Tumor Suppressor Protein p53 Promotes 2-Methoxyestradiol-Induced Activation of Bak and Bax, Leading to Mitochondria-Dependent Apoptosis in Human Colon Cancer HCT116 Cells

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\textbf{Introduction}

A physiological metabolite of 17β-E\textsubscript{2}, 2-methoxyestradiol (2-MeO-E\textsubscript{2}) is known to be a promising anticancer drug candidate with apoptogenic and antiangiogenic activities \cite{27}. Recently, 2-MeO-E\textsubscript{2} has received much attention owing to its wide spectrum of anticancer activity, with a low level of undesirable side effects. The majority of tumor cell lines appear to be sensitive to the \textit{in vitro} antiproliferative properties of 2-MeO-E\textsubscript{2} at concentrations ranging from 0.08 to 5.0 µM \cite{31, 34}.

The anticancer actions of 2-MeO-E\textsubscript{2} at pharmacological concentrations are attributable to apoptotic cell death and cell cycle arrest. The 2-MeO-E\textsubscript{2}-induced apoptotic cell...
death in tumor cells appears to be mediated by several different mechanisms, such as the up-regulation of death receptor (DR5), p53, and p21, down-regulation of Bcl-2, phosphorylation of Bcl-2 and Bcl-XL, accumulation of ROS, activation of JNK, and mitochondrial cytochrome c release in an estrogen receptor (ER)-independent manner [4, 5, 13, 34]. In relation to 2-MeO-E₂-induced cell cycle arrest, the interference in cellular microtubule formation, which occurs via reducing the tubulin polymerization rate by competitively inhibiting colchicine-binding sites, has been implicated [9]. Moreover, the G₂/M-promoting doses of 2-MeO-E₂ and paclitaxel (a microtubule-polymerizing drug) have been shown to possess similar effects on the cell cycle and apoptosis in human prostate cancer cells [30]. These previous data suggest that the mechanism underlying 2-MeO-E₂-induced cell cycle arrest may be similar to those by microtubule-targeting drugs, which commonly exhibit the disruption of the mitotic spindle, and loss of microtubule function, leading to blockade at the M phase due to activation of the mitotic spindle assembly checkpoint [20, 25]. However, several studies have shown that tumor cells following treatment with 2-MeO-E₂ undergo cell cycle arrest at the G₂/S phase rather than at the M phase, along with apoptosis. In addition, 2-MeO-E₂ appears to inhibit tubulin polymerization by interacting at the colchicine site, and the Kₐ value of 2-MeO-E₂ for inhibition of colchicine binding appears to be 22 µM, which is a much higher concentration than that required for inducing apoptosis [9]. Furthermore, it has been reported that Bcl-2 overexpression in Jurkat T cells by retroviral transduction can prevent 2-MeO-E₂ (0.5–1.0 µM)-induced apoptosis via p2⁷⁰⁶-mediated G₁/S arrest and NF-κB activation, suggesting that Jurkat T cells might provoke G₁/S arrest prior to undergoing apoptosis in the presence of 2-MeO-E₂ (0.5–1.0 µM) [4]. Although these previous studies raised the possibility that 2-MeO-E₂ at low doses (0.5–1.0 µM) could induce apoptosis independently of microtubule damage and resultant mitotic arrest of the cell cycle, the correlation between cell cycle arrest and apoptosis in tumor cells following 2-MeO-E₂ treatment needs to be studied further in order to clarify the anticancer activity of 2-MeO-E₂. Furthermore, although the expression level of tumor suppressors p53 and p2¹⁷⁰⁶/WAF/CIP1 appears to be enhanced during 2-MeO-E₂-induced apoptosis of tumor cells, the precise role of p53 and p2¹⁷⁰⁶/WAF/CIP1 in the induced death signaling pathway leading to apoptotic DNA fragmentation remains obscure.

In the present study, in order to elucidate further how 2-MeO-E₂-induced apoptotic events are regulated by the tumor suppressor protein p53, we investigated the effect of 2-MeO-E₂ (0.5–5 µM) on mitotic arrest, microtubule network organization, and the mitochondria-dependent apoptotic signaling pathway, using the human colorectal carcinoma cell lines HCT116 (p53⁺/⁻) and HCT116 (p53⁻/⁻).

Materials and Methods

Reagents, Antibodies, and Cells

An ECL plus western blotting kit was purchased from Thermo Scientific (Rockford, IL, USA), and Immobilon-P membrane was obtained from Millipore Corporation (Bedford, MA, USA). The anti-caspase-3 antibody was purchased from BD Biosciences (San Jose, CA, USA), and anti-p53, anti-poly (ADP-ribose) polymerase (PARP), anti-Bak, anti-Bax, anti-Bcl-2, anti-Bcl-xL, anti- lamin A/C, anti-Mcl-1, and anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-caspase-9, anti-lamin B2, anti-phospho-p53 (Ser-15), and anti-α-tubulin were purchased from Cell Signaling Technology (Beverly, MA, USA). The anti-Bak (Ab-1) and anti-Bax (6A7) were obtained from Calbiochem (San Diego, CA, USA). The monoclonal anti-p21 antibody was purchased from Neomarkers (Freemont, CA, USA). Human colorectal adenocarcinoma cell lines HCT116 (p53⁺/⁻) and HCT116 (p53⁻/⁻) were provided by Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA). Both HCT116 cells were maintained in DMEM (Hyclone, Gaithersburg, MD, USA) containing 10% FBS (Hyclone) and 100 µg/ml gentamycin.

Cytotoxicity Assay

The cytotoxic effect of 2-MeO-E₂ on HCT116 cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [22]. Briefly, HCT116 cells (4 × 10⁶/well) were incubated with a serial dilution of 2-MeO-E₂ in 96-well plates. At 20 or 44 h after incubation, 50 µl of MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. The colored formazan crystal produced from MTT was dissolved in DMSO, and the OD values of the solutions were measured at 540 nm by a plate reader.

Flow Cytometric Analysis

Flow cytometric analysis for the cell cycle of HCT116 cells exposed to 2-MeO-E₂ was done as described elsewhere [21]. The extent of necrosis was detected using an Annexin V-FITC apoptosis kit as previously described [21]. Changes in the mitochondrial membrane potential (Δψm) following treatment with 2-MeO-E₂ were measured after staining with 3,3'-dihexylox acarbocyanine iodide (DiOC₆) [29]. Activation of Bak and Bax in HCT116 cells following treatment with 2-MeO-E₂ was measured by flow cytometry as previously described [24].

Immunofluorescence Microscopy

HCT116 cells adhered onto glass coverslips were fixed with cold methanol for 3 min [19]. The cells were rinsed four times with

cold PBS containing 0.5% Triton X-100, and blocked with 10% goat serum for 30 min. The cells were then incubated with monoclonal anti-α-tubulin (1:2,500) and rabbit-polycyclonal anti-lamin B2 (1:600) overnight at 4°C. For detection, the cells were treated with Alexa Fluor 488-labeled goat anti-mouse IgG and Alexa Fluor 568-labeled goat anti-rabbit IgG for 1h at room temperature. Thereafter, the cells were stained with 4',6-diamidino-2-phenylindole (DAPI) to label the nuclei. Images were visualized and photographed using a Carl Zeiss MicroImaging Confocal Laser Scanning Microscope overnight.

Transfection of Bak- and/or Bax-Specific Small Interference RNA (siRNA) into HCT116 (p53<sup>++</sup>) Cells

HCT116 (p53<sup>++</sup>) cells were cultured in DMEM containing 10% FBS at a concentration of 0.2 × 10<sup>6</sup>/2 ml using 35 mm culture plates at 37°C for 20 h. The cells were transfected with a mixture of 5.0 µl of 20 µM siRNA and 2.5 µl of Lipofectamine 2000 (Invitrogen) and 500 µl of Opti-MEM (Gibco, Grand Island, NY, USA) for 20 min. Sequentially, 2 ml of DMEM containing 10% FBS was added. After incubation for 24 h, the transfected cells were harvested, resuspended in the culture medium at a density of 1.5 × 10<sup>6</sup>/3 ml using 60 mm culture plates, and then incubated with or without 2-MeO-E<sub>2</sub> for 24 h. The cells were trypsinized and harvested for cell number counting as well as preparation of cell lysates.

Both the Bak- and Bax-specific siRNAs, and control siRNA were obtained from Invitrogen. The sense and antisense sequences for Bak siRNA (HSS184087) were 5'-CAGUUUGUGGUACGAAGAUUCUUA-3' and 5'-UGAAGAAUCUUCGUACCACAAACUG-3', respectively. The sense and antisense sequences for Bax siRNA (HSS141355) were 5'-ACUUUGCCAGCAACUGGUCUCA-3' and 5'-UUGGACCACGUUGUGGCAAGU-3', respectively. Stealth RNAi negative control low GC was used as the control. All of the transfections were run in triplicates, and each siRNA was tested in at least three independent experiments.

Preparation of Cell Lysate and Western Blot Analysis

Cell lysates were prepared by suspending 3 × 10<sup>6</sup> HCT116 cells in 200 µl of the lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 25 mM MOPS, 1 mM PMSF, and 2.5 µg/ml proteinase inhibitor E-64, pH 7.2). Cells were disrupted by sonication and extracted for 30 min at 4°C. An equivalent amount of protein lysate (20 µg) was electrophoresed on a 4–12% NuPAGE gradient gel and then electrotransferred to an Immobilon-P membrane. The detection of each protein was carried out with an ECL plus western blotting kit, according to the manufacturer’s instructions.

Statistical Analysis

Unless otherwise indicated, each result in this paper is representative of at least three separate experiments.

Results and Discussion

Comparison of Cytotoxic Effect of 2-MeO-E<sub>2</sub> Between HCT116 (p53<sup>++</sup>) and HCT116 (p53<sup>-/-</sup>) Cells

To examine whether 2-MeO-E<sub>2</sub>-induced apoptotic cell death is affected by tumor suppressor protein p53, the cytotoxic effects of 2-MeO-E<sub>2</sub> (0.5–5.0 µM) on HCT116 (p53<sup>++</sup>) and HCT116 (p53<sup>-/-</sup>) cells were compared using MTT assay. The viability of HCT116 (p53<sup>++</sup>) cells following treatment with 2-MeO-E<sub>2</sub> (0.5, 1.0, and 5.0 µM) for 24 h was 88.2%, 72.1%, and 70.8%, respectively, whereas that following treatment with 2-MeO-E<sub>2</sub> (0.5, 1.0, and 5.0 µM) for 48 h was 50.4%, 35.2%, and 32.0%, respectively (Fig. 1).

Under these conditions, the viability of HCT116 (p53<sup>-/-</sup>) cells appeared to decline to the level of 96.8%, 94.7%, and 83.9% following treatment with 2-MeO-E<sub>2</sub> (0.5, 1.0, and 5.0 µM) for 24 h, and 84.3%, 56.9%, and 45.3% following treatment with 2-MeO-E<sub>2</sub> (0.5, 1.0, and 5.0 µM) for 48 h.

![Fig. 1](image_url)  
Cytotoxic effect of 2-MeO-E<sub>2</sub> on human colorectal carcinoma cell clones HCT116 (p53<sup>++</sup>) and HCT116 (p53<sup>-/-</sup>). After individual cells (4 × 10<sup>3</sup>/well) were incubated with 2-MeO-E<sub>2</sub> at various concentrations (0, 0.5, 1.0, and 5.0 µM) in 96-well plates for 24 h (A) and 48 h (B), the cell viability was determined using the MTT assay as described in Materials and Methods. Each value is expressed as the mean ± SD (n = 6). *p < 0.05 compared with the control. A representative study is shown and two additional experiments yielded similar results.
with 2-MeO-E₂ (0.5, 1.0, and 5.0 µM) for 48 h. These results indicate that the cytotoxic effect of 2-MeO-E₂ on HCT116 cells was exerted in a dose- and time-dependent manner, and show that HCT116 (p53−/−) cells were more sensitive to the cytotoxicity of 2-MeO-E₂ than were HCT116 (p53+/+) cells. The reliance of the cytotoxicity of 2-MeO-E₂ on p53 status was further tested by the MTT assay using human breast cancer cell lines such as MCF7 (WT p53) and MDA-MB-231 cells (mutant p53), along with other human colon cancer cell lines, including RKO (p53−/−) and RKO-E6 (disrupted p53). As shown in Table 1, the IC₅₀ values of 2-MeO-E₂ toward HCT116 (p53+/+), HCT116 (p53−/−), RKO (p53+/+), RKO-E6 (disrupted p53), MCF7 (WT p53), and MDA-MB-231 cells (mutant p53) were 0.5, 1.8, 0.5, 2.0, 1.0, and 2.3 µM, respectively. These results indicate that the sensitivity of human colon cancer cell lines toward the cytotoxicity of 2-MeO-E₂, which was augmented in the presence of WT-p53 can be extended to human breast cancer cells.

To compare the 2-MeO-E₂-induced change in cell cycle

<table>
<thead>
<tr>
<th>Cancer cell line</th>
<th>Origin</th>
<th>p53 status</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116 (p53+/+)</td>
<td>Colon</td>
<td>Wild-type</td>
<td>0.5</td>
</tr>
<tr>
<td>HCT116 (p53−/−)</td>
<td>Colon</td>
<td>Null</td>
<td>1.8</td>
</tr>
<tr>
<td>RKO</td>
<td>Colon</td>
<td>Wild-type</td>
<td>0.5</td>
</tr>
<tr>
<td>RKO-E6</td>
<td>Colon</td>
<td>Disrupted</td>
<td>2.0</td>
</tr>
<tr>
<td>MCF7</td>
<td>Breast</td>
<td>Wild-type</td>
<td>1.0</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>Breast</td>
<td>Mutant (point mutation)</td>
<td>2.3</td>
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For cytotoxicity assay, HCT116 cells (4 × 10⁴/well), RKO cells (1.5 × 10⁵), MCF7 cells (1.0 × 10⁵), or MDA-MB-231 cells (6 × 10⁴) were incubated with a serial dilution of 2-MeO-E₂ in 96-well plates. At 44 h after incubation, 50 µl of MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. The colored formazan crystal produced from MTT was measured using a colorimetric method as described in Materials and Methods.

**Table 1.** Cytotoxic effect of 2-MeO-E₂ on various human cancer cell lines.

**Fig. 2.** Flow cytometric analysis of the cell cycle distribution (A) and apoptotic cell death (B) in HCT116 (p53+/+) and HCT116 (p53−/−) cells following exposure to 2-MeO-E₂ (0, 0.5, 1.0, and 5.0 µM) for 24 h. Cell cycle distribution and apoptotic cell death were determined by flow cytometric analyses after PI staining and Annexin V-FITC/PI double staining, respectively, as described in Materials and Methods. A representative result is presented, and two additional experiments yielded similar results.
distribution of HCT116 (p53+/+) and HCT116 (p53−/−) cells, the cells were treated with 2-MeO-E₂ (0.5, 1.0, and 5.0 μM) for 24 h, and then their cell cycle profiles were analyzed by flow cytometry. As shown in Fig. 2A, although HCT116 (p53+/+) and HCT116 (p53−/−) cells after 2-MeO-E₂ treatment commonly exhibited an enhancement in the level of sub-G₁ cells, representing apoptotic cells in a dose-dependent manner, the proportion of apoptotic sub-G₁ cells appeared to be higher in HCT116 (p53+/+) cells than in HCT116 (p53−/−) cells. Along with an enhancement in the proportion of sub-G₁ cells, the level of cells accumulated in the G₂/M phase were also elevated in both HCT116 (p53+/+) and HCT116 (p53−/−) cells after 2-MeO-E₂ treatment in a dose-dependent manner. In HCT116 (p53+/+) cells treated with 2-MeO-E₂ (5.0 μM) for 24 h, the level of G₂/M cells was enhanced to a level of 42.9%, whereas the level of G₂/M cells appeared to increase to a level of 68.9% in HCT116 (p53−/−) cells. Under the same conditions, analysis by Annexin V-FITC and PI staining of HCT116 cells treated with 5.0 μM 2-MeO-E₂ for 24 h revealed that the number of early apoptotic cells stained only with Annexin V-FITC was enhanced, but late apoptotic cells stained with both Annexin V-FITC and PI, as well as necrotic cells stained only with PI, were rarely detected (Fig. 2B), indicating that 2-MeO-E₂-induced apoptosis was not accompanied by necrosis. These results demonstrated that the cytotoxicity of 2-MeO-E₂ (0.5–5.0 μM) in HCT116 cells was attributable to not only the cytostatic effect mediated by G₂/M arrest, but also apoptotic cell death, which occurred more dominantly in the presence of p53.

It is noteworthy that 2-MeO-E₂-induced apoptotic cell death in HCT116 cells occurred more significantly in the presence of p53, whereas 2-MeO-E₂-induced G₂/M arrest in HCT116 cells occurred more dominantly in the absence of p53. Because 2-MeO-E₂-induced apoptosis has been shown to be preceded by cell cycle arrest at G₂/M in several previous studies [15, 23, 26, 36], these previous and current

![Fig. 3. Effect of 2-MeO-E₂ on the organization of the microtubule network in HCT116 (p53+/+) and HCT116 (p53−/−) cells.](image-url)

After treatment with 5.0 μM 2-MeO-E₂ for 24 h, the cells were fixed with cold methanol, permeabilized, and blocked with 10% goat serum for 30 min. Sequentially, the cells were incubated with mouse monoclonal anti-tubulin (1:2,500) and rabbit monoclonal anti-lamin B2 (1:600), and then treated with Alexafluor 488-conjugated goat anti-mouse immunoglobulin and Alexafluor 568-conjugated goat anti-rabbit immunoglobulin. The cells were then stained with DAPI to label the nuclei. Images were obtained using a Carl Zeiss Micro Imaging Confocal Laser Scanning Microscope. A representative study is shown and two additional experiments yielded similar results. The scale bar represents a length of 10 μm in each of the images.
Effect of 2-MeO-E₂ on the Cellular Microtubule Network in HCT116 (p53⁺⁻) and HCT116 (p53⁻⁻) Cells

Because the 2-MeO-E₂-induced apoptotic sub-G₁ peak was more apparently induced in HCT116 (p53⁺⁻) than in HCT116 (p53⁺⁺) cells, and because 2-MeO-E₂-induced G₂/M arrest was more dominantly induced in HCT116 (p53⁺⁻) than in HCT116 (p53⁺⁺) cells, we decided to examine whether impairment of the mitotic spindle network, which can be caused by 2-MeO-E₂, is modulated by p53. The effect of 2-MeO-E₂ on the organization of the microtubule network and the integrity of the nuclear envelope in HCT116 (p53⁺⁺) and HCT116 (p53⁻⁻) cells was investigated by immunofluorescence microscopy using anti-α-tubulin and anti-lamin B2 antibodies.

Although the microtubule network in continuously growing HCT116 (p53⁺⁺) and HCT116 (p53⁻⁻) cells showed a normal arrangement, with the majority of cells in interphase, both cell types exhibited, in common, an aberrant bipolar array of microtubules as well as nuclear envelope breakdown following exposure to 5.0 µM 2-MeO-E₂ for 24 h (Fig. 3). In addition, DAPI staining revealed that most of the chromosomes in the cells treated with 5.0 µM 2-MeO-E₂ failed to align at the equator of the mitotic spindle, regardless of the presence of p53. These results show that the 2-MeO-E₂-induced defect in the organization of the mitotic spindle network, which is known to be a target for the apoptotic action of 2-MeO-E₂ [27], and subsequent mediation of incomplete alignment of the chromosomes at the equatorial plate, leading to prometaphase arrest, was not influenced by p53.

Effect of p53 on 2-MeO-E₂-Induced Activation of Bak and Bax, and Δψₘ Loss in HCT116 (p53⁺⁺) and HCT116 (p53⁻⁻) Cells

To examine whether p53 contributes to modulation of the downstream events of the 2-MeO-E₂-induced mitotic arrest, resulting from the mitotic spindle defects, the 2-MeO-E₂-induced activation of Bak and Bax, and Δψₘ loss were compared between HCT116 (p53⁺⁺) and HCT116 (p53⁻⁻) cells. When the Δψₘ loss of cells treated with 2-MeO-E₂ was measured by DiOC₆ staining, continuously growing HCT116 (p53⁺⁺) and HCT116 (p53⁻⁻) cells non-treated with 2-MeO-E₂ showed barely detectable levels of negative fluorescence (Fig. 4). After treatment with 5.0 µM 2-MeO-E₂ for 24 h, the proportions of negative fluorescence in both HCT116 (p53⁺⁺) and HCT116 (p53⁻⁻) cells were 28.0% and 13.3%, respectively. Since current results indicated that 2-MeO-E₂-induced Δψₘ loss was more dominant in HCT116 (p53⁺⁺) cells than in HCT116 (p53⁻⁻) cells, it was likely that p53 could positively modulate the 2-MeO-E₂-induced mitochondrial damage and thus mitochondria-dependent apoptotic events.

To examine this prediction further, the activation of the pro-apoptotic multidomain Bcl-2 family members, Bak and Bax, as evidenced by their N-terminal conformational changes detected using an active conformation-specific anti-Bak antibody (Ab-1) and an active conformation-specific anti-Bax antibody (6A7), were compared between HCT116 (p53⁺⁺) and HCT116 (p53⁻⁻) cells after treatment with 5 µM 2-MeO-E₂. As shown in Fig. 5, 2-MeO-E₂-induced activation of Bak appeared to be more significant in HCT116 (p53⁻⁻) cells than in HCT116 (p53⁺⁺) cells. In
particular, whereas 2-MeO-E₂-induced Bax activation was easily detected in HCT116 (p53⁺⁺) cells, it was not detected in HCT116 (p53⁻⁻) cells, suggesting that the presence of p53 was a prerequisite for the 2-MeO-E₂-induced activation of Bax in HCT116 cells. Previously, it has been reported that either Bak activation or Bax activation can mediate permeabilization of the mitochondrial outer membrane to trigger mitochondrial cytochrome c release into the cytoplasm, leading to the caspase cascade activation [6, 8]. Consequently, these previous and current results indicated that p53-mediated enhancement in the 2-MeO-E₂-induced ∆ψm loss was due to the promotion in the activation levels of Bak and Bax in the presence of p53.

Numerous studies have reported that chemotherapeutic agent-induced apoptotic signaling pathways are frequently associated with mitochondria-dependent apoptotic events [3, 10, 11]. In addition, it has been reported that an alteration in the expression ratio of Bak to Bcl-2 and/or Bax to Bcl-2, resulting in an enhancement in the ratio of Bak to Bcl-2 and/or Bax to Bcl-2, is often associated with provoking the activation of Bak and/or Bax during the mitochondria-dependent apoptosis induced by chemotherapeutic agents [1, 7, 14]. To examine the upstream pro-apoptotic events that mediate 2-MeO-E₂-induced activation of Bak and Bax, the levels of Bcl-2 family proteins, such as the pro-apoptotic Bcl-2 family members (Bak and Bax) and anti-apoptotic Bcl-2 family members (Bcl-2, Bcl-xL, and Mcl-1), the activation of caspase-9 and -3, and the cleavage of lamin A/C were compared, by western blot analysis, between HCT116 (p53⁺⁺) and HCT116 (p53⁻⁻) cells treated with 2-MeO-E₂. As shown in Fig. 6, p53 expression was easily detected in HCT116 (p53⁺⁺) but not in HCT116 (p53⁻⁻) cells. In HCT116 (p53⁺⁺) cells treated with 0.5–5.0 µM 2-MeO-E₂, the levels of phosphorylated p53 at Ser-15 and total p53 were enhanced by 8.4-fold and 1.8-fold, respectively. Although the up-regulation of p21 expression level, which acts as a negative cell cycle regulator for G₁ arrest and/or G₂ arrest [2, 28], was remarkable in HCT116 (p53⁺⁺) after 2-MeO-E₂ treatment, it was not detected in HCT116 (p53⁻⁻) cells. Under these conditions, HCT116 (p53⁺⁺) after 2-MeO-E₂ treatment exhibited a higher proportion of G₁ cells compared with HCT116 (p53⁻⁻) cells, suggesting that the 2-MeO-E₂-induced up-regulation of the p21 expression level in HCT116 (p53⁺⁺) cells might contribute to G₁ arrest rather than G₂ arrest. The expression level of Bcl-2 was reduced by 0.5-fold in HCT116 (p53⁻⁻) following exposure to 2-MeO-E₂, whereas the Bcl-2 expression level remained relatively constant in HCT116 (p53⁺⁺) cells. The

**Fig. 5.** Flow cytometric analysis of Bak activation (A) and Bax activation (B) in HCT116 (p53⁺⁺) and HCT116 (p53⁻⁻) cells after 5 µM 2-MeO-E₂ treatment.

After cells were incubated with 5.0 µM 2-MeO-E₂ for 24 h, the cells were harvested and subjected to flow cytometric analysis of Bak activation and Bax activation, as described in Materials and Methods. A representative study is shown and two additional experiments yielded similar results.
expression levels of Bcl-xl, Mcl-1, and Bak appeared to be similar between HCT116 (p53<sup>−/−</sup>) and HCT116 (p53<sup>+/−</sup>) cells, regardless of 2-MeO-E<sub>2</sub> treatment. In contrast, the expression level of Bax increased by 2.6-fold in HCT116 (p53<sup>−/−</sup>) cells after 2-MeO-E<sub>2</sub> treatment, whereas the Bax expression level, which was barely detected in HCT116 (p53<sup>−/−</sup>) cells, failed to increase in HCT116 (p53<sup>−/−</sup>) cells following treatment with 2-MeO-E<sub>2</sub>. Under these conditions, the caspase-9 and -3 activations, and the lamin A/C cleavage, which is known to be catalyzed by active caspase-3 and -6 [32], were more significantly detected in HCT116 (p53<sup>−/−</sup>) than in HCT116 (p53<sup>−/−</sup>) cells. Previously, it has been shown that p53 can negatively regulate the expression of Bcl-2 via repression of the Bcl-2 promoter [12, 17, 35]. It has also been reported that p53 can exert a pro-apoptotic role at the mitochondria through direct interaction of p53 with anti-apoptotic Bcl-2 protein, which results in induction of mitochondria-dependent apoptosis [16, 33]. These previous and current results suggested that the differential expression pattern of Bcl-2, which declined following 2-MeO-E<sub>2</sub> treatment and exhibited a relatively lower level in HCT116 (p53<sup>−/−</sup>) than in HCT116 (p53<sup>−/−</sup>) cells, along with up-regulation of the Bax expression level, might also render HCT116 (p53<sup>−/−</sup>) cells more susceptible to the onset of Bak and Bax activations.

To confirm the critical role for Bak and Bax activations in the 2-MeO<sub>2</sub>-induced apoptosis, we examined whether the siRNA approach-mediated depletion of Bak and/or Bax could inhibit 2-MeO-E<sub>2</sub>-induced apoptotic events in HCT116 (p53<sup>−/−</sup>) cells. As shown in Figs. 7A and 7B, the transfection of cells with either Bak-specific siRNA or Bax-specific siRNA did not reduce the cellular levels of Bak or Bax completely, but could reduce the proteins levels by approximately 70% compared with those in the control cells. Under these conditions, either Bak down-regulation or Bax down-regulation by each siRNA failed to suppress the induced cytotoxicity, caspase-3 activation, and lamin A/C cleavage, whereas the concomitant down-regulation of Bak and Bax could completely abrogate 2-MeO-E<sub>2</sub>-induced apoptotic events such as caspase-3 activation and lamin A/C cleavage, the induced cytotoxicity was prevented only by ~30%, confirming that prometaphase arrest rather than apoptotic cell death might be more critical for exerting the cytotoxicity of 2-MeO-E<sub>2</sub> (5.0 µM) in HCT116 (p53<sup>−/−</sup>) cells. Consequently, these results indicated that the 2-MeO-E<sub>2</sub>-induced activation of Bak and Bax, which occurred more dominantly in the presence of p53, was exerted by not only a transcription-dependent mechanism of p53 via up-regulation of the Bax level and down-regulation of Bcl-2, but also by a transcription-independent mechanism of p53 via direct binding to Bcl-2.

In conclusion, these results show that HCT116 (p53<sup>−/−</sup>) cells were more sensitive to the cytotoxicity of 2-MeO-E<sub>2</sub>, which is attributable to the more potent induction of
positive modulation of 2-methoxyestradiol-induced apoptosis by p53

apoptotic cell death, as compared with HCT116 (p53\(^{-/-}\)) cells. Although the 2-MeO-E\(_2\)-induced defect in the organization of the mitotic spindle network and the subsequent mediation of incomplete alignment of the chromosomes at the equatorial plate, causing prometaphase arrest, were not influenced by p53, the downstream events including both Bak and Bax activations and \(\Delta\psi_m\) loss were observed more potently in the presence of p53. In HCT116 (p53\(^{+/+}\)) cells following 2-MeO-E\(_2\) treatment, the levels of phosphorylated p53 at Ser-15, total p53, and Bax were markedly enhanced in a dose-dependent manner, suggesting that the 2-MeO-E\(_2\)-induced activation of Bak and Bax might be positively modulated by the pro-apoptotic action of p53. These results provide insight into the molecular and cellular mechanisms underlying the pro-apoptotic role of p53 in Bak and Bax activations, provoked by a microtubule-damaging drug, 2-MeO-E\(_2\).

Acknowledgments

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References


