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Upregulated Cyclin B1/CDK1 Mediates Apoptosis Following 2-Methoxyestradiol-Induced Mitotic Catastrophe: Role of Bcl-X_L Phosphorylation

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Abstract

2-Methoxyestradiol is an endogenous nonpolar metabolite of 17β-estradiol with a strong antitubulin activity. Earlier we showed that 2-methoxyestradiol increases the level and activity of cyclin B1/CDK1, which subsequently induces mitotic prometaphase arrest. In the present study, we demonstrate that upregulation of cyclin B1/CDK1 is responsible for the increased phosphorylation of the anti-apoptotic proteins Bcl-2 and Bcl-XL in 2-methoxyestradiol-induced, mitotically-arrested cancer cells. Additional analysis shows that only the increase in phosphorylation of Bcl-XL, but not Bcl-2, is associated with activation of the mitochondrial cell death pathway. We find that MAD2 is an important upstream mediator of the antitubulin function of 2-methoxyestradiol, resulting in activation of the MKK4-JNK1 pathway. JNK1 activation then leads to cyclin B1/CDK1 upregulation, which further increases Bcl-2 and Bcl-XL phosphorylation. Together, these results indicate that cyclin B1/CDK1 upregulation in cancer cells undergoing 2-methoxyestradiol-induced mitotic catastrophe causes apoptosis via Bcl-XL phosphorylation.

Keywords: 2-Methoxyestradiol; prometaphase arrest; cyclin B1; CDK1; Bcl-2; Bcl-XL
Introduction

2-Methoxyestradiol, a nonpolar endogenous metabolite of 17β-estradiol (E₂) [1], has a strong anticancer activity in various human cancer cell lines in culture [1-8], by inducing cell cycle arrest and apoptosis [9-12]. The ability of 2-methoxyestradiol to induce cell cycle arrest is consistent with its ability to bind to tubulins and microtubules, thereby causing microtubule disruption [13-16]. Earlier studies with other microtubule inhibitors have shown that microtubule disruption would lead to cell cycle arrest and ultimately apoptosis (reviewed in Refs. [17]). It is of note that most earlier studies, which were based on flow cytometric analysis of different cell populations, reported that treatment of human cancer cells with 2-methoxyestradiol increases the combined G₂/M cell populations [18, 19]. However, our earlier study based on morphological analysis revealed that 2-methoxyestradiol induces predominantly mitotic prometaphase arrest in human cancer cells, but not G₂ phase arrest [20].

In a normal cell cycle, activation of the cyclin B1-dependent CDK1 (also called "Cdc2") is a key event in triggering the transition from G₂ phase to mitotic phase, by promoting the breakdown of the nuclear membrane, chromatin condensation, and microtubule spindle formation [21]. Cells with a suppressed CDK1 activity often tend to be arrested in G₂ phase, whereas cells with an elevated CDK1 activity would be favored to proceed through mitosis [22]. However, we recently showed, for the first time, that during 2-methoxyestradiol-induced mitotic prometaphase arrest, cyclin B1 and CDK1 protein levels as well as CDK1 phosphorylation (at T161) are upregulated, and their increase is crucial for the induction of prometaphase arrest [20].

In the present study, we provide evidence to show that the upregulated cyclin B1/CDK1 in 2-methoxyestradiol-induced, mitotically-arrested two human cancer cell lines (MDA-MB-435s and
MCF-7) is also responsible for the increased phosphorylation (presumably resulting in functional inactivation) of the anti-apoptotic Bcl-2 and Bcl-X<sub>L</sub>, and the increased phosphorylation of Bcl-X<sub>L</sub> (but not Bcl-2) is associated with activation of mitochondrial apoptotic pathway. In addition, we show that MAD2 is an important upstream mediator of the anti-tubulin action of 2-methoxyestradiol, which results in cyclin B1/CDK1 upregulation in 2-methoxyestradiol-treated cancer cells via activation of the MKK4-JNK1 pathway.
Materials and methods

Materials

2-Methoxyestradiol, nocodazole, roscovitine, Iscove’s modified minimum essential medium, and Eagle’s minimum essential medium (EMEM) were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS) was obtained from HyClone (South Logan, UT), the antibiotics solution (containing 10,000 U/mL penicillin and 10 mg/mL streptomycin) from Invitrogen (Grand Island, NY), and the trypsin-versene mixture from Lonza Walkersville (Walkersville, MD). SP600125 (a JNK1/2 inhibitor) was obtained from Calbiochem (La Jolla, CA). The anti-p--Jun, anti-p-SEK/MKK4(Thr261), anti-p-ERK1/2, anti-ERK1/2, anti-p-p38, anti-p38, anti-p-Bcl-2(Ser70), anti-Bcl-2, anti-Bcl-XL, anti-Bax, anti-CDK1 (Cdc2), anti-cyclin B1, anti-p-CDK1 (Thr161), anti-PARP, anti-MAD2, and anti-GAPDH antibodies were purchased from Cell Signaling Technology (Beverly, MA), and the anti-c-Jun NH2-terminal kinase (JNK1/2) antibody was purchased from Biosource (Camarillo, CA).

Cell culture

The MDA-MB-435s and MCF-7 human cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). While MCF-7 cells are well-known estrogen receptor-positive human breast cancer cells, MDA-MB-435s cells, which were originally described as estrogen receptor-negative metastatic human breast cancer cells, are presently considered as human melanoma cancer cells [23]. MDA-MB-435s cells were maintained in Iscove’s modified minimum essential medium supplemented with 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 3.024 g/L NaHCO3 and incubated at 37°C under 5% CO2. MCF-7 cells were maintained in EMEM containing 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 μg/mL insulin, 0.5 mM sodium pyruvate, 10 mM nonessential amino acids, and 2
mM L-glutamine. The MDA-MB-435s and MCF-7 cells were cultured at 37°C under 5% CO₂, and sub-cultured once every 3 to 4 days.

**MTT assay**

The MTT assay was used to reflect gross cell viability. MDA-MB-435s and MCF-7 cells were seeded into 96-well plates at a density of 5000 cells per well and incubated overnight before the start of drug treatments. The stock solution of 2-methoxyestradiol (prepared in 200-proof ethanol at a concentration of 5 mM and stored at −20°C) was diluted in the culture medium immediately before addition to each well at desired final concentrations, and the treatments usually lasted 24 h. Following the treatments, cell viability was determined (MTT assay) by adding to each well 10 µL of the MTT solution (final concentration 500 µg/mL) and followed by incubation at 37°C for 4 h. An aliquot (100 µL) of the solubilizing solution (DMSO:ethanol = 1:1, v:v) was then added to each well, and the absorbance was read with a UVmax microplate reader (Molecular Device, Palo Alto, CA) at the 560-nm wavelength. The relative cell density is expressed as a percentage of the control that is treated with vehicle alone.

**Nuclear and cytoplasmic extracts**

Nuclear and cytosolic fractions were prepared using the Cytosolic/Nuclear Fractionation Kit obtained from Biovision (Mountain View, CA) according to manufacturer’s instructions, and the detailed procedures were described in our earlier study [20]. The nuclear and cytoplasmic extracts were stored in aliquots at −80°C until used in experimentation.

**Western blotting**

For Western blotting, cells were washed first with phosphate-buffered saline (PBS, pH 7.4) and then suspended in 100 µL lysis buffer (containing 20 mM Tris–HCl, 150 mM NaCl, 1 mM
EDTA, 1% Triton X-100, and 10 μL/mL protease inhibitor cocktail, pH 7.5). The amount of proteins was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). The detailed procedures for the Western blotting analysis were described in our earlier study [20].

**Flow cytometric analysis**

After treatment with 2-methoxyestradiol, cells were harvested by trypsinization and washed once with PBS. After the cells were resuspended in 0.9 % NaCl (1 mL), ice-cold 90% ethanol (2.5 mL) was added. After the cells were incubated at room temperature for 30 min, they were centrifuged and the supernatants were removed. Then the cells were resuspended in 1 mL PBS containing propidium iodide (PI; 50 μg/mL) and ribonuclease A (100 μg/mL), and incubated at 37°C for 30 min. After centrifugation and resuspension in PBS, the cells were undergone flow cytometric analysis on a BD LSR II flow cytometer (BD Bioscience, San Jose, CA).

**Immunoprecipitation**

Total cell lysates were incubated for 1 h at 4°C with the anti-Cdc2 or anti-cyclin B1 antibody. The immuno-complex was collected on protein A-Sepharose beads (Sigma-Aldrich) for 1 h and washed 5 times with the TNN buffer (containing 40 mM Tris-HCl, 120 mM NaCl, 0.5% NP-40, 0.1 mM sodium orthovanadate, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 100 μg/mL phenylmethylsulfonyl fluoride, pH 8.0) prior to boiling in the SDS buffer. The precipitated proteins were then separated on the SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membrane, and the protein level was determined by Western blotting analysis.

**Small interfering RNA (siRNA) treatment**

The role of JNK1 in mediating the effect of 2-methoxyestradiol was examined using the JNK1-siRNAs to silence the JNK1 gene expression. The JNK1-siRNAs (siJNK1, catalog # AM16704) and
the negative control siRNAs (siCon; catalog # AM4611) were obtained from Ambion (Austin, TX). Similarly, the role of Bcl-2, Bcl-X\textsubscript{L}, MKK4, CDK1 and cyclin B1 in mediating the effect of 2-methoxyestradiol was examined using the following specific siRNAs (obtained from Santa Cruz, CA) to selectively silence the expression of each of these genes: siBcl-2 (catalog # sc-29214), siBcl-X\textsubscript{L} (catalog # sc-43630), siMKK4 (catalog # sc-35910), siCDK1 (catalog # sc-29252), siCyclin B1 (catalog # sc-29284), plus the negative control siRNAs (catalog # sc-37007). MDA-MB-435s and MCF-7 cells were seeded the night before transfection, and they would reach 30–50% confluence by the time of transfection. Sixty pmol of siJNK1 or 40 nmol of siBcl-2, siBcl-X\textsubscript{L}, siMKK4, siChk2, siBcl-2, or siCon were used for transfection with Lipofectamine-2000 (Invitrogen) according to the instructions of the manufacturer. Transfected cells were cultured for 2 days before they were harvested for analysis. The efficiency of siRNA knockdown was estimated according to Western blot analysis of the target proteins.

**Transient transfection**

One day before transfection, cells were seeded at 10\textsuperscript{5} cells/well in the 6-well tissue culture plates. Cells were transfected using the FuGENE6 Transfection Reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's recommendations. Three \(\mu\)L FuGENE6 and 1 \(\mu\)g pCMV-CDK1 (Cdc2) were diluted individually in 100 \(\mu\)L aliquots of OptiMEM Serum I Reduced-Serum Medium (Invitrogen). Twenty-four h after transient transfection, cells were incubated in fresh complete medium with or without 2-methoxyestradiol for additional 24 h, and then the cells were harvested and assayed by Western blot analysis.

**Statistical analysis**

Quantitative data were obtained from three or more experiments and expressed as mean \(\pm\) S.D. Comparisons between two different treatment groups were analyzed using analysis of
variance (ANOVA) followed by a multiple comparison test with a Bonferroni adjustment. A $P$ value of below 0.05 is considered to be statistically significant.
Results

2-Methoxyestradiol sequentially induces mitotic prometaphase arrest and cell death

Earlier we showed that 2-methoxyestradiol induces predominantly prometaphase arrest in human cancer cell lines MDA-MB-435s and MCF-7, but not G2 phase arrest [20]. Based on flow cytometric analysis of the sub-G1 cell population (dead cells), we found that 2-methoxyestradiol-induced death of MDA-MB-435s and MCF-7 cells lags behind the induction of prometaphase arrest (Fig. S1). While accumulation of prometaphase-arrested cells peaks at about 12 h following 2-methoxyestradiol exposure, induction of cell death starts around 24 h and peaks at 48–72 h. This data suggests that the dead cells likely arise predominantly from those cells undergoing mitotic catastrophe following 2-methoxyestradiol exposure.

2-Methoxyestradiol induces Bcl-2 and Bcl-XL phosphorylation

Bcl-2 and Bcl-XL are members of the Bcl-2 family anti-apoptotic proteins that prevent the release of mitochondrial content such as cytochrome c, which then leads to caspase activation and ultimately apoptosis [24, 25]. It has been suggested that phosphorylation of Bcl-2 and Bcl-XL would lead to inactivation of their anti-apoptotic function [24, 25]. As shown in Fig. 1A and 1B, treatment of MDA-MB-435s and MCF-7 cells with 2-methoxyestradiol (2 μM) induces, in a time-dependent manner, phosphorylation of Bcl-2(Ser70), with peak levels detected at 12 and 24 h (in MDA-MB-435s cells; Fig. 1A) or at 12 h (in MCF-7 cells; Fig. 1B) following the treatment, whereas the total Bcl-2 protein level is not markedly changed. In a similar time-dependent manner, 2-methoxyestradiol also induces phosphorylation (inactivation) of Bcl-XL in both cell lines (Fig. 1A, 1B). For comparison, we also determined the change in Bax protein level (a pro-apoptotic protein), and we found that its level is not significantly altered by 2-methoxyestradiol treatment (Fig. 1A, 1B).
Supplementary Figure S1. 2ME2 sequentially induces mitotic arrest and cell death in MDA-MB-435s cells (A) and MCF-7 cells (B). The MDA-MB-435s and MCF-7 cells were seeded at $5 \times 10^4$ cells/mL and cultured for 24 h before they were treated with 2 μM 2ME2 for 3, 6, 12, 24, 48 and 72 h. For detection of mitotic prometaphase arrest, cells were stained with Hoechst (nuclear staining), and the morphology of cells arrested in mitotic prometaphase (based on counting ≥200 nuclei for each of the cell culture wells) was manually scored under a fluorescence microscope. Similarly, the time-dependent changes in sub-G1 cell population following treatment with 2ME2 were analyzed using flow cytometry as described in the Material and Methods section. Each data point is the mean ± S.D. from three replicate measurements.
Figure 1. 2-methoxyestradiol (2ME₂) alters the expression of apoptotic proteins in MDA-MB-435s cells (A) and MCF-7 cells (B). The MDA-MB-435s and MCF-7 cells were treated with 2ME₂ (2 μM) for different length of time as indicated. Total cell lysates were prepared and equal amounts of protein lysates were separated on 10% SDS-polyacrylamide gel. Western blotting analysis was used to detect specific proteins by using specific antibodies against p-Bcl-2(Ser70), Bcl-2, Bcl-X₅, Bax, and GAPDH (as a loading control).
Our recent study showed that cyclin B1 and CDK1 accumulation is mostly seen in 2-methoxyestradiol-treated, prometaphase-arrested cells, and they appear to be associated with the condensed chromatin structures [20]. In this study, we compared cellular distribution of Bcl-2 and Bcl-XL with that of cyclin B1/CDK1 in 2-methoxyestradiol-treated MCF-7 cells in an effort to show that the p-Bcl-2 and p-Bcl-XL proteins are predominantly present in prometaphase-arrested cells. We found that accumulation of p-Bcl-2 and p-Bcl-XL proteins is only seen in prometaphase-arrested cells but not in untreated control cells.

To determine the involvement of Bcl-2 and Bcl-XL in mitotically-arrested cells following 2-methoxyestradiol treatment is responsible for initiating apoptosis, we individually knocked down the expression of each of these two proteins and then tested the sensitivity of the cells to 2-methoxyestradiol-induced apoptosis. Experimentally, cells were transfected with the siBcl-2 or siBcl-XL, whereas the control cells were transfected with the negative control siRNAs (siCon). Twenty-four h after transfection, cells were treated with 2-methoxyestradiol. The degree of Bcl-2 and Bcl-XL knockdown was confirmed by Western blotting analysis of these two proteins. As shown in Fig. 2A, knockdown of Bcl-2 or Bcl-XL strongly reduces their total protein levels, and it also abrogates 2-methoxyestradiol-induced phosphorylation of these two proteins. For comparison, we also examined the effect of nocodazole (a prototypical microtubule inhibitor), and similar observations were made in cells with Bcl-2 or Bcl-XL knockdown (Fig. 2A). Flow cytometric analysis showed that the 2-methoxyestradiol-induced accumulation of the combined G2/M populations (which is used here as an indicator for mitotic prometaphase arrest) is reduced in cells with Bcl-XL knockdown (Fig. S2), but the appearance of sub-G1 cells (dead cells) is markedly increased (Fig. 2B). Similarly, knockdown of Bcl-XL also increases the number of 2-methoxyestradiol-induced annexin V-positive cells (which is used here to indicate the appearance of apoptotic cells) (Fig. 2C). By contrast, knockdown of Bcl-2 does not increase the population of
dead cells, but instead it has a modest protective effect against 2-methoxyestradiol-induced cell death (Fig. 2B, 2C). Collectively, these data suggest that treatment of human cancer cells with 2-methoxyestradiol increases the phosphorylation of the anti-apoptotic proteins Bcl-2 and Bcl-XL in mitotically-arrested cells, but it appears that only the increased Bcl-XL phosphorylation is associated with the induction of apoptosis in these cells.

**Figure 2. Effect of knockdown of Bcl-2 or Bcl-XL on 2ME2-induced cell death in MCF-7 cells.** A. The MCF-7 cells in culture were transfected with Bcl-2 siRNAs (siBcl-2), Bcl-XL siRNAs (siBcl-XL), or control siRNAs (siCon). Twenty-four h later, cells were exposed to 2 µM 2ME2 for additional 12 h. Then the whole cell lysates were prepared and analyzed.
for the levels of Bcl-2, p-Bcl-2(Ser70) and Bcl-XL by Western immunoblotting. The loading control is GAPDH. B, C. Cells were first transfected with siBcl-2, siBcl-XL, or siCon, and then further treated with 2 µM 2ME2 for 48 h. After incubation, cells were analyzed for cell cycle change (B) and apoptosis (C) using flow cytometric analysis after they were stained with PI alone (B) or with annexin V-FITC + PI (C). Experiments were repeated three times, and similar observations were made. The values in panel B are the mean ± S.D. (n = 3).

Supplementary Figure S2. Effect of knockdown of Bcl-2 or Bcl-XL on 2ME2-induced cell death in MCF-7 cells. Cells were first transfected with siBcl-2, siBcl-XL, or siCon, and then further treated with 2 µM 2ME2 for 48 h. Cells were stained with Hoechst (nuclear staining) for detection of mitotic prometaphase cells. The morphology of cells arrested in mitotic prometaphase (based on counting ≥200 nuclei for each of the cell culture wells) was manually scored under a fluorescence microscope. Each data point is the mean ± S.E.M. from three replicate measurements. * P < 0.05 versus vehicle control; # P < 0.05 versus 2ME2 treatment.

Earlier we showed that cyclin B1 and CDK1 protein level is upregulated in human cancer cells treated with 2-methoxyestradiol, and this upregulation is critical for 2-methoxyestradiol-induced prometaphase arrest [20]. This observation is confirmed in this study. Here we sought to test a hypothesis that the elevated levels of cyclin B1 and CDK1 in mitotically-arrested cells are linked to the increased phosphorylation of the anti-apoptotic Bcl-2 and Bcl-XL proteins as well as the subsequent cell death. A series of experiments were conducted to test this hypothesis (data are described below).

First, we probed the functional role of CDK1 in Bcl-2 and Bcl-XL phosphorylation in 2-
methoxyestradiol-treated MCF-7 cells by comparing the modulating effect of roscovitine (a known inhibitor of CDKs) on Bcl-2 and Bcl-X₅ phosphorylation. As shown in Fig. 3A, treatment of cells with roscovitine suppresses 2-methoxyestradiol-induced Bcl-2 and Bcl-X₅ phosphorylation, which supports the postulated functional relationship between the upregulated CDK1 and the elevated Bcl-2 and Bcl-X₅ phosphorylation. Second, based on flow cytometric analysis of the cell populations in different phases of the cell cycle, we observed that treatment of cells with roscovitine also abrogates 2-methoxyestradiol-induced mitotic arrest (Fig. 3B; the combined G₂/M population is decreased from 75.7% to 36.8% at 12 h) as well as the subsequent cell death (Fig. 3B; the sub-G₁ population is decreased from 80.4% to 19.0% at 72 h). Here it is of note that treatment with roscovitine alone causes a small increase in the G₂ phase cell population (Fig. 3B), but it does not increase the prometaphase cell population (based on morphological analysis; data not shown). Our observation is consistent with the well-known role of Cdc2 in G₂→M transition [26-27]. Collectively, these data suggest that while treatment with roscovitine (an inhibitor of CDKs) alone can selectively increase G₂ phase cells, co-treatment of cells with 2-methoxyestradiol + roscovitine abrogates 2-methoxyestradiol-induced prometaphase arrest and also prevents the onset of subsequent apoptosis.
Figure 3. Effect of rescorvitine (Rosc) on 2ME₂-induced Bcl-Xₐ phosphorylation and cell death in MCF-7 cells. A. Cells were first pre-treated with roscovitine (20 µM) alone for 2 h and then jointly treated with roscovitine + 2ME₂ (2 µM) for additional 12 h. Total cell lysates were analyzed by Western immunoblotting for p-Bcl-2(Ser70), Bcl-Xₐ, Bcl-2 and GAPDH. B. Cells were first pre-treated for 2 h with roscovitine (20 µM) and then co-treated with rescovitine + 2ME₂ (2 µM) for additional 12, 24, 48, and 72 h. Cells were analyzed using flow cytometry for the cell cycle populations. Shown are the data for G₂/M and sub-G₁ cells. Experiments were repeated three times, and similar observations were made.

To provide more definitive evidence for the involvement of cyclin B1/CDK1 in the phosphorylation of Bcl-2 and Bcl-Xₐ, we also used the selective knockdown approach in this study. Cells were transfected with the cyclin B1 siRNAs (sicyclin B1) and CDK1 siRNAs (siCDK1), whereas control cells were transfected with control siRNAs (siCon). Twenty-four h after transfection, cells were treated with 2-methoxyestradiol and then harvested for Western blot analysis of cyclin B1, CDK1, p-Bcl-2(Ser70), p-Bcl-Xₐ, total JNK1/2, and p-JNK1/2 protein levels.
(the reason for including JNK1/2 and p-JNK1/2 is discussed later). As shown in Fig. 4A and 4B, selective knockdown of cyclin B1 or CDK1 each abrogates 2-methoxyestradiol-induced increases in p-Bcl-2(Ser70) and p-Bcl-Xl compared to siCon-transfected cells, whereas the levels of total JNK1/2 and p-JNK1/2 are not altered. In addition, we have also examined the effect of cyclin B1/CDK1 upregulation on Bcl-Xl phosphorylation by using an alternative approach, i.e., using cells that selectively overexpress CDK1 protein. Cells were first transfected with the pCMV-CDK1 vector or the pCMV empty vector, and then treated with 2-methoxyestradiol for 12 h. As shown in Fig. S3, Bcl-2 and Bcl-Xl phosphorylation is enhanced in CDK1-overexpressing cells after 2-methoxyestradiol treatment. Lastly, we have also sought to provide evidence to show that cyclin B1/CDK1 can directly interact with Bcl-2 and Bcl-Xl during the development of 2-methoxyestradiol-induced mitotic arrest by using the co-immunoprecipitation method. We found that the association of cyclin B1/CDK1 with Bcl-2 and Bcl-Xl is very low in untreated cells, but treatment of cells with 2-methoxyestradiol results in significant increase in the binding of cyclin B1/CDK1 with Bcl-2 and Bcl-Xl (Fig. S4). Collectively, these data support the hypothesis that the upregulated cyclin B1/CDK1 is directly involved in the phosphorylation of Bcl-2 and Bcl-Xl in 2-methoxyestradiol-treated cancer cells.

Using flow cytometric analysis of 2-methoxyestradiol-treated cells that were stained with PI and annexin V, we showed that selective knockdown of cyclin B1 or CDK1 almost completely abrogates 2-methoxyestradiol-induced apoptotic cell death (Fig. 4C). This piece of data, in conjunction with the observation with roscovitine (Fig. 3B), indicates that the upregulated cyclin B1/CDK1 is involved in triggering apoptosis following 2-methoxyestradiol-induced mitotic catastrophe.
Figure 4. Effect of cyclin B1 or CDK1 knockdown on 2ME$_2$-induced Bcl-X$_L$ phosphorylation and cell death in MCF-7 cells. A. Cells were transfected with cyclin B1 siRNAs (siB1) or the negative control siRNAs (siCon), and 24 h later, cells were exposed to 2ME$_2$ (2 µM) for additional 12 h. Then the whole cell lysates were analyzed for p-Bcl$_2$(Ser70), Bcl-2, p-JNK1/2, JNK1/2, cyclin B1, and Cdc2 levels using Western immunoblotting. B. Cells were transfected with CDK1 siRNAs (siCDK1) or the negative control siRNAs (siCon), and 24 h later, cells were exposed to 2ME$_2$ (2 µM) for additional 12 h. Then the whole cell lysates were analyzed for p-Bcl$_2$(Ser70), Bcl-2, p-JNK1/2, JNK1/2, cyclin B1, and CDK1 levels using Western immunoblotting. C. Cells were transfected with cyclin B1 siRNAs (siB1) or CDK1 siRNAs (siCDK1) or the negative control siRNAs (siCon) alone, and 24 h later, cells were exposed to 2ME$_2$ (2 µM) for additional 48 h. After incubation, cells were analyzed for cell death by using flow cytometry after they were stained with annexin V-FITC + PI.
Supplementary Figure S3. Effect of CDK1 overexpression on 2ME₂-induced Bcl-2 and Bcl-Xₐ phosphorylation in MCF-7 cells. Cells were transfected with pCMV or pCMV-CDK1 vector and then treated with 2ME₂ for 24 h. Bcl-Xₐ, p-Bcl-2, and CDK1 levels were determined by Western blotting as described in Material and Methods.

Supplementary Figure S4. Interaction between Bcl-2 or Bcl-Xₐ with CDK1 and cyclin B1 in 2ME₂-treated cells in MCF-7 cells. A. The co-immunoprecipitation of Bcl-Xₐ or Bcl-2 with CDK1 (Cdc2) or cyclin B1 was carried out as described in the Materials and Methods section. Total cell lysates were immunoprecipitated with anti-CDK1 (Cdc2) or anti-cyclin B1 antibody, respectively, and then analyzed by Western blotting for p-Bcl-2(Ser70) or Bcl-Xₐ. B. The relative Bcl-Xₐ and p-Bcl-2(Ser70) levels were determined by densitometric scanning of the blots and calculated by averaging the results from three separate experiments.
Role of cyclin B1/CDK1 in mediating JNK1-induced Bcl-2 and Bcl-XL phosphorylation

Our earlier study showed that treatment of human cancer cells with 2-methoxyestradiol results in activation of the mitogen-activated protein kinases (MAPKs), particularly JNK and p38 [9], and activation of the JNK pathway (but not p38 and ERK) promotes apoptosis in these cells [9]. It is likely that JNK may be involved in Bcl-2 and/or Bcl-XL phosphorylation in 2-methoxyestradiol-treated cancer cells. While there are studies supporting the direct involvement of JNK in Bcl phosphorylation [28, 29], there are also studies that do not appear to provide support for its involvement [28, 30-31]. In this study, therefore, we sought to answer the following two questions: First, is JNK involved in 2-methoxyestradiol-induced phosphorylation of Bcl-2 and Bcl-XL? Second, if it does, then does the JNK-mediated phosphorylation of Bcl-2 and Bcl-XL involve upregulation of cyclin B1/CDK1?

To answer these questions, first we confirmed the earlier observation that 2-methoxyestradiol can induce the phosphorylation of JNK1 (not JNK2), ERK, and p38 in MCF-7 cells, beginning as early as at 30 min after 2-methoxyestradiol treatment (Fig. 5A, left panel). Additional analysis showed that the level of JNK1 phosphorylation remains significantly elevated at 24 h after 2-methoxyestradiol treatment (Fig. 5A, right panel).

Next, we probed the possible role of JNK, ERK and p38 signaling pathways in 2-methoxyestradiol-induced Bcl-2 and Bcl-XL phosphorylation by studying the effect of the commonly-used pharmacological inhibitors of these MAPKs. As shown in Fig. 5B, treatment with any of these inhibitors alone does not cause a significant change in the basal levels of Bcl-2 and Bcl-XL phosphorylation. However, pre-treatment of these cells with a JNK1/2 inhibitor SP600125 abrogates 2-methoxyestradiol-induced Bcl-2 and Bcl-XL phosphorylation, although MEK-1 and p38 inhibitors does not similarly alter their phosphorylation. Therefore, these data suggest that JNK,
but not ERK and p38, is involved in the phosphorylation of Bcl-2 and Bcl-X\textsubscript{l}. Moreover, the JNK1/2 inhibitor can effectively protect the cells against 2-methoxyestradiol-induced cell death (Fig. 5C) as well as prometaphase arrest (Fig. 5D), whereas the ERK and p38 inhibitors do not have a similar protective effect (Fig. S5). In addition, flow cytometric analysis of cells stained with annexin V-FITC and PI further confirmed that JNK1/2 inhibition can significantly suppress 2-methoxyestradiol-induced apoptosis (Fig. 5E).

To provide more definitive evidence for the involvement of JNK1 in mediating Bcl-2 and Bcl-X\textsubscript{l} phosphorylation, we employed the siRNA approach to selectively knock down JNK1 expression. We did not choose to study the role of JNK2 because this protein is not altered following 2-methoxyestradiol treatment. Twenty-four h after transfection with siJNK1, cells were treated with 2-methoxyestradiol and then harvested for Western blot analysis of the levels of cyclin B1, CDK1, p-Bcl-2, p-Bcl-X\textsubscript{l} and cleaved PARP. As shown in Fig. 5F, JNK1 knockdown abrogates 2-methoxyestradiol-induced increases in Bcl-2 and Bcl-X\textsubscript{l} phosphorylation and PARP cleavage, whereas the total Bcl-2 and Bax are not altered. These changes were accompanied by decreases in the number of apoptotic cells compared to corresponding controls. Moreover, cells with JNK1 knockdown has a blunted upregulation of the cyclin B1/CDK1 in 2-methoxyestradiol-treated cells (Fig. 5F). Notably, knockdown of cyclin B1 and CDK1 does not affect 2-methoxyestradiol-induced phosphorylation of JNK1/2, while 2-methoxyestradiol-induced phosphorylation of Bcl-2 and Bcl-X\textsubscript{l} and cell death are mostly abrogated (shown in Fig. 4A, 4B). Collectively, these data clearly show that the cyclin B/CDK1 complex is a downstream signaling target of JNK1 which mediates 2-methoxyestradiol-induced Bcl-2 and Bcl-X\textsubscript{l} phosphorylation as well as cell death.
Figure 5. 2ME₂-induced activation of the JNK signaling pathway during the induction of prometaphase arrest and Bcl-Xₐ phosphorylation in MCF-7 cells. A. The MCF-7 cells were first incubated with 2ME₂ (2 µM) for the indicated lengths of time, and then the whole cell lysates were prepared and analyzed for the levels of p-JNK1/2, JNK1/2, P-ERK1/2, ERK1/2, p-p38, p38, and GAPDH by Western immunoblotting. B, C. Cells were pre-treated with 20 µM MAPK inhibitors, which included SP600125 (a JNK inhibitor, SP), PD98059 (an ERK inhibitor, PD) and SB202190 (a p38 inhibitor, SB), for 2 h, and then co-treated with or without 2ME₂ (2 µM) for additional 12 h (for Western Blot; B) or 24 h (for MTT assay; C). Total cell lysates were analyzed by Western blotting for p-Bcl-2(Ser70) and Bcl-Xₐ expression (B), and cell viability was measured by the MTT assay (C). Each bar is a mean ± S.E.M. from triplicate measurements. D, E. Cells were pre-treated for 2 h with a JNK inhibitor (SP600125) and then co-treated with 2ME₂ (2 µM) for additional 12 h (D) or 48 h (E). Cells were stained with Hoechst (nuclear staining) for detection of mitotic prometaphase cells. The morphology of cells arrested in mitotic prometaphase (based on counting ≥200 nuclei for each of the cell culture wells) was manually scored under a fluorescence microscope. Each data point is the mean ± S.E.M. from three replicate measurements (D). * P < 0.05 versus vehicle control; # P < 0.05 versus 2ME₂ treatment. At 48 h after treatment, cells were stained with annexin V-FITC and PI for detection of apoptotic cells as described in the Material and Methods section (E). F. Cells in culture were transfected with JNK1 siRNAs (siJNK1) and control siRNAs (siCon). Cells were exposed to 2ME₂ for 12 h, and then the whole cell lysates were prepared and analyzed for the levels of JNK1/2, Bcl-Xₐ, p-Bcl-2(Ser70), Bcl-2, Bax, PARP, cyclin B1, CDK1 and GAPDH (as a loading control) by Western immunoblotting.
Supplementary Figure S5. 2ME2-induced activation of the JNK signaling pathway during the induction of prometaphase arrest in MCF-7 cells. Cells were first transfected with siBcl-2, siBcl-XL, or siCon, and then further treated with 2 µM 2ME2 for 48 h. Cells were stained with Hoechst (nuclear staining) for detection of mitotic prometaphase cells. The morphology of cells arrested in mitoticprometaphase (based on counting ≥200 nuclei for each of the cell culture wells) was manually scored under a fluorescence microscope. Each data point is the mean ± S.E.M. from three replicate measurements. * P < 0.05 versus vehicle control.

MAD2 is an upstream mediator of 2-methoxyestradiol-induced mitotic prometaphase arrest and cell death

It is known that MAD2, a key spindle checkpoint protein, can block the progression through the metaphase-to-anaphase transition by binding to unattached kinetochores [32-34]. In addition, during the development of prometaphase arrest, MAD2 can inhibit the activity of the anaphase promoting complex (APC) by sequestering Cdc20 until all chromosomes are attached by microtubules and properly aligned at the metaphase plate. To probe how 2-methoxyestradiol-induced microtubule disruption subsequently leads to JNK1 activation, we examined the role of MAD2 in regulating the phosphorylation of JNK1 and its downstream signaling molecules in human cancer cells by using the selective knockdown approach. As shown in Fig. 6A, knockdown of MAD2 abrogates 2-methoxyestradiol-induced increases in the protein levels of p-JNK1 and p-c-Jun (a downstream kinase activated by JNK1). In addition, the 2-methoxyestradiol-induced
upregulation of the cyclin B1/CDK1 proteins as well as CDK1 phosphorylation (activation) are both markedly reduced in cells with MAD2 knockdown. As expected, the levels of 2-methoxyestradiol-induced Bcl-2 and Bcl-X, phosphorylation are also abrogated by MAD2 knockdown. These data jointly show that MAD2 is an important upstream protein that senses the anti-tubulin action of 2-methoxyestradiol, which then leads to JNK1 activation. JNK1 activation further leads to upregulation of cyclin B1/CDK1 protein levels and CDK1 activity (via phosphorylation). Our recent study showed that the increased functionality of cyclin B1/CDK1 at early stages following 2-methoxyestradiol treatment is responsible for the development of prometaphase arrest [17]. The results of the present study further show that upregulation of cyclin B1/CDK1 following a prolonged mitotic arrest results in heightened phosphorylation of Bcl-2 and Bcl-X, which then triggers the intrinsic cell death pathway.

To probe how MAD2 leads to JNK1 activation in 2-methoxyestradiol-treated cells, we studied M KK4, which is an upstream kinase that directly phosphorylates (activates) JNK1. We found that M KK4 phosphorylation is increased at 6 h after 2-methoxyestradiol treatment (Fig. 6B). M KK4 knockdown abrogates 2-methoxyestradiol-induced phosphorylation of JNK1 and c-Jun, upregulation of cyclin B1/CDK1, and the phosphorylation of Bcl-2 and Bcl-X, (Fig. 6C). These data show that M KK4 is a key mediator between MAD2 and JNK1 activation following 2-methoxyestradiol-induced MAD2 activation.
Figure 6. Effect of M KK4 or MAD2 knockdown on the development of 2ME\textsubscript{2}-induced prometaphase arrest and Bcl-X\textsubscript{L} phosphorylation in MCF-7 cells. A, C. Cells were transfected with siMAD2 (A) and siMKK4 (C) or siRNA negative control and then treated with 2ME\textsubscript{2} for 12 h. MAD2, p-JNK\textsubscript{2}/MMK4(Thr261), p-JNK1, p-c-jun, Cyclin B1, CDK1, p-CDK1(T161), Bcl-X\textsubscript{L}, p-Bcl-2 and Bcl-2 levels were determined by Western blotting. Membrane was stripped for determining the levels of GAPDH which was used as a loading control. B. Cells were first incubated with 2 μM 2ME\textsubscript{2} for 6, 12, 24, and 48 h, and then the cellular extracts were subjected to Western blotting for the levels of p-SEK1/MMK4(Thr261) and SEK1/MMK4(Thr261). Membrane was stripped for determining the levels of GAPDH which was used as a loading control.
DISCUSSION

Upregulated cyclin B1/CDK1 mediates Bcl-2 and Bcl-X\textsubscript{L} phosphorylation in 2-methoxyestradiol-induced, mitotically-arrested cells

The results of this study, along with data from our earlier study [20], showed that treatment of human cancer cells (MDA-MB-435s and MCF-7) with 2-methoxyestradiol induces, in a sequential manner, prometaphase arrest and then apoptotic cell death, and the dead cells arise predominantly from those cells arrested in mitotic prometaphase following exposure to 2-methoxyestradiol. In addition, we observed that 2-methoxyestradiol-induced mitotic arrest is accompanied by extensive phosphorylation of two anti-apoptotic proteins, Bcl-2 and Bcl-X\textsubscript{L}. However, only the increased phosphorylation of Bcl-X\textsubscript{L} (but not Bcl-2) is associated with the induction of apoptotic cell death in 2-methoxyestradiol-treated, mitotically-arrested cancer cells.

It was reported earlier that 2-methoxyestradiol can induce a strong early upregulation of cyclin B1/CDK1 protein level as well as CDK1 activation in human cancer cells, and these changes are critical to 2-methoxyestradiol-induced prometaphase arrest [20]. In this study, we provided further evidence to show that the upregulated cyclin B1 and CDK1 in 2-methoxyestradiol-induced, mitotically-arrested cells are also responsible for the induction of subsequent apoptosis in these cells via increased phosphorylation of Bcl-X\textsubscript{L}. As briefly discussed below, a number of experiments were conducted to provide support for this hypothesis.

First, we show that knockdown of cyclin B1 and CDK1 is associated with abrogation of 2-methoxyestradiol-induced phosphorylation of Bcl-2 and Bcl-X\textsubscript{L}. By using the co-immunoprecipitation method, we also showed that the cyclin B1/CDK1 complex can directly interact with Bcl-2 and Bcl-X\textsubscript{L} during the development of 2-methoxyestradiol-induced cell death, which supports the hypothesis that the cyclin B1/CDK1 complex directly catalyzes the
phosphorylation of Bcl-2 and Bcl-X\textsubscript{L} proteins.

Second, the role of cyclin B1/CDK1 in Bcl-2 and Bcl-X\textsubscript{L} phosphorylation in mitotically-arrested cells is also supported by the parallel timing of cyclin B1/CDK1 upregulation and Bcl-2/Bcl-X\textsubscript{L} phosphorylation. Furthermore, p-Bcl-2(Ser70) and Bcl-X\textsubscript{L} have a similar cellular distribution pattern in mitotically-arrested cells as cyclin B1 and CDK1, and their co-localization occurs at the time when these proteins reach high levels.

Third, it was confirmed in this study that Bcl-2 and Bcl-X\textsubscript{L} phosphorylation is selectively increased in 2-methoxyestradiol-treated cells. In addition, we showed that the increased phosphorylation of Bcl-X\textsubscript{L}, but not Bcl-2, in 2-methoxyestradiol-treated, mitotically-arrested cells is a trigger of cell death. The observation that increased phosphorylation of Bcl-2 in 2-methoxyestradiol-treated cancer cells does not contribute to cell death is not surprising, and is in line with many other observations indicating that Bcl-2 and Bcl-X\textsubscript{L} have overlapping but different biological functions.

Fourth, we have tested this hypothesis by studying for comparison the effect of roscovitine, an inhibitor of the cyclin-dependent kinases. Pre-treatment of MCF-7 cells with roscovitine inhibits 2-methoxyestradiol-induced Bcl-2 and Bcl-X\textsubscript{L} phosphorylation. Moreover, it also strongly suppresses 2-methoxyestradiol-induced prometaphase arrest and particularly, the subsequent cell death. Interestingly, treatment with roscovitine alone causes a modest G\textsubscript{2}-phase arrest, but not mitotic prometaphase arrest. This observation is in agreement with the known function of CDK1 in G\textsubscript{2}→M transition and the ability of roscovitine to inhibit CDK1 function [27-28, 35-36].

**Role of JNK1 and MAD2**

The results of this study show that while JNK1, ERK and p38 are all activated by 2-
methoxyestradiol treatment, only JNK1 activation is associated with increased Bcl-2 and Bcl-XL phosphorylation. In addition, the JNK1-dependent pathway regulates 2-methoxyestradiol-induced mitotic prometaphase arrest and apoptosis, through upregulation of cyclin B1/CDK1 and the subsequent phosphorylation of Bcl-2 and Bcl-XL. As summarized below, a number of supporting evidence is provided. First, we show that knockdown of JNK1 abrogates 2-methoxyestradiol-induced cyclin B1/CDK1 upregulation and Bcl-2 and Bcl-XL phosphorylation. These changes are accompanied by a decrease in PARP cleavage and annexin V-positive cells compared to the corresponding controls. Second, pre-treatment of cells with SP600125 (a JNK inhibitor) significantly inhibits 2-methoxyestradiol-induced Bcl-2 and Bcl-XL phosphorylation, and this effect is accompanied by a strong protection of these cells against 2-methoxyestradiol-induced prometaphase arrest and cell death. Third, we showed that phosphorylation of MKK4, a kinase that directly phosphorylates (activates) JNK1, is increased after 2-methoxyestradiol treatment. MKK4 knockdown results in decreases in the phosphorylation of JNK1, c-jun, the level of cyclin B1/CDK1, and the phosphorylation of Bcl-2 and Bcl-XL. Collectively, these data indicate that JNK1 activation is an upstream signal in 2-methoxyestradiol-induced upregulation of cyclin B1/CDK1 and the subsequent phosphorylation of Bcl-2 and Bcl-XL.

Progression from metaphase to anaphase is marked by sister chromatid separation. As a safeguard against chromosome segregation errors, the spindle assembly checkpoint (SAC) delays anaphase until all sister chromatid pairs have become bipolarly attached [37]. Once microtubules attach to kinetochores, chromosomes are aligned on the metaphase plate, and proper bi-orientation has been achieved, the SAC stopping mechanisms are removed [37]. Entrance into anaphase is mediated by activation of APC-Cdc20, which is a multi-protein complex with E3 ligase that tags cyclins and other mitotic protein for destruction; without Cdc20, APC is inactive and anaphase would not ensue [37]. MAD2, an important SAC protein, can block the metaphase-to-
anaphase transition by binding to unattached kinetochores [32-34, 38]. In the presence of 2-methoxyestradiol, the normal microtubule functions would be disrupted by preventing microtubule attachment to the kinetochores. Consequently, MAD2 would remain bound to the unattached kinetochores, resulting in the sequestration of Cdc20 in a ternary complex [39, 40].

In this study, we found that the kinetochore-bound MAD2 proteins serve as an upstream mediator of 2-methoxyestradiol's anti-tubulin action by resulting in the activation of the MKK4-JNK1 pathway and subsequently cyclin B1/CDK1 upregulation. The exact mechanism by which MAD2 activates MKK4 is not clear at present. The results of this study showed that the increased functionality of cyclin B1/CDK1 following 2-methoxyestradiol exposure not only plays an important role in the induction of prometaphase arrest, but it also mediates apoptotic cell death via increased phosphorylation of the apoptotic protein Bcl-X<sub>L</sub>.

Conclusions and Implications

As depicted in Fig. 7, the results of this study show that MAD2 is an important upstream protein that mediates the anti-tubulin action of 2-methoxyestradiol by sensing the unattached kinetochores, and subsequently leads to cyclin B1/CDK1 upregulation and CDK1 activation in 2-methoxyestradiol-treated cancer cells via activation of the MKK4-JNK1 pathway. As shown in our recent study, the increased functionality of the cyclin B1/CDK1 complex in 2-methoxyestradiol-treated cells plays a critical role in the induction of mitotic prometaphase arrest. The sustained high functionality of the cyclin B1/CDK1 complex due to 2-methoxyestradiol-induced, prolonged mitotic arrest would then cause increased phosphorylation of Bcl-2 and Bcl-X<sub>L</sub> proteins. The increased phosphorylation of Bcl-X<sub>L</sub>, but not Bcl-2, then activates the mitochondrial apoptotic cell death cascade. The results of this study, along with observations made in our recent study, suggest
that upregulation of the cyclin B1/CDK1 function in 2-methoxyestradiol-treated cells is not only a key event for the induction of mitotic prometaphase arrest, but also an important initial event that couples mitotic prometaphase arrest to apoptotic cell death via phosphorylation (inactivation) of the anti-apoptotic Bcl-XL protein in these cells.

Lastly, it is of note that two very different types of human cancer cells (MDA-MB-5435s and MCF-7) happened to be used in our earlier studies [10, 20] as well as in the present study. The similar observations regarding the anticancer effect of 2-methoxyestradiol in these two different types of human cancer cells may suggest a broader anticancer spectrum. This possibility needs further testing in the future.
Figure 7. The proposed mechanism of 2-methoxyestradiol-induced G2/M cell cycle arrest in human cancer cells.
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Graphical abstract
Highlights

- Upregulation of cyclin B1/CDK1 induced by 2ME₂ is coupled with phosphorylation of Bcl-2 and Bcl-Xₐ and apoptosis.
- Phosphorylation of Bcl-Xₐ, but not Bcl-2, causes mitochondrial cell death in 2ME₂-treated cells.
- JNK1 activation results in cyclin B1/CDK1 upregulation and subsequently phosphorylation of Bcl-2 and Bcl-Xₐ.
- MAD2 is a key mediator of 2ME₂’s action, resulting in MKK4-JNK1 activation and cyclin B1/CDK1 upregulation.