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Activation of Chk2 by 3,3'-Diindolylmethane is required for causing G2/M cell cycle arrest in human ovarian cancer cells

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Running title: Activation of Chk2 by DIM in ovarian cancer cells.

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Abbreviations: DIM, 3,3´-diindolylmethane; Chk2, check point kinase 2; DN-Chk2, dominant negative check point kinase 2; ROS, reactive oxygen species; NAC, N-acetyl cysteine.

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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 dose and time-dependent manner with effective concentrations ranging from 40μM to 100μM. 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 $H_2A.X$ at Ser 139 and activation of Chk2 in all the three cell lines. Other G2/M regulatory molecules such as Cdc25C, Cdk1, Cyclin B1 were downregulated 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 knock out DT40 lymphoma cells where 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. Further our results show that DIM treatment cause ROS generation. Blocking ROS generation by NAC protect 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.

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Ovarian cancer is a major cause of deaths among 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 (McPherson, et al., 2004; Goodheart, et al., 2002). 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 as 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 molecular level are needed.

Epidemiological studies continue to indicate inverse relation between the consumption of cruciferous vegetables and risk of cancer of ovary, breast, lung and pancreas (Pan, et al., 2004; Zhang, et al., 2002; Bosetti, et al., 2001). 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 activity against various cancers (Chen, et al., 2006; Bhatnagar, et al., 2009; Hong, et al., 2002a; Kong, et al., 2007; Rahman, et al., 2006). DIM has been shown to suppress cancer growth by inhibiting oncogenic molecules such as NF-KB, Akt, β-Catenin in breast and prostate cancers (Chen, et al., 2006; Kong, et al., 2007; Rahman, et al., 2006). Anti-apoptotic molecules such as Bcl-2 and pro-

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apoptotic proteins like Bax were also regulated by DIM (Hong, et al., 2002a). Inhibition of H^+ATP synthase by DIM leads to induction of P21 in breast cancer cells (Gong, et al., 2006). DIM potentiates the effect of erlotinib, an EGFR 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 G2/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 using Chk2 KO cells, abrogated DIM mediated G2/M cell cycle arrest and protected the cells from apoptosis, indicating Chk2 as a potent molecular target of DIM in ovarian cancer cells.

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Materials and methods:

***Chemicals*-** BR-DIM was a kind gift from Dr. Michael Zeligs (Bio Response, Boulder, CO). We have referred BR-DIM as DIM in our studies. Sulphorhodamine B, RNase A, Propidium iodide, Ampicillin, Luria broth, Actin antibody, *N*-Acetyl Cysteine (NAC), trichloro acetic acid (TCA), Medium 199 and MCDB 105 were obtained from Sigma-Aldrich (St. Louis, MO). Cycloheximide was obtained from Pierce (Rockford, IL), MG132 and Chk2 inhibitor 2-[4-(4-Chlorophenoxy)phenyl]-H-benzo-imidazole-5-carboxylic acid amide (Catalog no. 220486) was bought from Calbiochem (New Jersey). Electrophoresis reagents were from Bio-Rad laboratories (Richmond, CA). Antibodies against checkpoint kinase 2 (Chk2), phospho-Chk2 (Thr-68), cell division cycle 25C (Cdc25C), phospho-Cdc25C (Ser-216), phospho-$H_2A.X$ (Ser-139), CyclinB1, Cdk1, P21, Cdk2, Cyclin D1, DNA pol β1 were from Cell Signaling Technology (Danvers, MA). RPMI, McCOY 5A, F12K, trypsin, heat inactivated fetal bovine serum (FBS) and Pencillin/streptomycin antibiotic mixture was from Mediatech Inc (Manassas, VA). DMEM was from ATCC (Manassas, VA). Lipofectamine, Optimem and DCFDA were obtained from Invitrogen (Carlsbad, CA). Plasmid Midi kit to extract DNA was from Qiagen (Germantown, MD). The DeadEnd Flurometric TUNEL System kit was purchased from Promega (Madison, WI). Agarose A beads were obtained from Santa Cruz (Santa Cruz, CA). Apoptosis kit was purchased from EMD Biosciences (Gibbstown, NJ).

***Cell culture*-** SKOV-3, OVCAR-3 and TOV-21G cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). All the three cell lines were well-differentiated epithelial ovarian adenocarcinoma's obtained from Caucasian females. SKOV-3 cells were maintained in McCoy 5A medium supplemented with 10% FBS. OVCAR-3 was maintained in

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RPMI medium supplemented with 20% FBS, 1mM sodium pyruvate, 10mM HEPES, 10mg/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% PSN antibiotic mixture was added to all the above media. Chk2 knockout DT40 lymphoma cells, a kind gift from Dr. Mark Walker (University of Glasgow, U.K), were maintained in DMEM medium with 10% FBS, 1% chick serum, 10μM β-mercaptoethanol, 1% PSN and 2mM L-glutamine. In addition, MDA-MB-231 and PC-3 cells were also obtained from ATCC and cultured in RPMI and F12K medium respectively. All the cell lines were maintained in a humidified incubator with 5% CO_2 and 95% air. A 100mM stock solution of DIM in 1:4 H_2O:DMSO mixture was prepared freshly before the experiment.

Sulphorhodamine cell survival Assay- About 5,000 cells in 0.1ml medium were plated in 96 well plates and allowed to attach overnight. Desired concentrations of DIM were added to the cells and incubated at 37^0C for 24h, 48h, and 72h. The cells were then processed and stained with 0.4% SRB solution and the absorbance was read at 570 nm using a Biotek plate reader as described by us 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 (PI). Stained cells were analyzed by using a BD FACS Caliber flow cytometer as described by us previously (Sahu, et al., 2009). Cell cycle data was reanalyzed using Cell Quest Pro software. Approximately, 20,000 cells were evaluated for each sample. In all determination, cell debris and clumps were excluded from the analysis.

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

Cdk1 kinase activity- Cdk1 kinase activity was performed using Cdk1 kinase assay kit (Upstate) with some modifications as described by us previously (Zhang *et al*, 2006). Control and DIM treated SKOV-3 cells were lysed as described by us previously (Sahu and Srivastava, 2009). Approximately 500µg of control and treated cell lysate was incubated with 3µg Cdk1 antibody for 2h at 4^{o}C followed by the addition of 35µl protein A agarose and the complex was left on a rocker overnight at 4^{o}C. The kinase activity was determined using the kit according to manufacturer's instructions.

Immunoprecipitation- About, 1x10^{6} cells were plated and treated with different concentrations of DIM for 24 hours. Control and treated cells were washed twice with ice-cold PBS and lysed on ice with NP-40 lysis buffer (50mM Tris-HCl, pH, 7.4, 150mM NaCl, 5mM EDTA and 1% NP40) containing 2 mmol/L Na_3VO_4, 2 mmol/L EGTA, 12 mmol/L β-glycerol phosphate,10 mmol/L NaF, 16 mg/L benzamidine hydrochloride, 10mg/L phenanthroline, 10 mg/L aprotinin, 10 mg/L leupeptin, 10 mg/L pepstatin and 1 nmol/L phenyl methyl sulfonyl fluoride. The cell lysate was cleared by centrifugation at 14,000g for 15 min. About 10µg of Chk2 antibody was

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added to 300µg of lysate obtained from control and treated cells and incubated over night at 4^0C. 50µl of Agarose A beads were added to the above mixture and incubated for five hours with gentle rocking. Lysates were then collected by centrifuging at 1000g for 1min and supernatant was separated. Beads were then washed 5 times with lysis buffer according to the protocol provided by Cell Signaling Technology. Chk2 protein from each sample was eluted with 40 µL of 1% SDS and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and the proteins were blotted onto PVDF membrane. After blocking with 10% nonfat dry milk in Tris buffered saline, the membrane was incubated overnight with P-Chk2 (Thr-68) antibody. Subsequently, the membrane was incubated with appropriate secondary antibody, and the immunoreactive bands were visualized using enhanced chemiluminescence kit (PIERCE) according to the manufacturer's instructions.

Chk2 inhibitor, Cycloheximide, MG132 and NAC treatments- In a separate experiment, cells were treated with either 10µM MG132 or 10µg/ml cycloheximide or 30µM Chk2 inhibitor for 2h or with 10mM *N*-acetyl cysteine (NAC) for 1h at 37^0C and then exposed to 100µM DIM for 24h. Subsequently, cells were processed for either flowcytometric 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), was transfected into ovarian cancer cells. Briefly $5x10^5$ cells were transfected with 1.5 µg of DN Chk2 plasmid diluted in Opti-MEM serum-free medium containing lipofectamine reagent. Cells were incubated with plasmid-lipofectamine mixture for 5

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hours and then replenished with normal growth medium for 24 hours. Transfected cells were treated with 100μM DIM for 24 hours. Cells were then processed for flowcytometric 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 (dbs) 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 180-200bp DNA fragments of cells undergoing apoptosis were labeled with fluorescein-12dUTP-labled DNA and quantitated by flow cytometry.

Determination of ROS generation- About 5000 cells were plated per well in a 96 well plate and allowed to attach overnight. The next day, media was completely removed and cells were washed with Hanks buffer and incubated with 10μM DCFDA in Hanks buffer for one hour. 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 up to 3 hours at excitation wavelength of 485nm and emission wavelength of 535nm using Tecan spectra fluor plus plate reader (Research Triangle Park, NC).

Statistical analysis- All statistical calculations were performed using GraphPad Prism 4.0. Results were expressed as mean ± standard error of the means 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 comparisions. Differences were considered statistically significant at $p<0.05$.

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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 around 100µM, 75µM and 65µM in SKOV-3 and OVCAR-3 cells at 24h, 48h and 72h respectively (Fig. 1A). On the other hand, IC_{50} of DIM in TOV-21G cells was about 60µM, 50µM and 40µM at 24, 48 and 72h treatment respectively (Fig. 1A, lower panel). These results indicate that DIM treatment reduces the survival of ovarian cancer cells in both 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 suppress 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 (Sahu, et al., 2009; Singh, et al., 2004; Zhang, et al., 2006). In order 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 100µM DIM treatment causes significant accumulation of cells in G2/M phase in all the three cell lines (Fig. 1B-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 when compared to control in both SKOV-3 and OVCAR-3 cells (Fig. 1C). However, G2/M arrest by DIM was relatively less in TOV-21G cells as compared to the other two cell lines (Fig. 1C, lower panel). We did not observe any G2/M arrest in MDA-

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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 100μM DIM for our subsequent experiments since we observed maximum G2/M arrest at this concentration.

DIM causes DNA damage and activates Check point 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 $H_2A.X$ 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 see if 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 as compared to 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 the three ovarian cancer cell lines in response to DIM treatment (Fig. 2A, C-D), but not in MDA-MB-231(breast cancer) and PC-3 (prostate cancer) cells (Suppl. Fig. 1C-D). Interestingly, protein level of Chk2 was also upregulated by DIM treatment in ovarian cancer cells (Fig. 2A, C-D). Activation of Chk2 by DIM was associated with inactivation of Cdc25C as indicated by inactivating phosphorylation of Cdc25C at Ser 216 in SKOV-3 cells (Fig. 2A). In a time dependent study,

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phosphorylation of H_2AX (Ser 139) and Chk2 (Thr-68) was as early as 8h after DIM treatment in SKOV-3 cells, although maximum effects were observed at 24h (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 (Thr 68). Our results show an almost 4 fold increase in the phosphorylation of Chk2 in DIM treated cells as compared to control (Fig. 2B).

Further G2/M regulatory molecules such as Cdc25C, Cyclin B1 and Cdk1 were significantly down regulated with DIM treatment in all the three cell lines, however, cyclin B1 level initially increased at 50 and 75μM DIM treatment but was substantially reduced at 100μM in OVCAR-3 cells (Fig. 2A, C-D). Cdk inhibitor P21 was up-regulated with DIM treatment in SKOV-3 and OVCAR-3 cells but not in TOV-21G cells (Fig. 2A, C-D). Since Cdk1-cyclin B1 complex is the rate limiting factor for the cells to enter into mitosis and its inactivation results in G2/M arrest, we further wanted to see whether down regulation of Cdk1 and cyclin B1 expression by DIM treatment affects the kinase activity of Cdk1. Treatment of SKOV-3 cells to DIM for 24h resulted in the inhibition of ~45% of Cdk1 kinase activity as compared to 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 the cell lines and the expression was down regulated modestly by DIM treatment (Suppl. Fig. 1A-B). On the other hand, expression of Cdk2 was substantially decreased by DIM treatment (Suppl. Fig. 1A-B), suggesting the role of Cdk2 in G2/M arrest in agreement with a recently published study (Chung, et al., 2010).

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Cycloheximide blocks DIM-mediated Chk2 activation – Because we observed significantly enhanced protein expression of Chk2 by DIM treatment, we wanted to see whether suppressing induced Chk2 protein expression can block the activation of Chk2 and hence G2/M arrest. In order to do that, we treated SKOV-3 cells with 10µg/ml cycloheximide (CHX), a known protein synthesis inhibitor, for 2h before treating the cells with 100µM DIM for 24h. 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). Interestingly, we observed that CHX treatment completely blocked DIM mediated G2/M arrest (Fig. 3A).

MG132 inhibits proteasomal degradation of Cdc25C – Since the expression of Cdc25C was drastically reduced by DIM treatment in SKOV-3 cells, we wondered if 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 10µM MG-132, a specific proteasome inhibitor prior to 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 MG-132 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 30µM Chk2 inhibitor for 2h prior

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to 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 Figure 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. Similarly, Chk2 inhibitor blocked Chk2 activation and abrogated DIM mediated G2/M arrest. But, 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 about 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 the three cell lines (Fig. 4A-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 100μM DIM for 24h. Interestingly, 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. 4A-C). In addition, Cdc25C phosphorylation at Ser 216 by DIM treatment was completely blocked in SKOV-3 cells transfected with DN-Chk2, suggesting that

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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 knock out cells. We tested our hypothesis in DT40 Chk2 knock out (Chk2 KO) lymphoma cells. We treated DT40 Chk2 KO cells with different concentrations of DIM for 24h. 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 knock out cells establishing Chk2 to be critical player in inducing G2/M arrest by DIM.

DIM treatment results in induction of apoptosis **-** Cell cycle arrest is usually followed by apoptosis. In order to see whether DIM induces apoptosis, we analyzed the cells by flowcytometry for: (i) annexin V/FITC staining, (ii) TUNEL staining, and (iii) propidium iodide staining. As shown in Figure 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 double strand breaks (dbs) are detected by TUNEL assay. DIM treatment resulted in significant TUNEL positive cells suggesting dbs in apoptotic cells (Fig 5B). The apoptosis-inducing effects of DIM were further confirmed by detecting sub-G1/G0 cell

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population by flow cytometry. Our results show concentration dependent apoptosis in all the three ovarian cancer cell lines. For example, we observed about 2-3 fold increase in apoptosis by 75μM and 100μM treatment respectively in SKOV-3 and OVCAR-3 cells (Fig. 5C). However, at these concentrations apoptosis was more in TOV-21G cells as compared to SKOV-3 or OVCAR-3 cells (Fig. 5A). Western blotting indicated an increase in the cleaved fragments of capase-3 and PARP in DIM treated ovarian cancer cells confirming apoptosis (data not shown). Interestingly, blocking the activation of Chk2 by CHX, Chk2 inhibitor or DN-Chk2 significantly blocked DIM induced apoptosis as compared to DIM treatment alone (Fig. 5D). These results suggest that Chk2 also plays a role in DIM induced apoptosis.

DIM treatment cause 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 10mM *N*-Acetyl Cysteine (NAC) prior to DIM treatment. NAC is a general antioxidant and a precursor of glutathione. NAC treatment was able to block about 50% of the phosphorylation of $H_2A.X$ at Ser 139 and Chk2 at Thr 68 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 about 50%

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protection to SKOV-3 cells from DIM-induced apoptosis indicating the involvement of other pathways in DIM induced apoptosis (Fig. 6C).

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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 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 where the activation of Chk2 was blocked, indicating its role in DIM-induced apoptosis. Further, 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.

Check points 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 (Sahu, et al., 2009; Zhang, et al., 2006; Zhou and Elledge, 2000). This protective mechanism provides time to either repair the DNA damage and proceed towards mitosis or enter into apoptosis if the DNA damage is not repaired (Zhang, et al., 2006; Norbury and Zhivotovsky, 2004). 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), effect of DIM in causing G2/M arrest in cancer cells was not reported so far. To ascertain

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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. Surprisingly, we did not observe any 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 (Zhang, et al., 2006; Bose, et al., 2005). 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 (Sahu, et al., 2009; Shukla and Gupta, 2008). 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 Ser-1981, 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 Thr-68 but not Chk1 by DIM treatment in all the three ovarian cancer cell lines. We did not observe significant activation of ATM by DIM treatment. This is not surprising as ATM independent activation of Chk2 and G2/M arrest and apoptosis has been documented (Hirao, et al., 2002; Cao, et al., 2007). A recent study reported increased expression of Chk2 in bleomycin treated human fibroblasts [Gire *et al*, 2004]. In

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agreement to 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 about 4 fold increased phosphorylation of Chk2 by DIM as compared to control. The time dependent studies revealed DNA damage and Chk2 activation by 8h 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 around 12h of DIM treatment (data not shown) which became more pronounced by 24h. 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 different mechanism. So far based on our observations, it appears 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 Cdc25C, which further inactivates Cdk1-CyclinB1 complex leading to blockade of cells in G2/M phase. Our results demonstrate increased phosphorylation of Cdc25C at Ser 216 and decreased protein expression of Cdc25C, Cyclin B1 and Cdk1. The phosphorylation of Cdc25C at Ser 216 by DIM treatment was abolished in the cells transfected with DN-Chk2. Further 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 decline 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 [Zhang *et al*, 2006, Singh *et al*, 2004].

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Interestingly, 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 (Molinari, 2000; Bunz, et al., 1998; 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 similar mechanism was observed in the induction of G2/M arrest (Zhang, et al., 2006). However, in contrast to what we observed in above two cell lines, DIM down regulated p21 in TOV-21G cells. Induction of p21 is known to be involved in apoptosis (Gartel, et al., 2002). However, on the contrary, few studies have documented that down-regulation of p21 may be necessary for the activation of apoptosis. In fact, cytoplasmic localization of over-expressed 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 DIM-induced decrease in cell survival and apoptosis in TOV-21G cells is mediated through other mechanism. 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. Since 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 as a very recent study demonstrated the involvement of Cdk2 in p53-independent G2/M checkpoint control (Chung, et al., 2010). Both SKOV-3 and OVCAR-3 cells do not have active p53. However, more studies are required to prove the correlation of Cdk2 with G2/M arrest.

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To establish the role of Chk2 in DIM mediated cell cycle arrest, we used chemical and genetic approach and also used a cell line from which Chk2 was stably knocked out. Cycloheximide pretreatment abrogated activation of Chk2 and also G2/M cell cycle arrest. In addition CHX treatment blocked the induction of P21. Although CHX is non-specific 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. 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 as Kinzler and Vogelstein indicated that Chk2 performs a “gate-keeper” activity that, when gets defective promotes tumorigenesis through the survival and proliferation of cells with compromised genomic integrity (Kinzler and Vogelstein, 1997). 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 is mediated via activation of Chk2.

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Taken together, our study reveals the chemotherapeutic effects of DIM on ovarian cancer cells. Our results established that DIM mediated G/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 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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Foot Notes:

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FIGURE LEGENDS

Figure 1. DIM is cytotoxic to human ovarian cancer cells and causes G2/M cell cycle arrest. Effect of varying concentrations of DIM at different time periods in SKOV-3, OVCAR-3 and TOV-21G (**A**) cells was determined by Sulphorhodamine B cell survival assay. Values are means ± SEM of 3 independent experiments with 8 replicates. *P < 0.05 when compared with control. Cell cycle analysis by flowcytometry. Representative cell cycle profiles of SKOV-3 cells treated with 75μM or 100μM DIM for 24h (**B**). FL2-H represents the intensity of propidium iodide and Y-axis represents the cell counts. Concentration-dependent effect of DIM was evaluated by flowcytometer to quantitate number of cells in G2/M phase in SKOV-3, OVCAR-3 and TOV-21G (**C**) cells. Values are means ± SEM of 3 independent experiments, each conducted in triplicate.

Figure 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 24h. In another experiment, SKOV-3 cells were treated for different time intervals with 100μM DIM (**A**). Cells were lysed and total lysate was prepared as described in Materials and Methods and used for western blotting. Representative immunoblots show the effect of DIM on the phosphorylation of $H_2A.X$ (Ser 139), Chk2 (Thr 68) and Cdc25C (Ser 216) as well as 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 3 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 (Thr 68) (**B**). The Cdk1 kinase activity was determined in control and DIM treated SKOV-3 cells using a kit (**B**). Values are

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means ± SEM of 3 independent experiments. P values less than 0.05 were considered to be significant when compared with control or DIM treatment.

Figure 3. Pharmacologically inhibiting Chk2 and Cdc25C block DIM mediated G2/M cell cycle arrest in SKOV-3 cells. Cells were pretreated with 10µg/ml protein synthesis inhibitor CHX for 2h followed by treatment with 100µM DIM for 24h. Phosphorylation of Chk2 at Thr 68 and protein level of Chk2 and P21 were evaluated by western blotting (A) and cell cycle was evaluated by flowcytometry (**A**) as described in Materials and Methods. In another experiment, cells were pretreated with 10µM MG132 for 2h followed by treatment with or without 100µM DIM for 24h. Representative western blots show the protein levels of Cdc25C and Cdk1 (**B**) and cell cycle by flowcytometry (**B**). As mentioned above, cells were also pretreated with 30µM Chk2 inhibitor for 2h followed by treatment with 100µM DIM for 24h. Phosphorylation of Chk2 at Thr 68 and protein level of Chk2 were evaluated by western blotting (**C**). Flow cytometry analysis (**C**) shows the blockade of G2/M arrest in presence of Chk2 inhibitor. Blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading. Values are means ± SEM of 3 independent experiments. P values less than 0.05 were considered to be significant when compared with control or DIM treatment.

Figure 4. Dominant negative Chk2 abrogates DIM induced G2/M arrest. A representative cell cycle profile of SKOV-3 cells that were transfected with 1.5µg of plasmid containing dominant negative Chk2 for 24h followed by treatment with or without 100µM DIM for another 24h (**A**) is shown. Cell cycle analysis of SKOV-3 (**A**), OVCAR-3 (**B**) and TOV-21G (**C**) cells that were

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transfected with dominant negative Chk2 as described above followed by treatment with or without DIM for 24h. Phosphorylation of Chk2 at Thr 68 and Cdc25C at Ser216 and protein levels of Chk2, Cdc25C, Cyclin B1 and Cdk1 were analyzed by western blotting in SKOV-3 (**A**, lower panel), OVCAR-3 (**B**, lower panel) and TOV-21G (**C**, lower panel) cells transfected with DN-Chk2 and treated with or without 100μM DIM for 24h. Blots were stripped and reprobed with anti-actin antibody to ensure equal protein loading. Values are means ± SEM of 3 independent experiments. P values less than 0.05 were considered to be significant when compared with control or DIM treatment. DIM does not induce cell cycle arrest in Chk2 knock out cells. DT40 Chk2 knock out lymphoma cells were treated with 75μM or 100μM DIM for 24h and its effect on cell cycle profile was analyzed by flow cytometry (**D,** upper panel). 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**, lower panel). Each blot was stripped and reprobed with anti-actin to ensure equal protein loading.

Figure 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 75μM and 100μM DIM for 24h. SKOV-3 cells were treated with either 10μM CHX for 2h (**D**), 30μM Chk2 inhibitor for 2h (**D**) or transfected with DN-Chk2 for 24h (**D**) followed by treatment with 100μM DIM for 24h and analyzed for apoptosis by flow cytometry. Values are means ± SEM of 3 independent experiments. P values less than 0.05 are considered to be significant when compared with control or DIM treatment.

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

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FIGURE 1

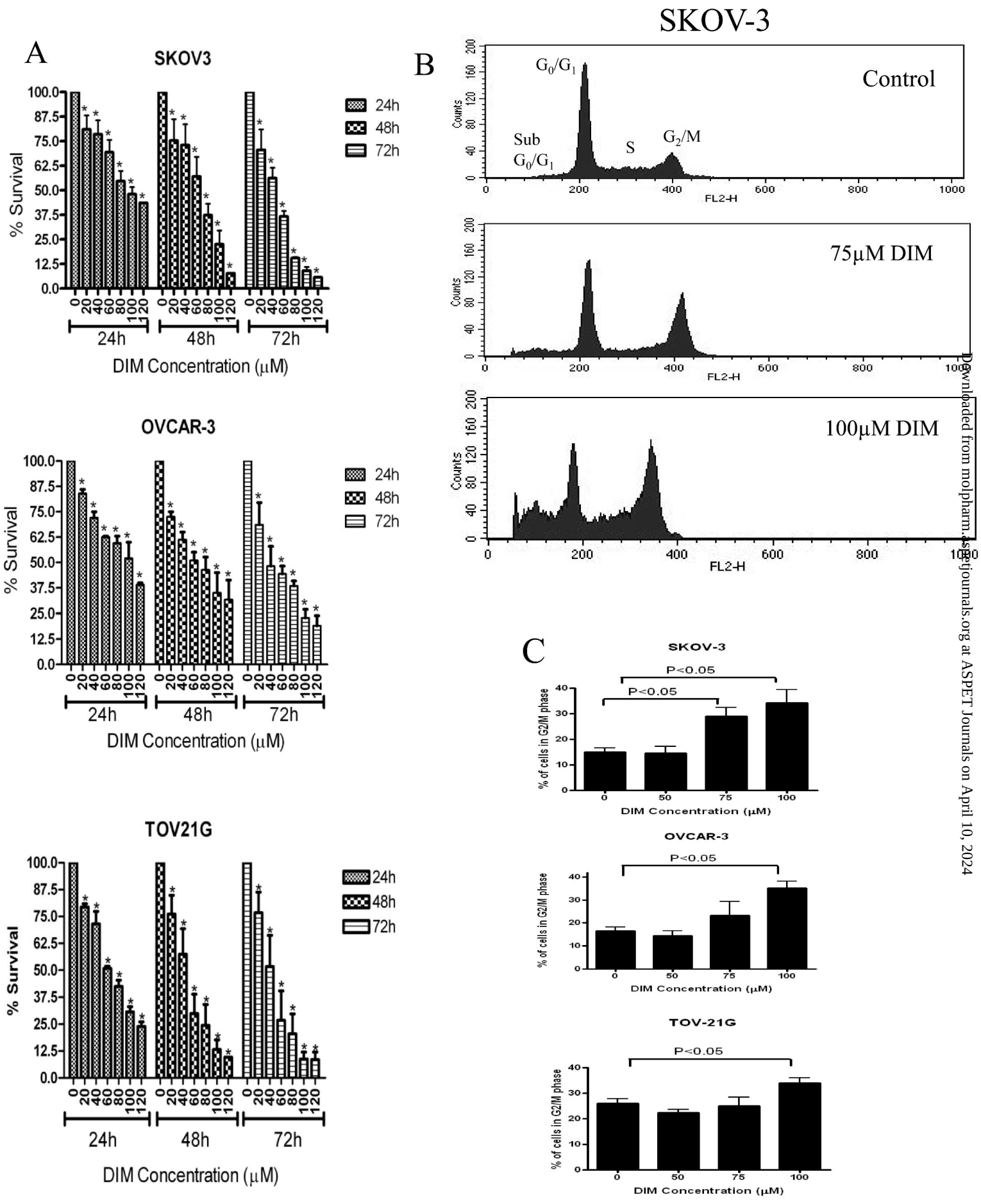

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FIGURE 2

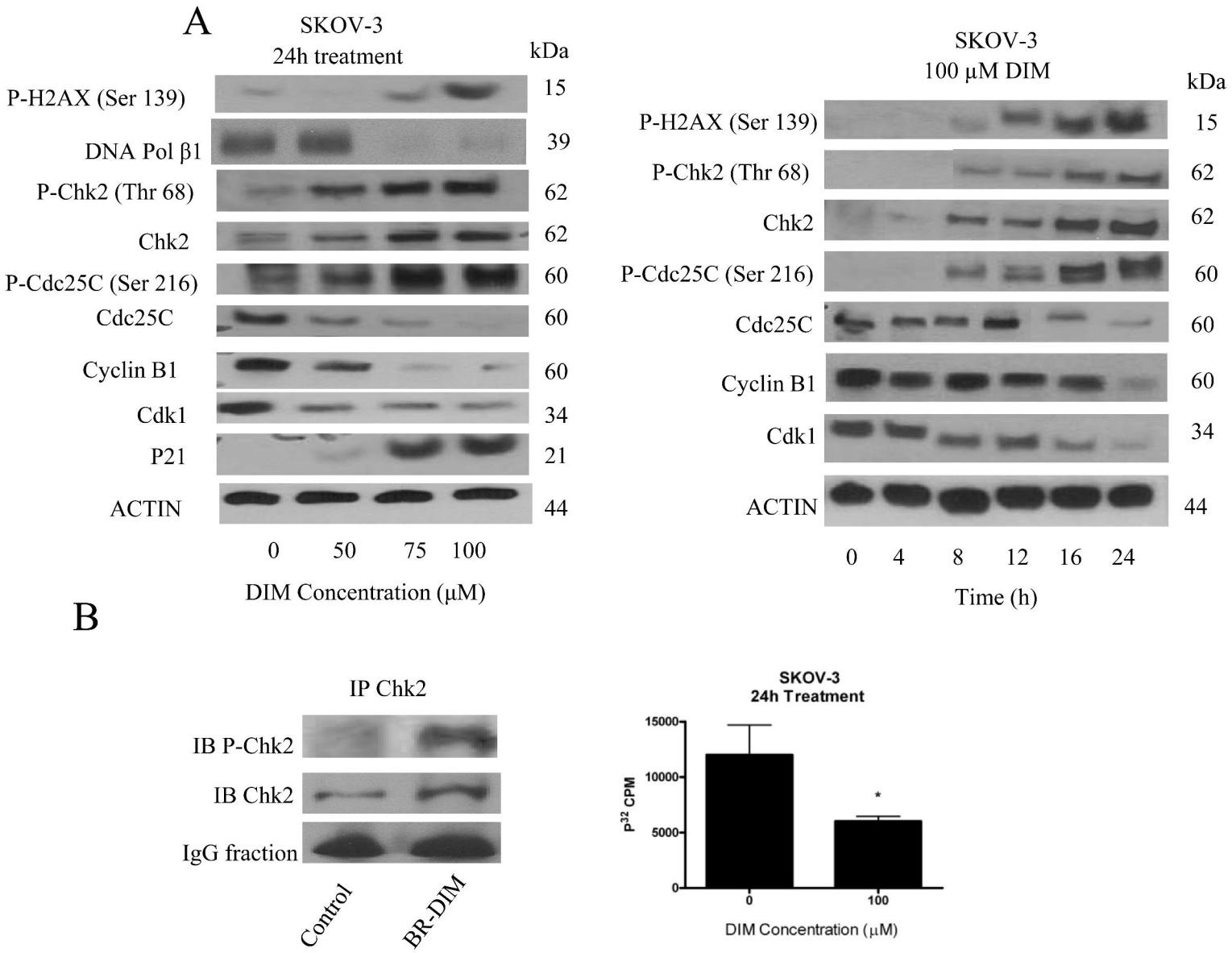

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FIGURE 3

SKOV-3

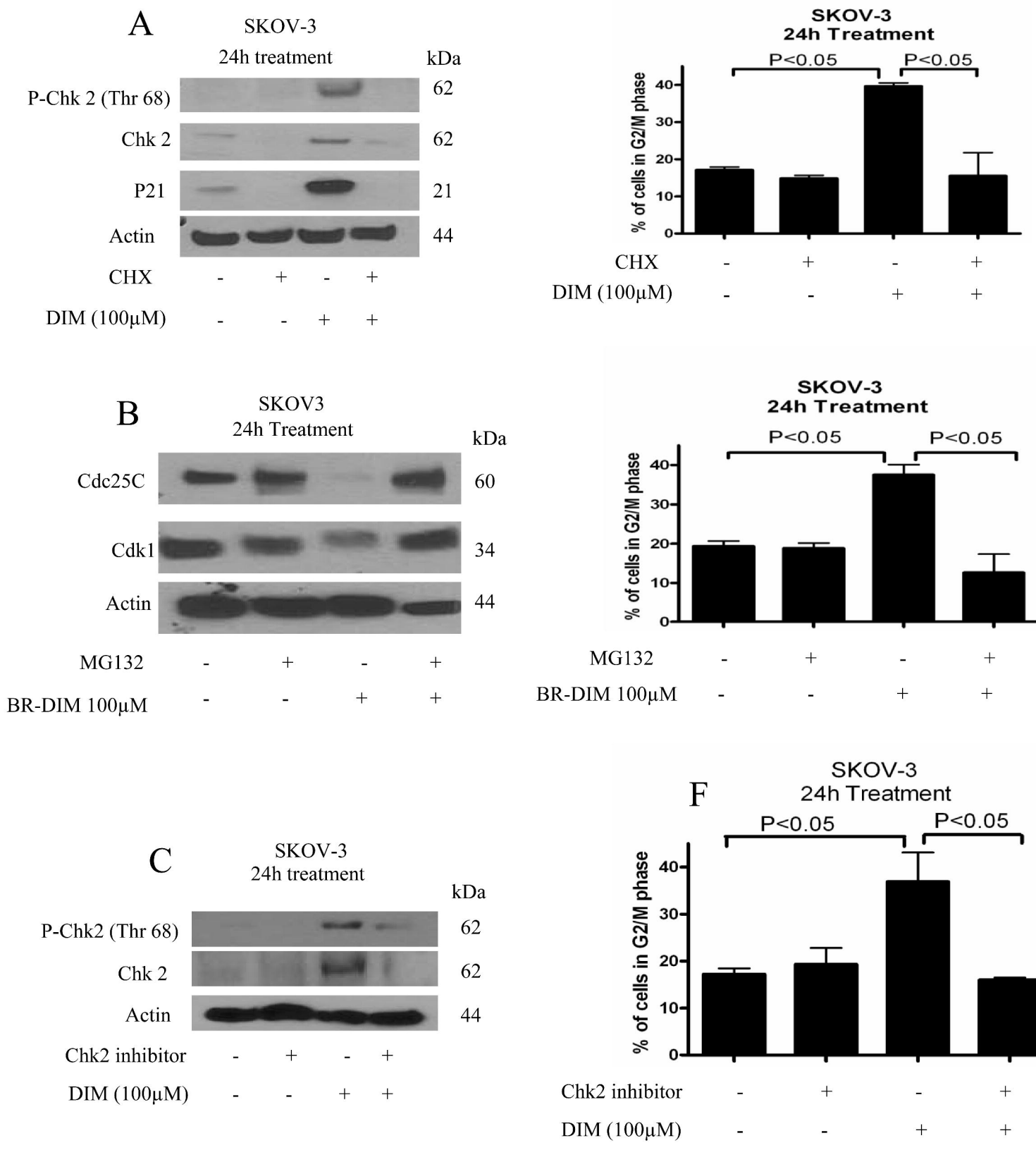

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FIGURE 4

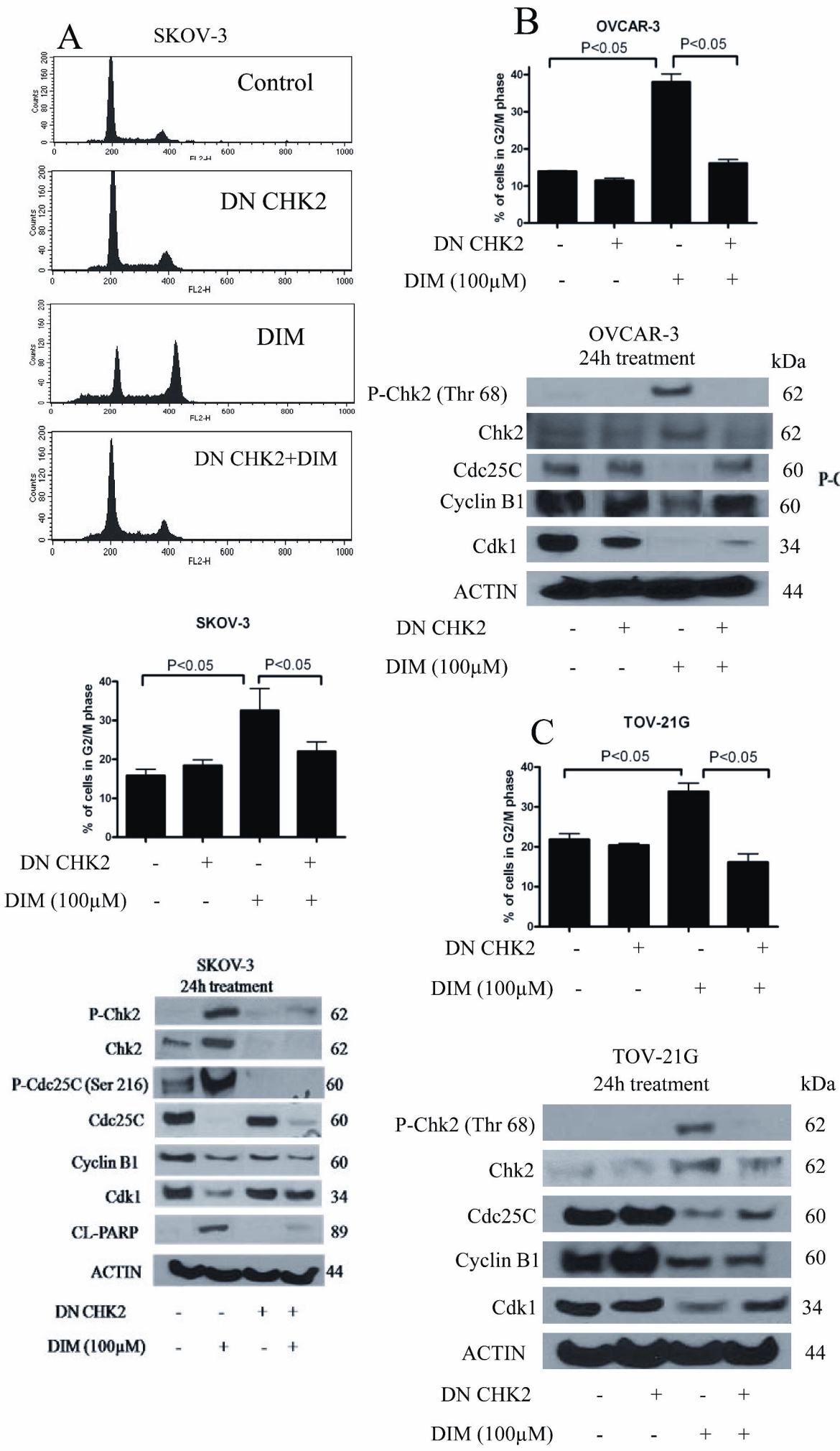

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FIGURE 5

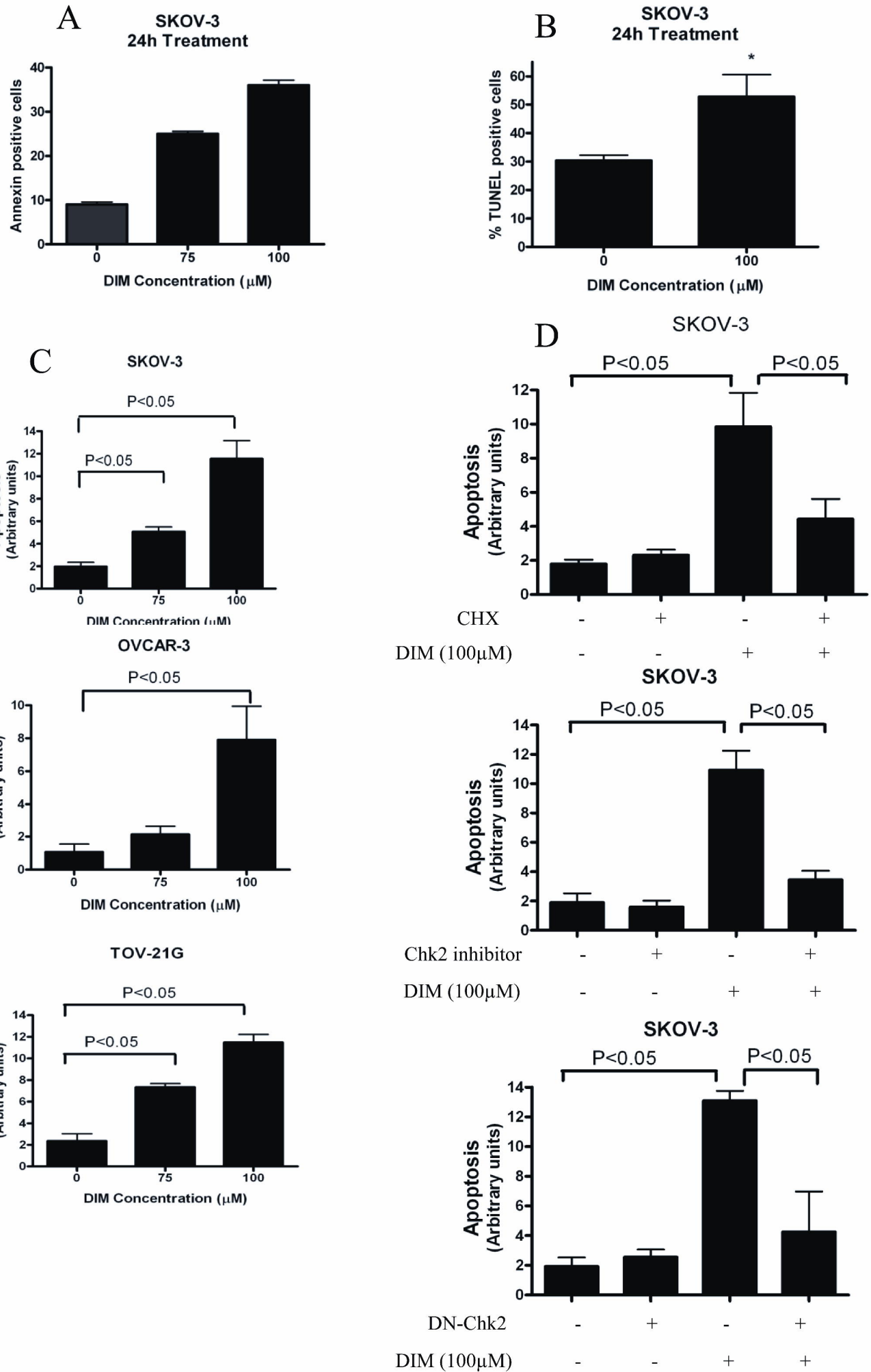

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FIGURE 6