDS gel electrophoresis followed by immunoblotting as described previously (Sahu and Srivastava, 2009).

Cdk1 Kinase Activity. Cdk1 kinase activity was performed using Cdk1 kinase assay kit (Millipore, Billerica, MA) with some modifications as described previously (Zhang et al., 2006). Control and DIM-treated SKOV-3 cells were lysed as described previously (Sahu and Srivastava, 2009). Approximately 500 μ g of control and treated cell lysate was incubated with 3 μ g of Cdk1 antibody for 2 h at 4°C followed by the addition of 35 μ l of protein A agarose, and the complex was left on a rocker overnight at 4°C. The kinase activity was determined using the kit according to manufacturer's instructions.

Immunoprecipitation. Approximately 10^6 cells were plated and treated with different concentrations of DIM for 24 h. Control and treated cells were washed twice with ice-cold phosphate-buffered saline and lysed on ice with Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH, 7.4, 150 mM NaCl, 5 mM EDTA, and 1% Nonidet P-40) containing 2 mM Na_3VO_4 , 2 mM EGTA, 12 mM β -glycerol phosphate, 10 mM NaF, 16 mg/l benzamidinium hydrochloride, 10 mg/l phenanthroline, 10 mg/l aprotinin, 10 mg/l leupeptin, 10 mg/l pepstatin, and 1 nM phenylmethylsulfonyl fluoride. The cell lysate was cleared by centrifugation at 14,000g for 15 min. Approximately 10 μ g of Chk2 antibody was added to 300 μ g of lysate obtained from control and treated cells and incubated overnight at 4°C. Agarose A beads (50 μ l) were added to the above mixture and incubated for 5 h with gentle rocking. Lysates were then collected by centrifuging at 1000g for 1 min, and supernatant was separated. Beads were then washed five times with lysis buffer according to the protocol provided by Cell Signaling Technology. Chk2 protein from each sample was eluted with 40 μ l of 1% SDS and then subjected to SDS-polyacrylamide gel electrophoresis, and the proteins were blotted onto polyvinylidene difluoride membrane. After blocking with 10% nonfat dry milk in Tris-buffered saline, the membrane was incubated overnight with P-Chk2 (Thr68) antibody. Subsequently, the membrane was incubated with appropriate secondary antibody, and the immunoreactive

bands were visualized using enhanced chemiluminescence kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Chk2 Inhibitor, Cycloheximide, MG132, and NAC Treatments. In a separate experiment, cells were treated with either 10 μ M MG132, 10 μ g/ml cycloheximide, or 30 μ M Chk2 inhibitor for 2 h or with 10 mM NAC for 1 h at 37°C and then exposed to 100 μ M DIM for 24 h. Subsequently, cells were processed for either flow cytometric analysis or Western blotting as described above.

Transfection of Cells with DN-Chk2. A plasmid containing mutant Chk2 (dominant-negative Chk2) cloned into pBabe Puro mammalian expression vector, a generous gift from Dr. Steve Elledge (Harvard Medical School, Boston, MA), was transfected into ovarian cancer cells. In brief, 5×10^5 cells were transfected with 1.5 μ g of DN-Chk2 plasmid diluted in Opti-MEM serum-free medium containing Lipofectamine reagent. Cells were incubated with a plasmid-Lipofectamine mixture for 5 h and then replenished with normal growth medium for 24 h. Transfected cells were treated with 100 μ M DIM for 24 h. Cells were then processed for flow cytometric analysis or Western blotting as described above.

Analysis of Apoptosis by the Detection of DNA Double-Strand Breaks by TUNEL Assay. DNA double-strand breaks (dsbs) were detected by DeadEnd Fluorometric TUNEL System (Promega) in control and DIM-treated cells by flow cytometry according to manufacturer's instructions. The multimers of 180- to 200-bp DNA fragments of cells undergoing apoptosis were labeled with fluorescein-12dUTP-labeled DNA and quantitated by flow cytometry.

Determination of ROS Generation. Approximately 5000 cells were plated per well in a 96-well plate and allowed to attach overnight. The next day, media were completely removed, and cells were washed with Hanks' buffer and incubated with 10 μ M DCFDA in Hanks' buffer for 1 h. DCFDA was then removed from the wells, and cells were washed with Hanks' buffer followed by treatment with DIM. Fluorescence readings were taken at different time points after treatment with DIM for up to 3 h at excitation wavelength of 485 nm and emission wavelength of 535 nm using SpectraFluor Plus plate reader (Tecan, Durham, NC).

Statistical Analysis. All statistical calculations were performed using Prism 4.0 (GraphPad Software Inc., San Diego, CA). Results were expressed as mean $\pm$ S.E.M. of at least three independent experiments, each conducted in triplicate. Data were analyzed by Student's *t* test or one-way analysis of variance followed by Bonferroni's post hoc analysis for multiple comparisons. Differences were considered statistically significant at *p* < 0.05.

Results

DIM Reduces the Survival of Ovarian Cancer Cells. We first determined the effect of DIM on the viability of ovarian cancer cells. To show that the effect of DIM is not specific to a single cell line, we used three different ovarian cancer cells. Exposure of SKOV-3, OVCAR-3, and TOV21G cells to different concentrations of DIM for different time periods resulted in significant reduction in the survival of cells (Fig. 1) with an IC_{50} of approximately 100, 75, and 65 μ M in SKOV-3 and OVCAR-3 cells at 24, 48, and 72 h, respectively (Fig. 1A). On the other hand, IC_{50} values of DIM in TOV-21G cells were approximately 60, 50, and 40 μ M at 24, 48, and 72 h of treatment, respectively (Fig. 1A, bottom). These results indicate that DIM treatment reduces the survival of ovarian cancer cells in both a time- and concentration-dependent manner.

DIM Induces G₂/M Cell Cycle Arrest in Ovarian Cancer Cells. From cell survival studies, it was clear that DIM suppresses the proliferation of ovarian cancer cells, but the mechanism behind its antiproliferative effects was not clear.

Anticancer agents are known to block the growth of cells in a particular phase of cell division, paving a path for apoptosis (Singh et al., 2004; Zhang et al., 2006; Sahu et al., 2009). To elucidate whether antiproliferative effects of DIM were mediated through cell cycle arrest, we performed cell cycle analysis in DIM-treated ovarian cancer cells using flow cytometry. Our results reveal that 100 μ M DIM treatment causes significant accumulation of cells in G₂/M phase in all three cell lines (Fig. 1, B and C) with a concomitant decrease in the number of cells in G₁ phase (Fig. 1B). Cell cycle arrest in G₂/M phase was more than 2-fold compared with control in both SKOV-3 and OVCAR-3 cells (Fig. 1C). However, G₂/M arrest by DIM was relatively less in TOV-21G cells compared with the other two cell lines (Fig. 1C, bottom). We did not observe any G₂/M arrest in MDA-MB-231 and PC-3 cells by DIM treatment (data not shown). Based on these observations, we hypothesized that the growth inhibitory effects of DIM in ovarian cancer cells was due to the perturbations in the cell cycle check points, and we systematically tested our hypothesis. We used 100 μ M DIM for our subsequent experiments because we observed maximum G₂/M arrest at this concentration.

DIM Causes DNA Damage and Activates Checkpoint Kinase 2. To elucidate the molecular mechanism leading to DIM-mediated G₂/M arrest, control and treated cells were subjected to Western blotting. Cell cycle arrest is usually initiated by checkpoint activation in response to DNA damage (Zhou and Elledge, 2000; Sahu et al., 2009). We observed that DIM treatment increased the phosphorylation of H₂A.X at Ser139, which is a marker of DNA double-strand breaks. We used neocarzinostatin as a positive control in SKOV-3 cells to confirm DNA damage (data not shown). We also determined the expression of DNA polymerase β to determine whether sustained DNA damage by DIM is due to the inability of cells to repair DNA damage. DNA polymerase β plays a critical role in the repair of DNA strand breaks. We observed that in response to DIM treatment, the expression of DNA polymerase β was drastically reduced compared with control in SKOV-3 cells (Fig. 2A). Our results further demonstrated that increased DNA damage was associated with substantially increased phosphorylation of Chk2 at Thr68 in all three ovarian cancer cell lines in response to DIM treatment (Fig. 2, A, C, and D) but not in MDA-MB-231 (breast cancer) and PC-3 (prostate cancer) cells (Supplemental Fig. 1, C and D). It is noteworthy that the protein level of Chk2 was also up-regulated by DIM treatment in ovarian cancer cells (Fig. 2, A, C, and D). Activation of Chk2 by DIM was associated with inactivation of Cdc25C as indicated by inactivating phosphorylation of Cdc25C at Ser216 in SKOV-3 cells (Fig. 2A). In a time-dependent study, phosphorylation of H₂A.X (Ser139) and Chk2 (Thr68) was as early as 8 h after DIM treatment in SKOV-3 cells, although maximum effects were observed at 24 h (Fig. 2A). To confirm the phosphorylation of Chk2, Chk2 protein was immunoprecipitated in control and DIM-treated SKOV-3 cells and immunoblotted against P-Chk2 (Thr68). Our results show an almost 4-fold increase in the phosphorylation of Chk2 in DIM-treated cells compared with control (Fig. 2B).

Further G₂/M regulatory molecules such as Cdc25C, cyclin B1, and Cdk1 were significantly down-regulated with DIM treatment in all three cell lines. However, cyclin B1 level initially increased at 50 and 75 μ M DIM treatment but was

Fig. 1. DIM is cytotoxic to human ovarian cancer cells and causes G₂/M cell cycle arrest. A, the effect of varying concentrations of DIM at different time periods in SKOV-3, OVCAR-3 and TOV-21G cells was determined by sulforhodamine B cell survival assay. Values are the means $\pm$ S.E.M. of three independent experiments with eight replicates; *, $p < 0.05$ compared with control. Cell cycle analysis by flow cytometry. B, representative cell cycle profiles of SKOV-3 cells treated with 75 or 100 μ M DIM for 24 h. FL2-H represents the intensity of propidium iodide, and the y axis represents the cell counts. C, concentration-dependent effect of DIM was evaluated by flow cytometer to quantitate the number of cells in G₂/M phase in SKOV-3, OVCAR-3, and TOV-21G cells. Values are means $\pm$ S.E.M. of three independent experiments, each conducted in triplicate.

Fig. 2. DIM treatment modulates cell cycle regulatory proteins. SKOV-3 (A), OVCAR-3 (C), and TOV-21G (D) cells were treated with different concentrations of DIM for 24 h. In another experiment, SKOV-3 cells were treated for different time intervals with 100 μ M DIM (A). Cells were lysed, and total lysate was prepared as described under *Materials and Methods* and used for Western blotting. Representative immunoblots show the effect of DIM on the phosphorylation of H₂A.X (Ser139), Chk2 (Thr68), and Cdc25C (Ser216) and the protein levels of Chk2, Cdc25C, cyclin B1, Cdk1, and p21. Each blot was stripped and reprobed with anti-actin antibody to ensure equal protein loading. Each experiment was performed at least three times independently, and the results were comparable. Total Chk2 protein was immunoprecipitated from control and DIM-treated SKOV-3 cell lysates and analyzed for P-Chk2 (Thr68) (B). B, the Cdk1 kinase activity was determined in control and DIM-treated SKOV-3 cells using a kit. Values are means $\pm$ S.E.M. of three independent experiments. *p* Values less than 0.05 were considered significant compared with control or DIM treatment.

substantially reduced at 100 μ M in OVCAR-3 cells (Fig. 2, A, C, and D). Cdk inhibitor p21 was up-regulated with DIM treatment in SKOV-3 and OVCAR-3 cells but not in TOV-21G cells (Fig. 2, A, C, and D). Because Cdk1-cyclin B1 complex is the rate-limiting factor for the cells to enter into mitosis, and because its inactivation results in G₂/M arrest, we further wanted to determine whether down-regulation of Cdk1 and cyclin B1 expression by DIM treatment affects the kinase activity of Cdk1. Treatment of SKOV-3 cells with DIM for 24 h resulted in the inhibition of ~45% of Cdk1 kinase activity compared with control (Fig. 2B). We also evaluated the expression of Cdk2 and cyclin D1 in DIM-treated SKOV-3 and OVCAR-3 cells. The constitutive level of cyclin D1 was very low in both of the cell lines, and the expression was down-regulated modestly by DIM treatment (Supplemental Fig. 1, A and B). On the other hand, expression of Cdk2 was substantially decreased by DIM treatment (Supplemental Fig. 1, A and B), suggesting the role of Cdk2 in G₂/M arrest in agreement with a study published recently (Chung and Bunz, 2010).

Cycloheximide Blocks DIM-Mediated Chk2 Activation. Because we observed significantly enhanced protein expression of Chk2 by DIM treatment, we wanted to determine whether suppressing induced Chk2 protein expression can block the activation of Chk2 and hence G₂/M arrest. To do that, we treated SKOV-3 cells with 10 μ g/ml cycloheximide (CHX), a known protein synthesis inhibitor, for 2 h before treating the cells with 100 μ M DIM for 24 h. Both DIM-mediated activation of Chk2 and increase in Chk2 protein levels were abrogated by CHX pretreatment (Fig. 3A). In addition, induction of p21 by DIM was also completely blocked by CHX (Fig. 3A). It is noteworthy that we observed that CHX treatment completely blocked DIM-mediated G₂/M arrest (Fig. 3A).

MG132 Inhibits Proteasomal Degradation of Cdc25C. Because the expression of Cdc25C was drastically reduced by DIM treatment in SKOV-3 cells, we wondered whether Cdc25C protein is degraded by proteasomal pathway, as shown in previous studies (Zhang et al., 2006). To address this, we pretreated SKOV-3 cells with 10 μ M MG132, a specific proteasome inhibitor, before DIM treatment. The drastic reduction in Cdc25C protein expression upon DIM treatment was completely prevented by MG132 (Fig. 3B). Furthermore, Cdk1 expression that was reduced by DIM treatment was also completely restored in SKOV-3 cells by MG132 treatment (Fig. 3B). Moreover, G₂/M arrest induced by DIM was also completely inhibited in SKOV-3 cells by MG132 treatment (Fig. 3B). These results indicate the involvement of Cdc25C and Cdk1 in DIM-mediated G₂/M arrest and that Cdc25C degradation by DIM is mediated by proteasomes.

Chk2 Inhibitor Blocks DIM-Mediated G₂/M Arrest. To confirm the role of Chk2 activation in DIM-mediated G₂/M arrest, SKOV-3 cells were treated with 30 μ M Chk2 inhibitor for 2 h before DIM treatment. Chk2 inhibitor 2-[4-(4-chlorophenoxy)phenyl]-1H-benzo-imidazole-5-carboxylic acid amide is a cell-permeable compound and is a potent ATP competitive inhibitor of Chk2. As shown in Fig. 3C, Chk2 inhibitor blocked the induction and phosphorylation of Chk2 and completely protected the cells from DIM-mediated G₂/M arrest (Fig. 3C).

Dominant-Negative Chk2 Blocks DIM-Induced G₂/M Arrest. CHX treatment blocked the induction and activation

of Chk2 and protected the cells from DIM-mediated G₂/M arrest. However, CHX is a general protein synthesis inhibitor and may have affected the synthesis of proteins other than Chk2 in SKOV-3 cells. Likewise, Chk2 inhibitor blocked Chk2 activation and abrogated DIM-mediated G₂/M arrest. However, chemical inhibitors are known to be associated with off-target effects. Therefore, to firmly establish the role of Chk2 in DIM-mediated G₂/M arrest, we transiently transfected SKOV-3, OVCAR-3, and TOV-21G cells with DN-Chk2 plasmid. We were able to silence approximately 90% of the induced expression of Chk2 by DN-Chk2 transfection. As expected, DN-Chk2 blocked the induction and activation of Chk2 by DIM treatment in all three cell lines (Fig. 4, A–C). Next, we asked whether blocking Chk2 activation can protect the cells from DIM-mediated G₂/M arrest. We performed cell cycle analysis of cells transfected with DN-Chk2 and treated with 100 μ M DIM for 24 h. It is noteworthy that the G₂/M arrest caused by DIM was completely abrogated in not only SKOV-3 cells (Fig. 4A) but also in OVCAR-3 and TOV-21G cells (Fig. 4B–C). Our Western blot results further revealed that the expression of G₂/M regulatory proteins such as Cdc25C, cyclin B1, and Cdk1, which were down-regulated by DIM treatment, were significantly attenuated in all the three cell lines transfected with DN-Chk2 (Fig. 4, A–C). In addition, Cdc25C phosphorylation at Ser216 by DIM treatment was completely blocked in SKOV-3 cells transfected with DN-Chk2, suggesting that Cdc25C phosphorylation was regulated by Chk2 in our model. Taken together, our studies demonstrate the critical role of Chk2 in DIM-mediated G₂/M arrest.

DIM Failed to Cause G₂/M Arrest in Chk2 Knockout Cells. CHX, Chk2 inhibitor, and DN-Chk2 data revealed the requirement of Chk2 in DIM-mediated cell cycle arrest. Based on these observations, we hypothesized that DIM treatment would fail to cause G₂/M cell cycle arrest in Chk2 knockout cells. We tested our hypothesis in DT40 Chk2 knockout (Chk2 KO) lymphoma cells. We treated DT40 Chk2 KO cells with different concentrations of DIM for 24 h. As hypothesized, we did not observe G₂/M arrest in DT40 cells (Fig. 4D). Our Western blot data showed no significant change in the expression of Cdc25C, cyclin B1, or Cdk1 in DIM-treated DT40 Chk2 KO cells (Fig. 4D). These results demonstrate that DIM failed to induce cell cycle arrest in Chk2 knockout cells, establishing Chk2 to be a critical player in inducing G₂/M arrest by DIM.

DIM Treatment Results in the Induction of Apoptosis. Cell cycle arrest is usually followed by apoptosis. To determine whether DIM induces apoptosis, we analyzed the cells by flow cytometry for Annexin V/fluorescein isothiocyanate staining, TUNEL staining, and propidium iodide staining. As shown in Fig. 5A, DIM treatment induced concentration-dependent apoptosis, as analyzed by flow cytometry for Annexin-positive SKOV-3 cells. In another assay, control and DIM-treated SKOV-3 cells were analyzed for TUNEL staining by flow cytometry. The apoptotic cells with DNA dsbs are detected by TUNEL assay. DIM treatment resulted in significant TUNEL-positive cells, suggesting dsbs in apoptotic cells (Fig. 5B). The apoptosis-inducing effects of DIM were further confirmed by detecting sub-G₁/G₀ cell population by flow cytometry. Our results show concentration-dependent apoptosis in all three ovarian cancer cell lines. For example, we observed an approximately 2- to 3-fold increase in apoptosis by

75 and 100 μM treatment, respectively, in SKOV-3 and OVCAR-3 cells (Fig. 5C). However, at these concentrations, apoptosis was more in TOV-21G cells compared with SKOV-3 or OVCAR-3 cells (Fig. 5A). Western blotting indicated an increase in the cleaved fragments of caspase-3 and poly(ADP-ribose) polymerase in DIM-treated ovarian cancer cells, con-

firmed apoptosis (data not shown). It is noteworthy that blocking the activation of Chk2 by CHX, Chk2 inhibitor, or DN-Chk2 significantly blocked DIM-induced apoptosis compared with DIM treatment alone (Fig. 5D). These results suggest that Chk2 also plays a role in DIM-induced apoptosis.

Fig. 3. Pharmacologically inhibiting Chk2 and Cdc25C block DIM-mediated G₂/M cell cycle arrest in SKOV-3 cells. Cells were pretreated with 10 $\mu\text{g}/\text{ml}$ protein synthesis inhibitor CHX for 2 h followed by treatment with 100 μM DIM for 24 h. A, phosphorylation of Chk2 at Thr68 and protein level of Chk2 and p21 were evaluated by Western blotting, and cell cycle was evaluated by flow cytometry as described under *Materials and Methods*. In another experiment, cells were pretreated with 10 μM MG132 for 2 h followed by treatment with or without 100 μM DIM for 24 h. B, representative Western blots show the protein levels of Cdc25C and Cdk1 and cell cycle by flow cytometry. As mentioned above, cells were also pretreated with 30 μM Chk2 inhibitor for 2 h followed by treatment with 100 μM DIM for 24 h. Phosphorylation of Chk2 at Thr68 and protein level of Chk2 were evaluated by Western blotting (C). Flow cytometry analysis (C) shows the blockade of G₂/M arrest in the presence of Chk2 inhibitor. Blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading. Values are means $\pm$ S.E.M. of three independent experiments. *p* Values less than 0.05 were considered significant compared with control or DIM treatment.

Fig. 4. Dominant-negative Chk2 abrogates DIM-induced G₂/M arrest. A representative cell cycle profile of SKOV-3 cells that were transfected with 1.5 µg of plasmid containing dominant-negative Chk2 for 24 h followed by treatment with or without 100 µM DIM for another 24 h (A) is shown. Cell cycle analysis of SKOV-3 (A), OVCA-3 (B), and TOV-21G (C) cells that were transfected with dominant-negative Chk2 as described above followed by treatment with or without DIM for 24 h. Phosphorylation of Chk2 at Thr68 and Cdc25C at Ser216 and protein levels of Chk2, Cdc25C, Cyclin B1, and Cdk1 were analyzed by Western blotting in SKOV-3 (A, bottom), OVCA-3 (B, bottom), and TOV-21G (C, bottom) cells transfected with DN-Chk2 and treated with or without 100 µM DIM for 24 h. Blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading. Values are means ± S.E.M. of three independent experiments. *p* Values less than 0.05 were considered to be significant compared with control or DIM treatment. DIM does not induce cell cycle arrest in Chk2 knockout cells. DT40 Chk2 knockout lymphoma cells were treated with 75 or 100 µM DIM

DIM Treatment Causes ROS Generation. Next we sought to determine the mechanism of Chk2 activation by DIM treatment. Previous studies, including those from our laboratory, have shown the involvement of ROS in causing DNA damage resulting in G₂/M arrest (Sahu et al., 2009). We therefore wanted to know whether DIM treatment causes ROS generation in ovarian cancer cells. We determined ROS generation by measuring the fluorescence of DCF, which is formed by the oxidation of DCFDA by peroxides. Our results demonstrate early and significant ROS generation by DIM treatment in SKOV-3 cells (Fig. 6A). To prove the involvement of ROS in DIM-mediated activation of Chk2 and G₂/M arrest, we pretreated the cells with 10 mM NAC before DIM treatment. NAC is a general antioxidant and a precursor of glutathione. NAC treatment was able to block approximately 50% of the phosphorylation of H₂A.X at Ser139 and Chk2 at Thr68 in SKOV-3 cells, suggesting the involvement of ROS in causing DIM-mediated DNA damage (data not shown). Further NAC treatment almost completely blocked DIM-mediated G₂/M arrest (Fig. 6B). Nevertheless, NAC treatment only afforded approximately 50% protection to SKOV-3 cells from DIM-induced apoptosis, indicating the involvement of other pathways in DIM-induced apoptosis (Fig. 6C).

Discussion

Our results demonstrate that DIM treatment significantly suppresses the viability of SKOV-3, OVCAR-3 and TOV-21G human ovarian cancer cells in a concentration- and time-dependent manner. The growth suppression of all of the cell lines by DIM treatment was associated with G₂/M arrest. Our results further show that DIM-mediated G₂/M arrest in ovarian cancer cells was linked with DNA damage-mediated induction and activation of Chk2. Blocking Chk2 induction and activation by protein synthesis inhibitor CHX, Chk2 inhibitor, or transfecting the cells with DN-Chk2 completely prevented G₂/M arrest mediated by DIM. In addition, DIM failed to cause G₂/M arrest in DT-40 Chk2 KO cells. Our results demonstrate that the apoptosis was also blocked in the cells in which the activation of Chk2 was blocked, indicating its role in DIM-induced apoptosis. Furthermore, our results show that DIM treatment causes ROS generation and that ROS were involved in causing DNA damage and activation of Chk2, leading to G₂/M arrest. To the best of our knowledge, this study for the first time establishes Chk2 as a critical molecular target of DIM in human ovarian cancer cells.

Checkpoints are important in ensuring the proper progression of cell cycle. Checkpoints are activated in response to DNA damage, resulting in the arrest of cells in G₂/M phase (Zhou and Elledge, 2000; Zhang et al., 2006; Sahu et al., 2009). This protective mechanism provides time to either repair the DNA damage and proceed toward mitosis or enter into apoptosis if the DNA damage is not repaired (Norbury and Zhivotovskiy, 2004; Zhang et al., 2006). Our results show a remarkable G₂/M arrest in SKOV-3, OVCAR-3, and TOV-21G cells by DIM treatment. Although, DIM has been shown

to cause G₁ arrest in breast cancer cells (Hong et al., 2002b), the effect of DIM in causing G₂/M arrest in cancer cells has not been reported so far. To ascertain whether DIM-mediated G₂/M arrest was specific to ovarian cancer cells, we evaluated the effect of DIM on breast (MDA-MB-231) and prostate (PC-3) cancer cells. We were surprised to observe no significant G₂/M cell cycle arrest by DIM in these cell lines. Our studies further show that DIM causes DNA double-strand breaks, suggesting that DIM-mediated G₂/M in our model is due to the DNA damage incurred by DIM. This is consistent with several other studies, including ours, showing a correlation between DNA damage and G₂/M arrest (Bose et al., 2005; Zhang et al., 2006). However, the exact mechanism by which DIM causes DNA damage was not clear. Previous studies have indicated the involvement of ROS in causing DNA damage in pancreatic and prostate cancer cells (Shukla and Gupta, 2008; Sahu et al., 2009). In agreement with these studies, our present results also show the generation of ROS by DIM and the involvement of ROS in causing DNA damage, leading to G₂/M arrest. Blocking ROS by NAC treatment prevented DIM-mediated G₂/M arrest.

DNA damage generally leads to the activation of DNA damage-mediated signaling pathways, such as ATM (Kurz and Lees-Miller, 2004). Activated ATM undergoes auto phosphorylation at Ser1981, is recruited at the site of DNA damage, and is involved in the phosphorylation of other DNA damage response cell cycle proteins such as Chk1 and Chk2 (Zhou and Elledge, 2000; Kurz and Lees-Miller, 2004). Most notably, our results demonstrate the significant activating phosphorylation of Chk2 at Thr68 but not Chk1 by DIM treatment in all three ovarian cancer cell lines. We did not observe significant activation of ATM by DIM treatment. This is not surprising, because ATM-independent activation of Chk2 and G₂/M arrest and apoptosis has been documented (Hirao et al., 2002; Cao et al., 2007). Gire et al., 2004 reported increased expression of Chk2 in bleomycin-treated human fibroblasts. In agreement with these studies, our results also show increased protein expression of Chk2 in response to DIM treatment. It is possible that DIM treatment stabilizes Chk2 in ovarian cancer cells. Our immunoprecipitation studies showed approximately 4-fold increased phosphorylation of Chk2 by DIM compared with control. The time-dependent studies revealed DNA damage and Chk2 activation by 8 h of DIM treatment. These results are in agreement with the fact that DNA damage leads to the activation of Chk2. Supporting these observations, we did observe modest G₂/M arrest at approximately 12 h of DIM treatment (data not shown), which became more pronounced by 24 h. However, we did not observe any Chk2 activation in MDA-MB-231 or PC-3 cells by DIM treatment, indicating that the growth-suppressive effects of DIM in these cell lines is probably mediated by a different mechanism. So far, based on our observations, it seems that activation of Chk2 by DIM is most likely specific to ovarian cancer cells; however, Chk2 activation in other cancer cell lines cannot be ruled out until an exhaustive screening is done.

Activated Chk2 in turn phosphorylates and inactivates

for 24 h, and its effect on cell cycle profile was analyzed by flow cytometry (D, top). Phosphorylation of Chk2 (Thr68) and Cdc25C (Ser216) and protein level of Chk2, Cdc25C, cyclin B1, and Cdk1 in DT-40 cells after treatment with DIM was evaluated by Western blotting (D, bottom). Each blot was stripped and reprobed with anti-actin to ensure equal protein loading.

Cdc25C, which further inactivates the Cdk1-cyclin B1 complex, leading to a blockade of cells in G₂/M phase. Our results demonstrate increased phosphorylation of Cdc25C at Ser216

and decreased protein expression of Cdc25C, cyclin B1, and Cdk1. The phosphorylation of Cdc25C at Ser216 by DIM treatment was abolished in the cells transfected with DN-

Fig. 5. DIM induces apoptosis in ovarian cancer cells. Apoptosis was evaluated by quantitating Annexin-positive SKOV-3 cells by flow cytometry (A), TUNEL-positive SKOV-3 cells by flow cytometry (B), and sub-G₀/G₁ SKOV-3, OVCAR-3, and TOV-21G cells by flow cytometry (C) cells treated with 75 and 100 μM DIM for 24 h. SKOV-3 cells were treated with either 10 μM CHX for 2 h (D), 30 μM Chk2 inhibitor for 2 h (E), or transfected with DN-Chk2 for 24 h (F) followed by treatment with 100 μM DIM for 24 h and analyzed for apoptosis by flow cytometry. Values are means ± S.E.M. of three independent experiments. *p* Values less than 0.05 are considered to be significant and were compared with control or DIM treatment.

Chk2. Furthermore, we did not observe any Cdc25C phosphorylation by DIM treatment in DT-40 Chk2 KO cells. These results clearly indicate that phosphorylation of Cdc25C was mediated by Chk2 in our model. The sharp decrease in the protein level of Cdc25C in SKOV-3 cells was proteasome-mediated, which was blocked once the cells were pretreated with MG132, a specific proteasome inhibitor. Proteasome-mediated degradation of Cdc25C has been reported earlier [Singh et al., 2004; Zhang et al., 2006]. It is noteworthy that DIM-induced G₂/M arrest was halted by MG132 pretreatment. This implies that Cdc25C, which is downstream of Chk2, is involved in cell cycle arrest. p21, which is

a cyclin-dependent kinase (CDK) inhibitor, is known to bind to Cdk1-cyclin B1 complex (Bunz et al., 1998; Molinari, 2000; Charrier-Savournin et al., 2004) and negatively regulate G₂/M phase. We observed significant induction of p21 by DIM treatment in SKOV-3 and OVCAR-3 cells. In several previous reports, a similar mechanism was observed in the induction of G₂/M arrest (Zhang et al., 2006). However, in contrast to what we observed in the above two cell lines, DIM down-regulated p21 in TOV-21G cells. Induction of p21 is known to be involved in apoptosis (Gartel and Tyner, 2002). However, on the contrary, few studies have documented that down-regulation of p21 may be necessary for the activation of

Fig. 6. DIM causes ROS generation. The time-dependent generation of ROS in SKOV-3 cells by DIM treatment was determined using 10 μM DCFDA as a probe as described under *Materials and Methods* (A). SKOV-3 cells were treated with 10 mM NAC for 1 h followed by treatment with 100 μM DIM for 24 h, and cell cycle analysis (B) or apoptosis (C) was performed. Values are means ± S.E.M. of three independent experiments. *, #, $p < 0.05$; values less than 0.05 were considered to be significant compared with control.

apoptosis. In fact, cytoplasmic localization of overexpressed p21 has been shown to correlate with inhibition of apoptosis (Asada et al., 1999), cancer cell survival, and poor prognosis of cancer patients. Therefore, the role of p21 is paradoxical and could be cell-specific as well. It is possible that a DIM-induced decrease in cell survival and apoptosis in TOV-21G cells is mediated through other mechanisms. G₁/S arrest by DIM has been reported previously (Chang et al., 2005). We therefore examined the effect of DIM on the expression of cyclin D1 and Cdk2, the key players in G₁/S arrest, in ovarian cancer cells. The constitutive levels of cyclin D1 were very low in both SKOV-3 and OVCAR-3 cells. Our result shows that DIM treatment significantly reduced Cdk2 levels and, to some extent, cyclin D1 levels in both SKOV-3 and OVCAR-3 cells. Because we did not observe any G₁/S arrest, it is possible that Cdk2 may be playing some role in G₂/M arrest in our model because a very recent study demonstrated the involvement of Cdk2 in p53-independent G₂/M checkpoint control (Chung and Bunz, 2010). Neither SKOV-3 nor OVCAR-3 cells have active p53. However, more studies are required to prove the correlation of Cdk2 with G₂/M arrest.

To establish the role of Chk2 in DIM-mediated cell cycle arrest, we used a chemical and genetic approach and a cell line from which Chk2 was stably knocked out. Cycloheximide pretreatment abrogated the activation of Chk2 and G₂/M cell cycle arrest. In addition, CHX treatment blocked the induction of p21. Although CHX is a nonspecific protein synthesis inhibitor, few previous studies used cycloheximide to inhibit Chk2 synthesis (Lukas et al., 2001; Jin et al., 2008; Yeh et al., 2009). Pharmacologically inhibiting Chk2 activation by Chk2 inhibitor also blocked DIM-mediated G₂/M arrest. We further confirmed our observations by transfecting the cells with Chk2 kinase dead mutant DN-Chk2. Our results demonstrate that blocking Chk2 activation by DN-Chk2 almost completely protected the cells from DIM-mediated G₂/M arrest and apoptosis. G₂/M regulatory molecules like Cdc25C, Cdk1, and Cyclin B1 that were down-regulated by DIM were also restored by DN-Chk2. The role of Chk2 was further strengthened by our studies in DT40 Chk2 knock out cells. Although, G₂/M arrest was observed in DT40 in previous studies (Rainey et al., 2008), DIM treatment failed to cause G₂/M arrest in this cell line. The cell cycle regulatory proteins were not modulated significantly in this cell line by DIM treatment. This strongly proves our point that DIM requires Chk2 activation for arresting the cells in G₂/M phase. Our results have a lot of significance, because Kinzler and Vogelstein (1997) indicated that Chk2 performs a "gatekeeper" activity that, when it gets defective, promotes tumorigenesis through the survival and proliferation of cells with compromised genomic integrity. Moreover, Chk2(-/-) cells have been shown to be radioresistant and show defects in irradiation-induced apoptosis (Hirao et al., 2002; Takai et al., 2002). Overall, our results suggest that DIM-induced G₂/M arrest and apoptosis are mediated via activation of Chk2.

Taken together, our study reveals the chemotherapeutic effects of DIM on ovarian cancer cells. Our results establish that DIM-mediated G₂/M arrest was due to DNA damage and activation of Chk2, which in part is caused by ROS. Blocking Chk2 activation completely protected the cells from DIM-induced G₂/M arrest and apoptosis. All of these observations are in agreement with the overall effectiveness of the growth-suppressive effects of DIM on ovarian cancer cells. Whether

or not inhibiting Chk2 plays any role in suppressing the growth of ovarian tumors in vivo by DIM treatment is the focus of our future studies.

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Address correspondence to: Dr. Sanjay K. Srivastava, Department of Biomedical Sciences, Texas Tech University Health Sciences Center, Amarillo, TX 79106. E-mail: sanjay.srivastava@ttuhsc.edu