Modulation of Cell-Cycle Regulatory Signaling Network by 2-Methoxyestradiol in Prostate Cancer Cells Is Mediated through Multiple Signal Transduction Pathways†

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ABSTRACT: 2-Methoxyestradiol (2-ME_2), a promising anticancer drug, induces growth arrest and apoptosis in various androgen-dependent (LNCaP) and -independent (DU145 and PC-3) prostate cancer cell lines. Moreover, flow cytometric analysis indicated a novel dual impact of 2-ME_2 on the cell division cycle of prostate cancer cells. Chronic exposure of high doses of 2-ME_2 enhance the accumulation of cells in S and G2/M phases, while cell numbers in the G1 phase were reduced significantly by this treatment. Because cyclin B1 overexpression, induction of cdc2 phosphorylation, and its regulatory proteins wee1 and phospho-cdc25C (interphase and mitotic forms) by 2-ME_2 treatment correlated with the induction of apoptosis, growth arrest at the G2/M phase, and accumulation of the S phase, we reasoned that cyclin B1 and cdc2 phosphorylation and its upstream regulatory molecular networks may be associated with the ultimate impacts of 2-ME_2. Because phosphorylation of cdc2 and upregulation of wee1 by 2-ME_2 can be abolished by both extracellular receptor kinase (ERK) inhibitor (U0126) and c-Jun N-terminal kinase (JNK) inhibitor (SP600125), our studies indicate that the 2-ME_2-induced upregulation of wee1 and subsequent cdc2 phosphorylation are mediated through mitogen-activated protein kinase (MAPK)−ERK−JNK signaling pathways.

2-Methoxyestradiol (2-ME_2)[1] is a physiological metabolite of natural and synthetic estrogen generated from hydroxylation at the 2-C position of 17β-estradiol by cytochrome P450 and from methylation at the same position by catechol-*O*-methyl transferase (COMT) (*1*). In recent years, 2-ME_2 has become a promising anticancer drug (*2*) because of its unique characteristics, such as orally active (*3*), nontoxic (*2*, *4*), a selective inhibitor of proliferating cells (*1*), antiangiogenic (*4*), able to overcome other conventional drug resistance to tumor cells (*5*), and its low binding affinity to estrogen receptor (*1*, *4*). Similar to colchicine, 2-ME_2 arrests the cell cycle at the G2/M phase (*1*) and binds to the colchicine-binding site of tubulin (*6*), resulting in tubulin depolymerization (*7*). 2-ME_2 also effectively induces apoptosis in actively proliferating cell in vitro and in vivo through various proposed mechanisms, such as the upregulation of p53 (*4*), death receptor (*8*), free-radical generations (*9*), activation of c-Jun N-terminal-activated kinase (JNK) and Bcl-xL and Bcl-2 phosphorylation (*10*−*12*), increased expression of FAS (*13*), and mitochondrial release of cytochrome *c* (*10*). When these studies are taken together, they indicate that the active molecular network of 2-ME_2 is a complex process that involves various pathways. However, the exact mechanism underlying 2-ME_2-mediated cell-cycle regulation including G2/M arrest has not been fully elucidated.

In the eukaryotic system, the entry of cells from the G2 to M phase depends upon the activity of several cell-cycle regulatory proteins, including cyclin B1, cdc2, wee1, cdc25C, and others (*14*−*16*). Inactivation of cdc2 by phosphorylation at threonine 14 (T14) and tyrosine 15 (Y15) normally allows the cell to be retained in the interphase, particularly the S−G2 phase (*17*). wee1, a nuclear tyrosine kinase, which along with Myt-1, facilitates these phosphorylation events (*18*). At the end of G2, dephosphorylation of cdc2 at T14 and Y15 is required for the mitotic entry of the cells (*19*). This dephosphorylation process is mediated through an active cdc25C dual phosphatase (*19*). Therefore, inactivation of cdc25C phosphatase by phosphorylation at serine 216 retains the cdc2−cyclin B1 complex in inactive conditions, resulting in cell-cycle arrest at the G2/M boundary (*20*) as well as the S-phase accumulation (*21*). Previously, we and others reported that cell-cycle regulatory proteins, such as cyclin B1 and cdc2 might play important roles in 2-ME_2-induced cell-cycle arrest and apoptosis in both breast and prostate tumor cells (*22*, *23*). In the present study, we reevaluated the impact of 2-ME_2 on cell proliferation and cyclin B1 and

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[1] Abbreviations: 2-ME_2, 2-methoxyestradiol; cdc2/cdk1, cyclin-dependent kinase 1; ERK, extracellular receptor kinase; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase, PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.

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cdc2 expression and activities, determining the underlying signaling molecules involved in 2-ME_2-induced regulation of cell-cycle regulatory proteins in prostate cancer cells. We report that 2-ME_2 arrests cells at the G2/M phase and increased the population in the S phase. Inactivation of the cdc2−cyclin B1 complex by phosphorylation of cdc2, which occurs via inactivation of cdc25C activity through the induction of its phosphorylation and wee1 overexpression, may be associated with cell-cycle alterations by 2-ME_2. Elevation of cyclin B1 and its accumulation in cytoplasm may also be associated with these events. Moreover, phosphorylation of cdc2 and upregulation of its regulatory molecule, wee1, are mediated through extracellular receptor kinase (ERK) 2 and JNK-dependent signaling pathways.

EXPERIMENTAL PROCEDURES

Reagents, Chemicals, and Antibodies. Dulbecco's modified Eagle's medium (DMEM), penicillin−streptomycin, trypsin EDTA solution, anti-actin monoclonal antibody, and 2-ME_2 were purchased from Sigma (St. Louis, MO). A 20 mM stock solution of 2-ME_2 was prepared in absolute ethyl alcohol and stored at −20 °C. Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT). Monoclonal mouse antibodies, such as cdc2 and cyclin B1 were purchased from NeoMarkers, Inc. (Fremont, CA), whereas, wee1 monoclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal phospho-(Thr) mitogen-activated protein kinase (MAPK)/CDK substrate antibody, rabbit polyclonal antibodies, such as phospho-Akt, Akt, cdc25C, phospho-cdc2 (Tyr15), ERK, phospho-ERK, phospho-JNK, JNK, p38 MAPK, and phospho-p38 MAPK were purchased from Cell Signaling Technologies, Inc. (Beverly, MA). Secondary antibodies, such as goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ERK inhibitor (U0126) and JNK inhibitor II (SP600125) were purchased from Promega (Madison, WI) and Calbiochem (San Diego, CA), respectively. All other chemical were obtained from either Sigma (St. Louis, MO) or Fisher Scientific (Fisher Scientific Co., Houston, TX).

Cell-Culture Conditions and Treatments. LNCaP, DU145 and PC-3 prostate cancer cell lines were obtained from American type culture and collection (ATCC) (Manassas, VA). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 2mM glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin in a 5% CO_2 incubator at 37°C. ~80% confluent cells were treated with 1, 5 and 10μM 2-ME_2 for 48 h or 10μg/mL colchicine for 1 h before harvesting.

Enzyme-Linked Immunosorbent Assay (ELISA) for Apoptosis. Photometric enzyme immunoassay for quantitative in vitro determination of cytoplasmic histone-associated DNA fragment (mono- and oligonucleosome) after apoptotic cell death was determined by using cell-death detection ELISA kits (Roche Diagnostic Corporation, Indianapolis, IN). Briefly, prostate cancer cell lines, DU145, PC-3, and LNCaP, were treated with 1, 5, and 10 μM 2-ME_2 for 48 h. Cells were harvested and lysed with lysis buffer. The cytoplasmic supernatant was collected, and the total protein was estimated. A total of 20 μL of cell lysate containing 10 μg of protein was added in the streptavidin-coated microplate and allowed to react with 80 μL of buffer mixture containing anti-histone-biotin and anti-DNA-peroxidase for 2 h with continuous shaking. Microplates were washed with incubation buffer 3 times. The ABTS [2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid)] chromogen substrate was added to get a color reaction that was measured in the ELISA reader at 405 nm.

Flow Cytometric Analysis. DU145 cells, after the treatment with 2-ME_2, were collected in 1.0 mL of phosphate-buffered saline (PBS, pH 7.4), and cells were counted. After centrifugation, the cells were resuspended in 150 μL of PBS, 375 μL of fluorescence-activated cell sorter (FACS) buffer containing 100 mM sodium acetate, 5.4 mM EDTA (pH 5.2), 250 μL of RNase (10 mg of 70 units/mg RNase plus 1.0 mL of FACS buffer), and 100 μL Triton-X 100. The cell suspension was incubated at 37 °C in 5% CO_2 for 30 min. After incubation, 100 μL of propidium iodide (0.5 mg/mL) solution was added to each tube and incubated at room temperature for an additional 30 min in the dark. The samples were then processed for flow cytometric analysis.

Extractions of Subcellular Fractionation. Cells were harvested by trypsinization from 70 to 80% confluent cultures; the membrane and cytosolic fractions were isolated using Mem-PER eukaryotic membrane protein extraction reagent kit (Pierce Biotechnology, Rockford, IL).

Western Blot Analysis. The Western blot analysis was the same as previously described by Banerjee et al. (*24*). Briefly, 2-ME_2-treated and untreated control cells were washed with ice-cold 1× PBS (pH 7.4), followed by incubation in cell lysis buffer for 30 min at 4 °C. Protein concentrations were measured by the method of Bradford using the Coomassie protein assay reagent (Pierce, Rockford, IL). A total of 50 μg of total protein was separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The fractionated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Micron Separation, Inc., MA) and subsequently probed with primary antibodies for a specific period of time as per the instructions of the manufacturer. Blots were washed in TBS-T and incubated with secondary antibodies conjugated with horseradish peroxidase for 30 min at room temperature. Immunoreactions (antigen−antibody) were detected by using enhanced chemiluminescence (ECL) (Pierce) followed by exposure to the Kodak Biomax ML Scientific Imaging Film system (Rochester, NY).

Cyclin B1−cdc2 Kinase Activity Assay. Cyclin B1−cdc2 kinase activity was performed by using a commercially available kit for immunoprecipitation (Kirkegaard and Perry Laboratories, Gaithersburg, MD). A 50% resin slurry was first prepared by repeated washing with lysis buffer containing 150 mM NaCl, 1.0% Triton X-100, and 50 mM Tris-HCl at pH 7.4. Protein lysates (1 mg/mL) were added to the resin slurry to preclear the protein sample by incubating at 4 °C for 1 h with constant gentle mixing. The supernatant was collected after centrifugation at 14000*g* for 20 s. A total of 5 μg of cdc2 monoclonal antibody was added to the precleared sample along with fresh 50% slurry. The mixture was incubated at 4 °C overnight. The pelleted resin was collected by centrifugation and washed with lysis buffer containing protease inhibitor cocktails. The samples were then incubated with 20 μL of kinase buffer (50 mM Tris-HCl at pH 7.5, 10 mM $MgCl_2$, 1.0 mM EGTA, and 2 mM DTT) at 30 °C for 5 min followed by incubation with 5 μg

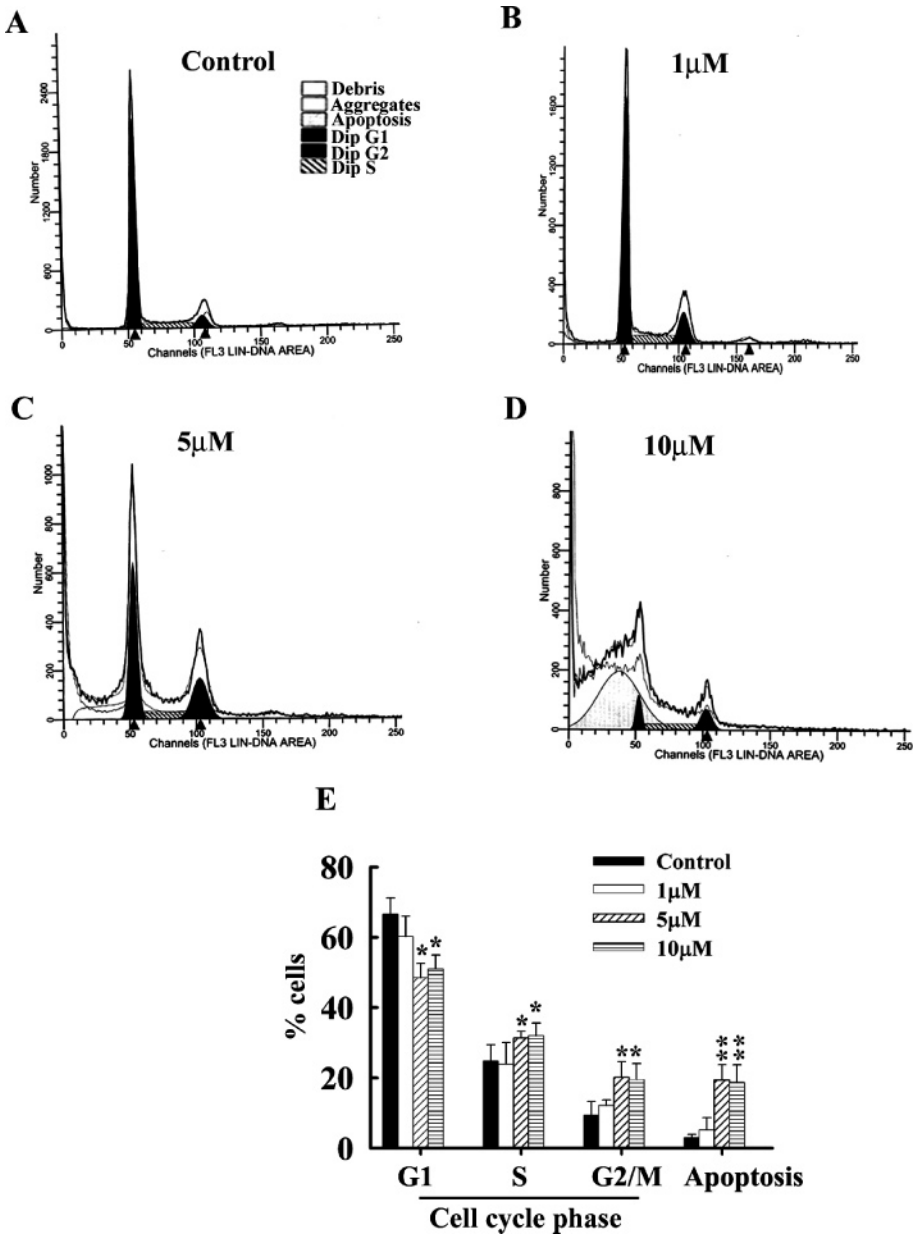

FIGURE 1: 2-ME$_2$-induced cell-cycle arrest and apoptosis in DU145 prostate cancer cells by flow cytometric analysis. (A−D) 2-ME$_2$-treated and vehicle-treated cells were harvested and incubated with propidium iodide and analyzed by flow cytometry. (E) Bar diagram showing comparative cell populations in G1, S, G2/M, and apoptotic cells in 2-ME$_2$-treated cells. (*) $p < 0.05$ and (**) $p < 0.01$ versus the vehicle-treated control (Student's *t* test).

of histone H1 and 500 μM ATP at 30 °C for 30 min. Proteins were separated by SDS−PAGE and transferred to PVDF membrane overnight at 4 °C. After blocking, the membrane was incubated with phospho-(Thr) MAPK/CDK for 1 h. The membrane was washed with TBS-T, and a secondary antibody (goat anti-mouse IgG-HRP) was applied for 30 min followed by ECL detections.

Statistical Analysis. All data are expressed as the mean ± standard deviation (SD). Statistically significant differences between groups were determined by using the nonpaired Student's two-tailed *t* test. A value of $p < 0.05$ was considered statistically significant.

RESULTS

2-ME$_2$ Induces S-Phase Accumulation, G2/M Arrest, and Apoptosis in Prostate Cancer Cells. We determined the effect of 2-ME$_2$ on cell-cycle progression in androgen-resistant DU145 prostate cancer cells by flow cytometric analysis (FACScan). Confluent cells (~70%) were exposed to 1, 5, and 10 μM 2-ME$_2$ for 48 h. The result shows a dose-dependent effect of 2-ME$_2$ on different phases of the cell cycle when asynchronous DU145 cells were exposed to 1, 5, and 10 μM 2-ME$_2$ for 48 h. The cell numbers in the G1 phase were reduced significantly, while the S and G2/M phases were significantly increased in 5 and 10 μM 2-ME$_2$-treated cells, respectively, as compared to the vehicle-treated controls (Figure 1). The impact of 2-ME$_2$ on cell-cycle division was identical in other prostate cancer cell lines, including PC-3 and LNCaP (data not shown). The flow cytometric analysis indicates that the frequency of apoptotic cells were also markedly elevated in 5 and 10 μM 2-ME$_2$-treated DU145 cells as compared to the controls. Similar to proliferation, 1 μM 2-ME$_2$ was unable to alter the apoptosis in these cells (Figure 1). This finding was further confirmed

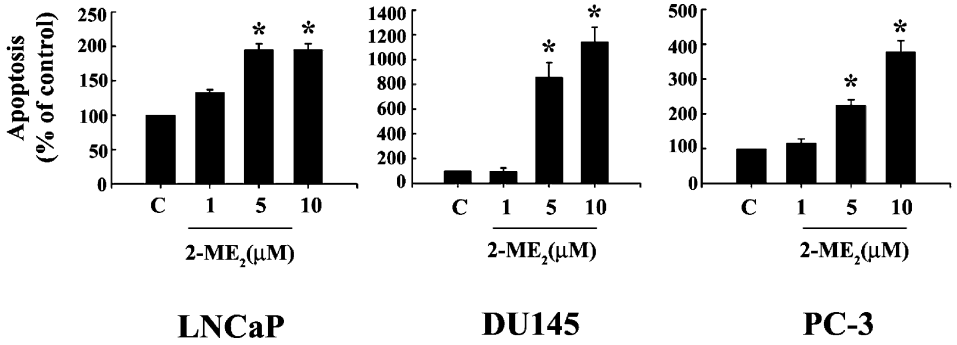

FIGURE 2: 2-ME_2-induced apoptosis in LNCaP, DU145, and PC-3 prostate cancer cells. Prostate cancer cells were grown asynchronously and treated with 1, 5, and 10 μM 2-ME_2 for 48 h. The enzyme immunoassay for quantitative in vitro determination of the cytoplasmic histone-associated DNA fragment for apoptotic cell death was determined by using the cell-death detection ELISA kit. Bar diagrams show apoptotic cell death in control (C) and treated cells. Results are expressed as a percent of the control ± SD from three separate experiments. (*) $p < 0.001$ versus the vehicle-treated control (Student's *t* test).

in other prostate cancer cell lines using cell-death detection ELISA. The studies showed that the apoptosis was increased in a dose-dependent manner in LNCaP, DU145, and PC-3 cells, and these inductions began significantly after 5 μM 2-ME_2 exposure (Figure 2). The effect of 1 μM 2-ME_2 was nonsignificant in these cell lines. The impacts of higher doses of 2-ME_2 on apoptosis are identical with the previous studies (*23*, *25*). Together, these studies provide a novel divergent effect of this estrogen metabolite on prostate cancer cells, which ultimately induces S-phase accumulation along with G2/M arrest and apoptosis.

Next, to corroborate the biphasic impact of 2-ME_2 on prostate cancer cells, we determined if 2-ME_2 is able to modulate the cell-cycle division of synchronously cycling prostate cancer cells. To investigate this, two experiments were carried out. In the first experiment, semiconfluent prostate cancer cells were brought to quiescence by serum starvation. Starved cells were then exposed to 10% serum or 5 μM 2-ME_2 alone for distinct times, and cell-cycle analysis was performed by propidium iodide staining followed by flow cytometry. The findings support the biphasic concept and indicate that, similar to serum, the addition of 2-ME_2 into the culture media differentially modulates the cell-cycle phases and is able to release the cell-cycle blockade induced by serum deprivation (Figure 3). 2-ME_2 significantly elevates S and G2/M phases after 6 h of exposure, and this induction was progressively increased with prolonged exposures (i.e., 12, 18, 24, and 48 h). The apoptotic cell death, which was induced by serum starvation, was decreased significantly immediately after the exposure of 2-ME_2 (Figure 3C). This immediate effect of 2-ME_2 was almost identical with the impact of serum. However, unlike serum, prolonged exposure (48 h) of 2-ME_2 induces apoptosis significantly in prostate cancer cells (Figure 3). Next, we determined the impact of 2-ME_2 on prostate cancer synchronized cycling cells after being released from the blockage by the addition of serum. As shown in Figure 3D, when cells were exposed to 2-ME_2 (5 μM) in the presence of serum, 2-ME_2 exhibits a synergistic effect on cell-cycle phases but induces a significant amount of apoptotic cell death after 24 h of exposure. Therefore, we anticipate that apoptotic cell death is associated with a cell-cycle progression in response to 2-ME_2.

2-ME_2 Differentially Regulates Cyclin B1 and cdc2 Expression in DU145 Cells. Dynamic shuttling of cyclin B1 between the nucleus and cytoplasm and overexpression of cyclin B1 has been considered as a marker of the interphase including S and G2 phases (*16*, *26*−*28*). On the other hand, activation of the cdc2−cyclin B complex kinase and accumulation of cyclin B1−cdc2 in the nucleus (*27*) is required for mitotic progression (*29*, *30*). Therefore, we sought to determine whether 2-ME_2 is able to modulate S−G2/M-phase regulatory proteins, such as the cyclin B1 and cdc2 expression and distribution of cyclin B1 in prostate cancer cells. Because the effects of 2-ME_2 on apoptosis in DU145, PC3, and LNCaP were identical, this and subsequent studies were carried out with DU145 cells with the use of various concentrations of 2-ME_2. Cyclin B1 protein levels were found to be significantly elevated by 1.6, 2.0, and 1.8-fold in 1, 5, and 10 μM 2-ME_2-treated cells, respectively, as compared to the untreated control (Figure 4). In contrast, total cdc2 protein levels were found unaltered in 2-ME_2-treated cells by Western blot analysis (Figure 4), which was not in agreement with the previous work (*23*). Next, we determined if 2-ME_2 controls the distribution of cyclin B1 in DU145 cells. To test this, cyclin B1 protein levels were determined in nuclear and cytoplasmic fractions of DU145 cells using Western blot analysis. We found that the cyclin B1 accumulation was increased markedly in the cytoplasm (Figure 5).

The product of the cdc2 gene (cdc2 or $p34^{cdc2}$) is a master regulator of cell-cycle progression (*29*). Studies show that increased phosphorylation on the T14 and Y15 residues of cdc2 reduces the kinase activity of the cdc2−cyclin B1 complex, which is essential for the DNA replication checkpoint in physiological and pathophysiological conditions (*31*). Tyrosine phosphorylation of cdc2 has also been implicated in cell-cycle arrest at G2 in response to DNA damage (*32*). Because preceding results showed that 2-ME_2 at a higher concentration arrests the cell cycle at the G2/M phase without altering the cdc2 protein levels, we, therefore, determined if 2-ME_2 modulates the cdc2 phosphorylation as well as its distribution in prostate cancer cells by immuno-Western blot analysis using phospho-cdc2 polyclonal antibody specific for tyrosine 15 phosphorylation. cdc2 phosphorylation at Y15 was significantly increased in 5 and 10 μM 2-ME_2-treated cells as compared to the vehicle-treated control (Figure 6), and the phosphorylated form of cdc2 was predominantly located in the nucleus of DU145 cells (Figure 5). As expected from previous study, a low dose of 2-ME_2 (1 μM) was unable

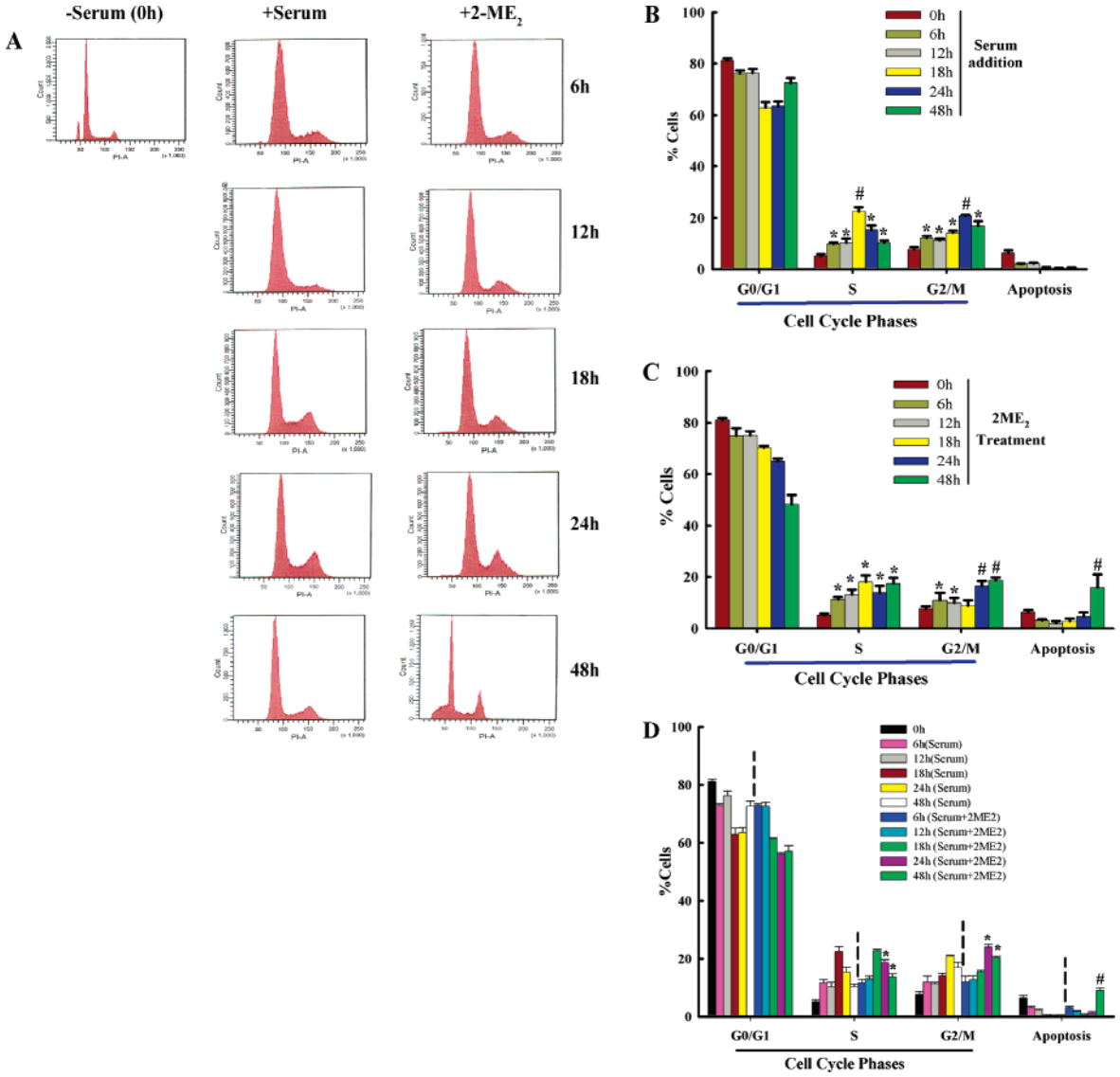

FIGURE 3: Effect of serum and 2-ME$_2$ on cell division cycle kinetics of synchronously cycling prostate cancer cells. Semiconfluent DU145 cells were brought to the quiescence by 3 days of serum starvation. Starved cells were then exposed to 10% serum, 5 μM 2-ME$_2$, or both for various times (i.e., 0, 6, 12, 18, 24, and 48 h), and cell-cycle analysis were performed by propidium iodide staining followed by flow cytometry. (A) Representatives of flow cytometric analyses of the cell division cycle in serum or 2-ME$_2$-treated prostate cancer cells. (B and C) Bar diagram shows the mean ± SD ratio of three experiments. (*) $p < 0.01$ versus the serum-starved control, and (#) $p < 0.001$ versus the serum-starved control (Student's *t* test). (D) Bar diagram exhibits the impact of 2-ME$_2$ on prostate cells after being released from the blockage by the addition of serum. (*) $p < 0.05$ versus the serum-exposed control, and (#) $p < 0.01$ versus the serum-exposed control (Student's *t* test).

to alter the phosphorylation of cyclin-dependent kinase 1 (cdc2/cdk1).

In mitosis, an active cdc2, after dephosphorylation at T14 and Y15, displays kinase activity by phosphorylating histone H1 as the substrate (*14*, *33*). We, therefore, verified whether phosphorylation of cdc2 by higher doses of 2-ME$_2$ eventually reduces the activity of cdc2 by determining the histone H1 kinase activity. As expected, cyclin B1−cdc2 kinase activity reduced significantly (~60%) in both 5 and 10 μM 2-ME$_2$-treated cells as compared to vehicle-treated cells (Figure 6). A 1 μM 2-ME2 exhibits no effect on the kinase activity of the cyclin B1−cdc2 complex (Figure 6). This study, therefore, further confirms that higher doses of 2-ME$_2$ inhibit the kinase activity of cdc2 in prostate cancer cells. Together with the previous study (*23*), our results suggest that the phosphorylation of cdc2 by 2-ME$_2$ at higher concentrations may be a unique effect of 2-ME$_2$ that leads the cells to accumulate at G2/M boundaries along with S-phase accumulations.

wee1 Protein Level Increased in 2-ME$_2$-Treated Prostate Cancer Cells. The human cell-cycle regulatory gene WEE1, a nuclear tyrosine kinase, inhibits entry into the M phase by mediating the phosphorylation of cdc2 at Y15 exclusively in the nucleus (*18*, *34*, *35*). Studies indicated that wee1 is overexpressed in human cells that are arrested at the G2 phase (*35*), and this overexpressed wee1 is localized in the nucleus and is able to block activated cyclin B1−cdc2 from inducing mitosis (*34*). Previous studies also indicated that wee1 activity increases at the S phase and decreases at the M phase (*36*). As we have shown in a previous study that 2-ME$_2$ at higher concentrations are capable of upregulating phosphorylation of cdc2 in the nucleus (Figures 5 and 6), we explored whether 2-ME$_2$ also modulates wee1 expression. The studies found that wee1 levels were elevated significantly

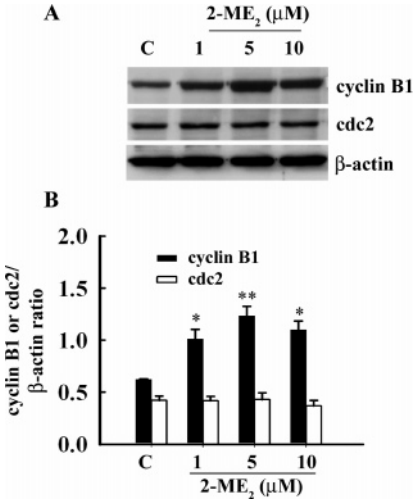

FIGURE 4: Effects of 2-ME_2 on cyclin B1 and cdc2 protein expression in DU145 prostate cancer cells. (A) Three single representative blots showing cyclin B1, cdc2, and β-actin (internal control) levels in controls (C) and 1, 5, and 10 μM 2-ME_2-treated cells. (B) Bar diagram showing mean $\pm$ SD of the cyclin B1/β-actin or cdc2/β-actin ratio from three independent experiments. (*) $p < 0.05$ and (**) $p < 0.01$ versus the vehicle-treated control (Student's t test).

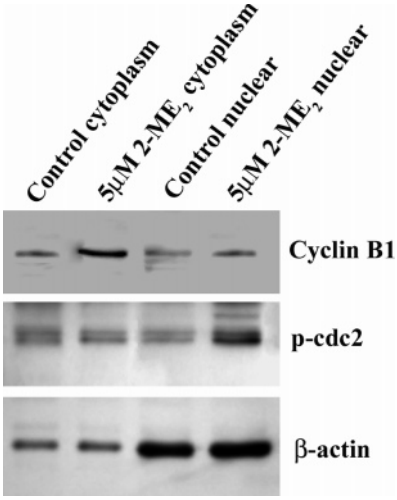

FIGURE 5: Distribution of cyclin B1 and cdc2 proteins in nuclear and cytoplasmic compartment of 2-ME_2-exposed DU145 cells. DU145 cells were exposed to 5 μM 2-ME_2 for 48 h, and then nuclear and cytoplasmic proteins were extracted as described in the Experimental Procedures. After gel electrophoresis and transfer to the membrane, the levels of cyclin B1 and cdc2 were detected by Western blot analysis using specific antibodies. The level of β-actin was also detected in the same membrane and used as a loading control.

in 5 and 10 μM 2-ME_2-treated cells when compared to the vehicle-treated controls (Figure 7). The study is directly correlated with the cdc2 phosphorylation and suggests that induction of the wee1 level by 2-ME_2 at higher concentrations in prostate cancer cells may be responsible for 2-ME_2-induced cdc2 phosphorylation in DU145 cells. Moreover, this induction of wee1 is also supportive of evidence that cells are more likely accumulated at the S−G2/M phase. Moreover, wee1 induction might be the cause of the reduced histone H1 kinase activity (*37*).

2-ME_2 Treatment Alters the cdc25C in Prostate Cancer Cells. cdc25C, a dual-specificity protein phosphatase, has been associated with both G1−S progressions and mitotic

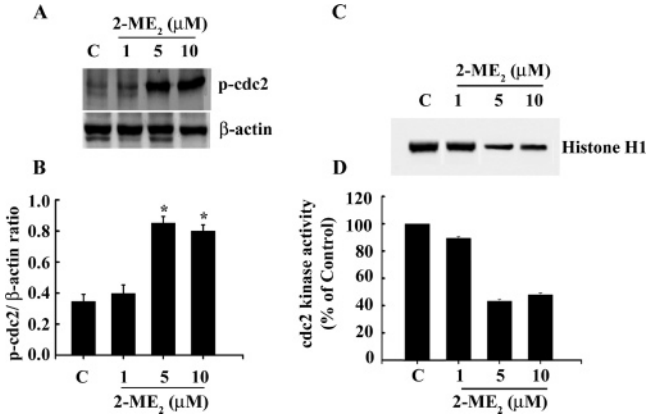

FIGURE 6: Modulation of phospho-cdc2 and histone H1 kinase activity by 2-ME_2. (A) Single representative immuno-Western blot showing phospho-cdc2 and β-actin (internal control) levels in prostate cancer cells. (B) Bar graphs represent mean $\pm$ SD of the phospho-cdc2/β-actin ratio from three independent experiments. (C) Cyclin B1−cdc2 (histone H1) kinase activities determined by immunoprecipitation followed by Western blot analysis using phospho-(Thr) MAPK/CDK antibody in DU145 prostate cancer cells. (D) Bar diagram represents mean $\pm$ SD of cdc2 kinase activities of three individual experiments. (*) $p < 0.01$ versus the vehicle-treated control (Student's t test).

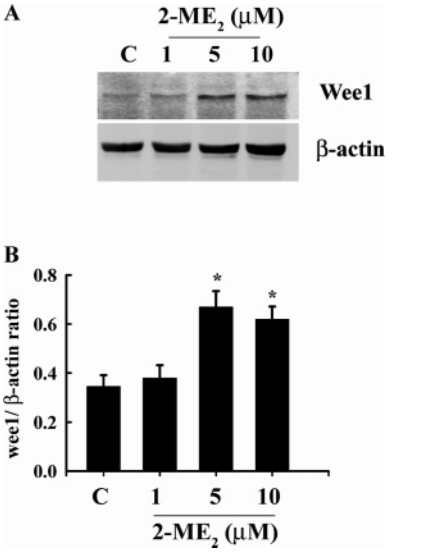

FIGURE 7: Differential expression of wee1 protein in the 2-ME_2-treated DU145 cell line. The total protein was isolated, and Western blot analysis was performed using mouse monoclonal antibody. (A) Single representative blot showing the wee1 and β-actin protein in the control (C) and 1, 5, and 10 μM 2-ME_2-treated cells. (B) Bar diagram shows the mean $\pm$ SD ratio of wee1 and β-actin from three independent experiments. (*) $p < 0.01$ versus the vehicle-treated control (Student's t test).

activation by altering the phosphorylation state of cdc2 (*21*, *38*). Three electrophoretic forms of cdc25C phosphorylation have been identified in asynchronous dividing cells (*39*). Two forms (57 and 60 kDa) are restricted to the interphase (i.e., G1−S−G2), while the other (80 kDa) is generally formed at G2/M (*39*). Phosphorylation of Ser^{216} and hyperphosphorylation on NH_2-teminal serine and threonine residues are involved in the formation of 60 and 80 kDa protein bands of cdc25C (*39*). After the serine 216 phosphorylation of cdc25C in the interphase, it becomes functionally inactive and binds to 14−3−3 family protein that abrogates the regulation of cdc2 (*32*). In response to DNA damage, cdc25C

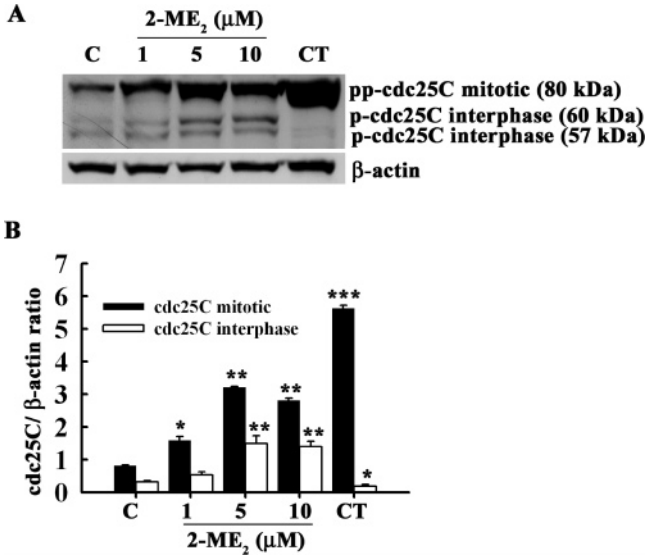

FIGURE 8: Differential expression of different forms of cdc25C levels by 2-ME$_2$ and colchicine in DU145 cells. The total protein was isolated, and cdc25C levels were determined in the control and 1, 5, and 10 μM 2-ME$_2$-treated and CT cells by immuno-Western blot analysis using cdc25C-specific polyclonal antibody. (A) Single representative blot showing cdc25C expression in the vehicle-treated control (C) and 1, 5, and 10 μM 2-ME$_2$-treated and CT cells with respect to the corresponding β-actin. (B) Bar diagram shows the mean ± SD ratio of three experiments for both mitotic and interphase forms of cdc25C expression after normalization with β-actin. (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ versus the vehicle-treated control (Student's t test).

has been found to be phosphorylated on serine 216 by a checkpoint kinase chk1, which leads to the binding of the 14−3−3 protein and inhibition of the ability of cdc25C to dephosphorylate and activate cdc2 (*40*, *41*). Because 2-ME$_2$ at 5 and 10 μM concentrations are capable of arresting cells at the G2/M phase and accumulation of cells in the S phase, we sought to determine the status of cdc25C in different concentration of 2-ME$_2$-treated prostate cancer cells using a specific polyclonal antibody that recognizes all of the three forms of cdc25C. As shown in Figure 8, the mitotic form is increased significantly in cells treated with 1, 5, and 10 μM 2-ME$_2$, as compared to the vehicle-treated controls, while the phosphorylated-interphase form (60 kDa) was found to be significantly elevated in 5 and 10 μM 2-ME$_2$-treated cells (Figure 8). A 1 μM 2-ME$_2$ has no effect on the phosphorylated interphase (Figure 8). In this study, we also determined the cdc25C status in colchicine-treated (CT) cells as a positive control because colchicine treatment increases the population of mitotic cells by arresting the cells at the G2/M checkpoint. We found that acute colchicine treatment increased the mitotic form of cdc25C in these cells, while it has no impact on interphase forms of cdc25C. Together, these studies suggest that the induction of mitotic cdc25C by 2-ME$_2$ or colchicine may be required to arrest the dividing cells at the mitotic phase by these two chemicals. The interphase phosphorylation of cdc25C may additionally support that cells may be accumulated at the S phase and arrested at the G2 phase. On the other hand, induction of the phosphorylated-interphase form of cdc25C by 2-ME$_2$ may block the dephosphorylation of T14 and Y15 of cdc2 (*20*), which may eventually potentiate cells to enter into the G1−S and arrested at the S/G2 phase (*21*).

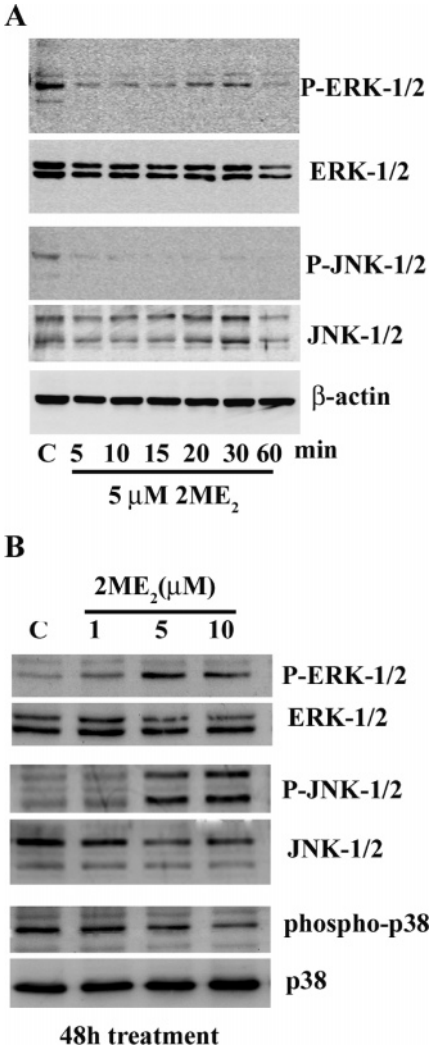

FIGURE 9: Differential changes in phosphorylated JNK 1/2, ERK 1/2, and p38 MAPK after the exposure of 2-ME$_2$. The total protein was isolated, and Western blot analysis was performed to determine total and phosphorylated forms of MAPK-specific antibodies. (A) Single representative blots showing total and phosphorylated JNK 1/2 and ERK 1/2 expression after a short-exposure time (0, 5, 10, 15, 20, 30, or 60 min) with 2-ME$_2$ (5 μM) in DU145 prostate cancer cells. β-Actin Western blot was done as an internal control to see the uniform protein loading. (B) Representative blots showing total and phosphorylated JNK 1/2, ERK 1/2, and p38 MAPK expression in the vehicle-treated control (C) and 1, 5, and 10 μM 2-ME$_2$-treated DU145 cells.

Effect of 2-ME$_2$ on MAPK Signaling Pathways. To investigate the effect of 2-ME$_2$ on cell-signaling molecules, we tested the consequence of the treatment of 2-ME$_2$ on prostate cancer cells. Thus, we exposed DU145 cells to 2-ME$_2$ for different times, and activities of MAPK were determined. We found that expressions of phospho-ERK 1/2 and phospho-JNK 1/2 were reduced after a short-exposure time with 5 μM 2-ME$_2$ (Figure 9A). However, when cells were exposed to 2-ME$_2$ (5 or 10 μM) for 48 h, phospho-ERK 2 and phospho-JNK 1/2 levels were elevated significantly (Figure 9B). The effect was undetected in 1 μM 2-ME$_2$-treated cells (Figure 9). Phospho-p38 MAPK was found to be downregulated in 5 and 10 μM 2-ME$_2$-treated cells as compared to the control (Figure 9B). This result is not in agreement with the previous study (*42*). This discrepancy may be due to the concentration of the chemical.

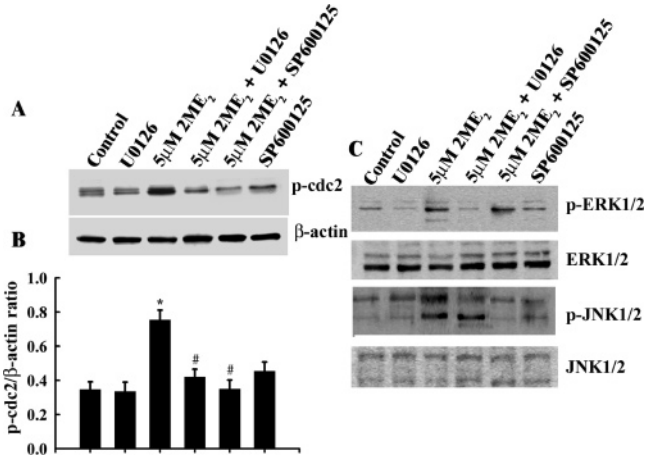

FIGURE 10: Effects of the MAPKK/ERK and JNK inhibitors on 2-ME_2-induced phosphorylated cdc2, ERK 1/2, and JNK 1/2 in DU145 prostate cancer cells. Cells were cultured in DMEM with 10% FBS, and ~70% confluent cells were treated with MAPKK/ERK inhibitor U0126 or JNK inhibitor SP600125 alone or in combination with 5 μM 2-ME_2. The total protein was isolated, and immuno-Western blot analysis was performed using a specific polyclonal antibody that recognizes P-cdc2 at tyrosine 15, P-ERK 1/2, ERK, P-JNK, and JNK proteins. Protein loading for the Western blot was done randomly. (A) Single representative immuno-Western blot analysis shows P-cdc2 levels in vehicle-treated controls, U0126, 5 μM 2-ME_2, and 5 μM 2-ME_2 in combination with U0126, 5 μM 2-ME_2 in combination with SP600125, or SP600125 alone in DU145 prostate cancer cells. (B) Bar diagram shows the mean ± SD ratio of P-cdc2 and β-actin of three separate experiments. (*) $p < 0.01$ versus the vehicle-treated control, and (#) $p < 0.01$ versus 5 μM 2-ME_2 (Student's t test). (C) Western blot analysis of the levels of phosphorylated and total ERK 1/2 and JNK 1/2 protein in DU145 cells treated with 2-ME_2 (5 μM) for 48 h.

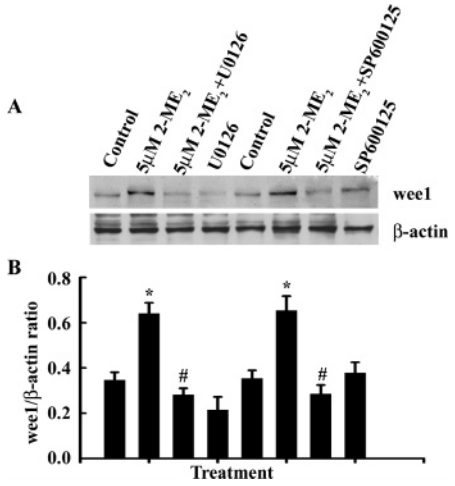

FIGURE 11: Impact of MAPK inhibitors on 2-ME_2-induced wee1 expression in DU145 prostate cancer cells. Cells were grown in DMEM with 10% FBS, and ~70% confluent cells were treated with MAPKK/ERK inhibitor U0126 or JNK inhibitor SP600125 alone or in combination with 5 μM 2-ME_2. wee1 protein levels were determined using immuno-Western blot analysis. (A) Single representative Western blot analysis illustrates wee1 levels in vehicle-treated controls, 5 μM 2-ME_2-treated cells, and U0126 or SP600125 alone or in combination with 5 μM 2-ME_2. (B) Bar diagram shows the mean ± SD ratio of wee1 and β-actin of three separate experiments. (*) $p < 0.01$ versus the vehicle-treated control, and (#) $p < 0.01$ versus 5 μM 2-ME_2 (Student's t test).

2-ME_2-Induced cdc2 Phosphorylation Is Mediated through MAPK Signaling Pathways. Because prolonged exposure of 2-ME_2 enhances the activities of MAPK signaling, it is feasible that cdc2 phosphorylation by 2-ME_2 is mediated through MAPK signaling pathways. To test the hypothesis, we examined whether MAPKK/ERK inhibitor U0126 and JNK inhibitor SP600125 suppress 2-ME_2-induced cdc2 phosphorylation. For this purpose, ~70% confluent DU145 cells were treated with 5 μM 2-ME_2 in the presence or absence of U0126 and SP600125 for 48 h and phospho-cdc2 (P-cdc2), phospho-ERK 1/2, and JNK/12 levels were evaluated. As expected, a significantly elevated cdc2 phosphorylation was found in 5 μM 2-ME_2-treated cells parallel with the inductions of ERK and JNK phosphorylations and these inductions can be blocked by both U0126 and SP600125 when exposed prior to 2-ME_2 treatment (Figure 10). Therefore, these observations suggest that cdc2 phosphorylation induced by 2-ME_2 is mediated through ERK 2 and JNK/SAPK signaling pathways.

2-ME_2-Induced Upregulation of wee1 Is Mediated through ERK 2 and JNK Pathways in DU145 Cells. In this study, we determined if MAPK signaling pathways are involved in mediating wee1 induced by 2-ME_2. To test this, ~70% confluent DU145 cells were treated with 5 μM 2-ME_2 alone or in combination with MAPKK/ERK inhibitor U0126 or JNK inhibitor SP600125 for 48 h and phosphorylation levels of wee1 were determined by immuno-Western blot analyses. As shown in Figure 11, the upregulation of wee1 by 2-ME_2 can be repressed significantly by both U0126 and SP600125.

Similar experiments were carried out to investigate if cdc25C induction by 2-ME_2 is mediated through MAPK or ERK signaling pathways. We found that none of the signaling pathways are involved in the induction of cdc25C by 2-ME_2 (data not shown).

DISCUSSION

The present study indicates that 2-ME_2 is a potent inhibitor of different prostate cancer cells as indicated by FACScan analysis and confirmed by apoptotic cell-death analysis (Figures 1 and 2). These studies provide evidence that 2-ME_2 at a higher concentration (5 and 10 μM) decreased the G1-phase progression and arrested cells at the G2/M checkpoint along with causing a significant accumulation of the S phase (Figures 1 and 3). A growing number of studies have established that 2-ME_2 is a potent cell-cycle inhibitor and induces apoptosis in various types of cancer cells (*2*, *4*). 2-ME_2 acts as colchicine (*43*, *44*) and blocks cells at the G2/M phase (*1*, *23*). Our studies, at one point, differ from existing concepts and demonstrated that the action of 2-ME_2 not only blocks the cells at the G2/M boundary but also increases the cell population in the S phase. Therefore, the present results suggest a biphasic action of 2-ME_2 on the cell-cycle regulation of prostate cancer cells.

The cyclin B1−cdc2 kinase is the master regulator in cell-cycle progression, and its mode of action is very intricate (*29*, *45*). During interphase (G1−S−G2), cyclin B1 is shifted to the cytoplasm from the nucleus and interacts with cdc2 (*27*, *28*, *45*). However, the cyclin B1−cdc2 kinase is held in an inactive state until the G2/M transition. The active complex of cdc2−cyclin B1 kinase accumulates mainly in the nucleus, which in turn allows the cells to enter into mitosis (i.e., transition from the G2 to M phases) in eukaryotes (*46*). Anomalous regulation of this complex or enhanced expression of cyclin B1 has been shown to be

associated with several cellular malfunctions, including G2/M arrest and apoptosis (*27*, *28*, *46*−*51*). On the basis of the papers, we analyzed whether 2-ME$_2$ is able to modulate these proteins during the induction of cellular arrest and apoptosis. Present studies have shown that 2-ME$_2$ significantly increased the cyclin B1 levels and enhanced its accumulation in the cytoplasm of DU145 prostate cancer cells parallel with the accumulation of S-phase cells, G2/M-phase cells, and apoptosis, as compared to the vehicle-treated controls (Figures 4 and 5). Although, the total cdc2 level was unaffected, the kinase activity of this protein, which is required by cells to enter mitosis, was reduced significantly in these cells when they were exposed to 2-ME$_2$ (Figure 6). Therefore, these studies suggest that the accumulation of the S phase, G2/M arrest, and induction of apoptosis by 2-ME$_2$ may be mediated by both upregulation of cyclin B1 and its accumulation in cytoplasm and inhibition of cdc2 kinase activity.

Phosphorylation of cdc2 residues have both positive and negative effects on its kinase activity (*46*). Phosphorylation at the threonine 161 (T161) residue of cdc2 is vital for the full kinase activity of this protein, while phosphorylation at T14 and Y15 residues of cdc2 inhibit its kinase activity even in the presence of phosphorylation of T161 (*46*, *52*). The wee1 kinase mediates T14 and Y15 phosphorylations of cdc2 in the nucleus (*18*, *34*, *36*, *53*). The present study indicates that 2-ME$_2$ at higher concentrations significantly increased the phospho-cdc2 (at Y15) level in the nucleus of DU145 cells (Figures 5 and 6), and it was supported by the evidence that histone H1 kinase activity was found to be significantly decreased in the 2-ME$_2$-treated cells (Figure 6). Moreover, studies also found that 2-ME$_2$ concurrently enhances the wee1 level in these cells. Therefore, these studies indicate that the inhibition of cdc2 kinase activity in the nucleus of DU145 cells by 2-ME$_2$ may be regulated by wee1 kinase-mediated phosphorylation of cdc2.

Dual-phosphatase cdc25C also plays a critical role in the regulation of T14 and Y15 phosphorylations of cdc2 (*21*, *38*, *54*). To make cdc2 kinase active and facilitate entry of cells from G2 to mitosis, these two residues are dephosphorylated by active cdc25C phosphatase (*46*, *55*). Thus, inactivation or alterations of cdc25C phosphatase activity arrests cells at the G2/M phase (*20*). There are three major forms (i.e., 57, 60, and 80 kDa) of cdc25C that have been identified in asynchronous dividing cells (*39*). 57 and 60 kDa protein bands are interphase forms (i.e., G1−S−G2), and the other is the mitotic form (*39*). Hyperphosphorylation of serine 216 (Ser^{216}) and hyperphosphorylation on NH_2-teminal serine and threonine residues are responsible for the shift in mobility of 60 and 80 kDa protein bands of cdc25C, respectively (*39*). The residue Ser^{216} of the interphase form of cdc25C is the primary site of phosphorylation, and it occurs throughout the interphase. However, hyperphosphorylation of this residue may contribute as a negative regulator of the cell cycle, thereby preventing the premature initiation of mitosis (*39*, *56*). It is evident from various studies and as depicted in Figure 12 that in the normal cell-cycle process of cdc25C (57 kDa form) is involved in mitotic entry by activating the cdc2−cyclin B1 complex through dephosphorylation of T14 and Y15 residues of the cdc2 protein (*20*). However, induction of phosphorylation of the Ser^{216} residue of cdc25C, which forms 60 kDa interphase cdc25C, by chk1

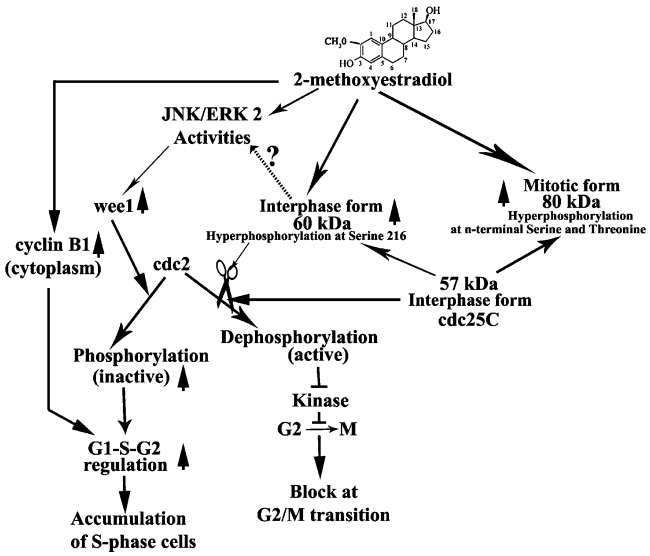

FIGURE 12: Diagram shows 2-ME$_2$-signaling pathways through which 2-ME$_2$ exerts its antitumor activity.

protein kinase inactivates or alters cdc25C phosphatase activity. This alternation eventually prevents dephosphorylation of cdc2 and maintains cdc2−cyclin B1 kinase as an inactive state (*20*). Thus, the DNA-damaging agents, which induces cdc25C hyperphosphorylation at Ser^{216}, inactivates the cdc2−cyclin B1 complex kinase activity and arrests cells at G2/M (*20*). The mitotic form, which is distinct from the interphase form and enriched in G2/M (*39*), may play a critical role in the G2/M phase. Present studies found that 2-ME$_2$ at higher doses induces significantly both mitotic (80 kDa) and interphase (60 kDa) forms of cdc25C in asynchronously growing prostate cancer cells. However, colchicine induces only the mitotic form of cdc25C (Figure 8). Therefore, these studies pointed out that 2-ME$_2$ not only induces wee1 expression for cdc2 phosphorylation but also may be able to enhance phosphorylation of cdc25C, which ultimately prevents dephosphorylation of cdc2. Consequently, these two crucial events finally facilitate in the accumulation of the interphase (S−G2) and G2/M arrest. The functional significance of the accumulation of the mitotic form of cdc25C by 2-ME$_2$ is not clear from this study. Therefore, further investigations are warranted.

MAPK signal transduction pathways have been implicated in a variety of cellular functions (*57*), including cell-cycle arrest (*58*, *59*) and apoptosis (*60*). In the present study, we found differential impacts of 2-ME$_2$ on the activities of ERK 1/2 and JNK 1/2 depending upon the duration of the treatment. Acute exposure of 2-ME$_2$ markedly reduces the activities of these signaling molecules without corresponding changes of cell-cycle progression and apoptosis (data not shown). However, prolonged exposure of this metabolite significantly increased the activities of ERK 2 and JNK-1/2 and decreased the p38 MAPK expression, parallel with the cell-cycle progression changes and apoptosis (Figures 1, 2, and 9). Therefore, we sought to determine whether 2-ME$_2$-induced modulations of cell-cycle regulatory proteins are mediated through signal transduction pathways. Our studies have shown that 2-ME$_2$-induced cdc2 phosphorylation and wee1 expression were mediated through both ERK 2 and JNK/SAPK signaling pathways, because the MAPKK and JNK inhibitors hampered the 2-ME$_2$ effect (Figures 9 and

10). cdc25C was not found to be regulated through any of the signal transduction pathways evaluated in this study.

The functional significance of downregulation of ERK and JNK phosphorylation by acute treatment and p38 phosphorylation by prolonged treatment of 2-ME_2 in DU145 prostate cancer cells is uncertain. Recently, we found that the acute treatment (i.e., 1−6 h) of 2-ME_2 is unable to modulate cell proliferation and expression of cell-cycle-related genes (Ray et al., unpublished data). Therefore, we anticipate that the inhibition of phosphorylation of these signaling molecules by this metabolite could be associated with additional events of cell-cycle progression, which have not yet been identified. Further studies are warranted.

In conclusion, the present study indicates that 2-ME_2 exhibits dual effects on the cell division cycle of prostate cancer cells. To accomplish these effects, multiple cell-cycle regulatory proteins including cyclin B1 and phospho-cdc2 and its regulatory proteins wee1 and cdc25C may be involved in these events (Figure 12). Finally, our studies manifested that molecular cross-talk between ERK 2 and JNK signaling pathways may be essential events to mediate 2-ME_2-induced upregulation of wee1 and phosphorylation of cdc2.

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