2-Methoxyestradiol (2-ME2) is an endogenous metabolite of estradiol (E2) with estrogen receptor-independent anti-cancer activity. The current study sought to determine the mechanism of anti-cancer activity of 2-ME2 in human acute T lymphoblastic leukemia CEM cells. Results showed that 2-ME2 markedly suppressed proliferation of CEM cells in a time- and dose-dependent manner. 2-ME2-treated CEM cells underwent typical apoptotic changes. Exposure to 2-ME2 led to G2/M phase cell-cycle arrest, which preceded apoptosis characterized by the appearance of a sub-G1 cell population. In addition, cytosolic cytochrome c release, increased procaspase-9 and -3 expressions, poly(ADP-ribose) polymerase (PARP) cleavage, and induced expression of caspase-8 were detected, suggesting that both the intrinsic apoptotic pathway and extrinsic apoptotic pathway were involved in 2-ME2-induced apoptosis. Moreover, the expression level of p21 protein was upregulated, whereas Bcl-2 and dysfunctional p53 protein were downregulated, which also contributed to 2-ME2-induced apoptosis. Our findings revealed that 2-ME2 might be a potent natural candidate for chemotherapeutic treatment of human acute T lymphoblastic leukemia when the precise effects of 2-ME2 were investigated further in other T leukemia cell lines and in primary T-cell leukemias.

Keywords 2-methoxyestradiol; human acute T lymphoblastic leukemia; cell proliferation; cell apoptosis; cell cycle

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Introduction

The percentage of long-term remitters and survivors in adult acute lymphoblastic leukemia has not improved significantly during recent decades, although several trials have attempted to intensify the induction and post-remission strategy, including early bone marrow transplantation [1]. Developing new effective drugs for human acute lymphoblastic leukemia is an important undertaking.

2-Methoxyestradiol (2-ME2) is a physiological metabolite of endogenous estradiol that cannot bind the estrogen receptor. It is produced by sequential 2-hydroxylation and O-methylation of the parent compound and is present in human blood and urine at picomolar to nanomolar levels [2–5]. The potential role of 2-ME2 as an anti-cancer agent has been intensively investigated. 2-ME2 was found to inhibit growth and induce apoptosis of tumors of Ewing sarcoma [6], chondrosarcoma [7], osteosarcoma [8], esophageal [9], hepatocellular [10], ovarian [11] and nasopharyngeal carcinomas [12], and prostate cancer [13] both in vitro and in vivo. 2-ME2 also showed anti-leukemic activity in vitro with therapeutic selectivity [14–16]. Several different mechanisms of action have been proposed, including the accumulation of cells in the G2/M phase [7–9], inhibition of tubule polymerization [17], inhibition of superoxide dismutase (SOD) [14], and accumulation of reactive oxygen species (ROS) [14,15], activation of c-jun N-terminal-activated kinase (JNK) [15], increased expression of p53 and p21 [7,8,10], Bcl-2 phosphorylation [11], and mitochondrial release of cytochrome c [14,15]. However, its exact mechanism on human acute T lymphoblastic leukemia remains unknown.

In the current study, we explored the effects of 2-ME2 on proliferation, cell-cycle progression, and apoptosis induction in human acute T lymphoblastic leukemia CEM cells and elucidated its underlying mechanism associated with its growth-inhibitory action.

Materials and Methods

Cell cultures and chemicals
Human acute T lymphoblastic leukemia CEM cells were obtained from the Fujian Institute of Hematology (Fuzhou, China) and were grown in RPMI-1640 medium.
After incubation, $1 \times 10^6$ cells were collected and washed twice with cold PBS and resuspended in 500 $\mu$l binding buffer solution (10 mM HEPES, pH 7.4, 140 mM NaOH, 2.5 mM CaCl$_2$). Then, 5 $\mu$l of annexin V-FITC and 10 $\mu$l of PI (5 $\mu$g/ml) solutions were added, and the resultant mixture was incubated for 15 min at room temperature in the dark. The apoptotic cells were determined using BD FACScan flow cytometry. For each sample, the fluorescence of 10,000 cells was gated and counted. Both early apoptotic (annexin V positive, PI negative) and late (annexin V positive and PI positive) apoptotic cells were included in cell death determinations.

**Measurement of mitochondrial transmembrane potential ($\Delta \Psi_m$)**

CEM cells were treated with different doses of 2-ME2 for 24 h, collected, and then resuspended in PBS and labeled with rhodamine-123 (final concentration 5 $\mu$g/ml). Cells were subsequently incubated at 37°C for 30 min. The mitochondrial membrane potential was determined using flow cytometry based on the fluorescence intensity of 10,000 cells.

**DNA fragmentation analysis**

The DNA fragmentation was analyzed by agarose gel electrophoresis. Treated or untreated cells ($1 \times 10^6$) were centrifuged, and cell pellets were resuspended with NP-40 lysis buffer (1% NP-40 in 20 mM EDTA and 50 mM Tris–HCl, pH 7.5). After lysis for a few seconds, the supernatants were collected by centrifugation for 5 min at 11,000 g. The extraction was repeated with the same lysis buffer. After centrifugation for 5 min at 1600 g, the supernatant was collected, treated with RNase A (final concentration 2.5 mg/ml) for 2 h at 56°C, brought to 1% (w/v) sodium dodecyl sulfate (SDS), and finally digested with proteinase K (final concentration 2.5 mg/ml) for 2 h at 37°C. Then, the mixtures were brought to 10 mM ammonium acetate prior to 100% ethanol precipitation for 30 min at $-20^\circ$C. The DNA was collected by centrifugation for 10 min at 11,000 g and suspended in TE buffer (10 mM Tris–HCl, 1 mM tetraacetic acid) and loaded on 2.0% agarose gel for electrophoresis. The gel was stained with ethidium bromide and photographed with ultraviolet (UV) illumination.

**Reverse transcription–polymerase chain reaction (RT–PCR)**

Total RNA was extracted from cells using the Trizol reagent (Invitrogen, Carlsbad, USA) and cDNA was synthesized using the Sensiscript RT kit (Promega, Madison, USA) following the manufacturer’s instructions. RT–PCR amplification was carried out for one cycle at 94°C for 5 min, followed by 32 cycles at 94°C for 30 s, (Tm $- 5^\circ$C) for 30 s, 72°C for 30 s, and then held at 4°C. The PCR
primers were designed by Premier Primer 5.0 software (Table 1). The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel. The gel was stained with ethidium bromide and photographed with ultraviolet (UV) illumination. In all cases, equivalent quantities of cDNA template were used, and the results were adjusted for variations in PCR efficiency by normalization to β-actin.

**Western blot analysis**
Actively growing CEM cells were treated with 2 μM 2-ME2 for 0, 24, 48, and 72 h, respectively. Then, cells were harvested, washed twice with cold PBS, and lysed with M-PER Mammalian Protein Extraction Reagent (Pierce). Cell lysates were kept on ice for 30 min and centrifuged at 12,000 g for 10 min at 4°C. For the detection of cytosolic cytochrome c release, NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Pierce) was used to obtain cytosolic fractions. Supernatants were collected and the protein content of each fraction was determined by BCA protein assay (Pierce). Samples were boiled in loading buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromphenol blue, and 100 mM dithiothreitol) and separated by 10% or 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, USA), which was incubated with blocking solution (5% nonfat dry milk in PBS containing 0.05% Tween-20) for 2 h at room temperature and immunoblotted, respectively, with anti-PARP, p21, p53, procaspase-9, -3, caspase-8, Bcl-2, cytochrome c, and β-actin antibodies overnight at 4°C. After washing three times in TBS for 10 min, membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (or goat anti-rabbit-IgG) in blocking solution. After washing three times in TBS for 10 min, the bound antibodies were detected by enhanced chemiluminescence using Supersignal West Pico Chemiluminescent Substrate (Pierce) following the manufacturer’s instructions.

**Statistical analysis**
Statistical significance and differences observed between experimental groups was determined using the Student’s t test. P < 0.05 was considered significant.

**Results**

**Inhibition of CEM cell proliferation by 2-ME2**
To determine the anti-proliferative effect of 2-ME2 in an *in vitro* model, exponentially growing CEM cells were treated with 0.25, 0.5, 1, 2, 4, and 8 μM 2-ME2 for 24, 48, and 72 h, respectively. As shown in Fig. 1, 2-ME2 inhibited the growth of CEM cells significantly in a time- and dose-dependent manner with an IC50 value of 2 μM. The data indicate that 2-ME2 is a potent inhibitor of CEM cell proliferation.

**Cell-cycle distribution of CEM cells following 2-ME2 treatment**
To determine the mechanism of growth inhibition by 2-ME2, we examined the effect of 2-ME2 on cell-cycle distribution by Fascine flow cytometry analysis. Figure 2 indicated that treatment of CEM cells with 2-ME2 for 24 h resulted in a significantly higher number of cells in the G2/M phase at all tested concentrations, 1 μM (45.09%), 2 μM (73.41%), and 4 μM (83.12%), compared with controls (6.20%). The dose-dependent effect of 2-ME2 on G2/M phase arrest in CEM cells was largely at the expense of the G1 and S phase compared with untreated CEM cells. In addition, a fraction of 2-ME2-treated cells appeared as a distinct peak below the G1 peak (sub-G1 population) compared with untreated control cells. The appearance of a sub-G1 population of cells is a potential indicator of apoptosis, but the sub-G1 population of cells is not increased in

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
<th>Product (bp)</th>
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<tr>
<td>β-actin</td>
<td>US: 5’-ATCTGGCACCACACCTTTCTACAATGAGCTGCG-3’ (F)</td>
<td>887</td>
</tr>
<tr>
<td></td>
<td>DS: 5’-CGTCATACTCTGTGCTCTGATCCACATCTGC-3’ (R)</td>
<td></td>
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<tr>
<td>bcl-2</td>
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<td></td>
</tr>
<tr>
<td>p21</td>
<td>US: 5’-GACACACAGGGGTAAGTCACT-3’ (F)</td>
<td>172</td>
</tr>
<tr>
<td></td>
<td>DS: 5’-CGGTCCACATGGTCTCTCT-3’ (R)</td>
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<tr>
<td>p53</td>
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<td>242</td>
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<tr>
<td></td>
<td>DS: 5’-CTCTCGGAACATCTCGAAGC-3’ (R)</td>
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</tbody>
</table>

US, upstream sequence; DS, downstream sequence.

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**Table 1 Primer sequences of p53 and others genes for PCR**

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2-ME2 blocks cell-cycle progression and induces apoptosis in CEM cells

A dose-dependent manner. We speculated that the G2/M block occurred prior to apoptosis.

**Induction of apoptosis in CEM cells following 2-ME2 treatment**

Using annexin V-conjugated FITC and PI staining to analyze the percentage of apoptotic cells induced by 2-ME2, we found that the late apoptotic cells (UR) and early apoptotic cells (LR) increased from 2.89% to 9.14% and 9.89% to 32.14% [Fig. 3(B–D)], respectively, when cells were treated for 24 h with 2-ME2 from 0.5 to 2 μM. Total percentage of apoptotic cells (UR + LR) increased from 12.78 to 41.28%. However, in untreated control CEM cells, UR and LR reached 1.02% and 4.81%, respectively [Fig. 3(A)].
Loss of mitochondrial transmembrane potential in 2-ME2 treated CEM cells
The integrity of the mitochondrial transmembrane of 2-ME2-treated cells was examined by measuring its ability to retain rhodamine-123, a fluorescent dye used to indicate the loss of mitochondrial transmembrane potential. Substantial loss of dye retention was observed in response to 2-ME2 (1–4 μM) for 24 h (Fig. 4).

DNA damage in 2-ME2-treated CEM cells
To confirm apoptosis induction by 2-ME2 in CEM cells, DNA fragmentation assay was performed using agarose gel electrophoresis. Genomic DNA was prepared from 1 × 10^6 CEM cells incubated in the absence or presence of different concentrations of 2-ME2 for 24 h. Agarose gel electrophoresis showed clear multiple DNA fragments were observed in 2-ME2-treated cells; however, only slight DNA fragmentation was observed in the untreated cell samples (Fig. 5).

Effect of 2-ME2 on the expression of cellular genes mRNA
To confirm the mechanism of the anti-proliferative activities of 2-ME2, the effects of 2-ME2 on the expressions of p21, bcl-2, p53 mRNA were examined. Figure 6 indicated that the expressions of bcl-2 and p53 mRNA were downregulated, whereas p21 mRNA expression was upregulated by 2 μM 2-ME2 treatment in a time-dependent manner. The results suggest that the downregulation of bcl-2 and p53 mRNA expressions and the upregulation of p21 mRNA expression are involved in the 2-ME2-mediated anti-proliferative activities on CEM cells.

Effect of 2-ME2 on the expressions of apoptosis-associated proteins
To explore the mechanism for the anti-proliferative activities of 2-ME2, we examined the effects of 2-ME2 on the expressions of apoptosis-associated proteins. The CEM cells, which were treated with 2 μM 2-ME2 for indicated times, were lysed and separated by 10% or 12% SDS–PAGE, and western blot analysis was performed with antibodies against PARP, p21, p53, procaspase-9, -3, caspase-8, Bcl-2, cytochrome c, and β-actin, respectively. As shown in Fig. 7, cytochrome c release, expression of procaspase-9, -3, and PARP cleavage were detected in CEM cells after exposure to 2-ME2 at 24, 48, and 72 h, while the expression of caspase-8 was increased at 48 h, which suggested that both the intrinsic and extrinsic apoptotic pathways were involved in 2-ME2-induced apoptosis. Figure 7 also showed that expression of p21 protein increased in response to 2-ME2 treatment at 24, 48, 72 h, whereas Bcl-2 and p53 protein expression decreased following 2-ME2 treatment at the same time points, which contributed to 2-ME2-mediated apoptosis.

Discussion
2-ME2, previously thought to be a physiologically inactive metabolite of 17β-estradiol (E2), is now considered to be a promising anti-cancer agent [18]. 2-ME2 exerts both in vitro and in vivo anti-cancer activity against a range of solid tumors [19]. Despite previous preclinical studies on human leukemia cells, the effect of 2-ME2 on human T lymphoblastic leukemia CEM cells has not been studied in detail. In the present study, we found that 2-ME2 significantly inhibited the proliferation of CEM cells in a
time- and dose-dependent manner. The half-maximal inhibitory concentration (IC50) was 2 μM at 48 h.

Apoptosis is the most common form of physiological cell death in multicellular organisms. Apoptosis signaling is classically composed of two principle pathways. One is a direct pathway from death receptor (CD95, TNF-R1, and TRAIL-R1/TRAIL-R2 [20]) ligation to caspase cascade activation and cell death. Death receptor ligation triggers recruitment of the precursor form of caspase-8 to a death-inducing complex through the adaptor protein Fas-associated death domain (FADD), which leads to caspase-8 activation. The other pathway triggered by stimuli such as drugs, radiation, infectious agents, and ROS is initiated in mitochondria. After cytochrome c is released from the mitochondria into the cytosol, it binds to Apaf1 and ATP, which then activate caspase-9. Subsequently, the activated caspase-9 triggers downstream caspases, such as caspase-3 [21]. Caspase-3, through the cleavage of several death substrates such as poly(ADP-ribose) polymerase (PARP), is believed to lead to the execution of cell death [21–24]. The effect of apoptosis induction and inhibition of cell proliferation has been frequently described [6–10,14,15]. In the present study, we found that exposure to 2-ME2 led to G2/M phase cell-cycle arrest, which preceded apoptosis characterized by the appearance of a sub-G1 population of cells. 2-ME2-treated CEM cells underwent typical apoptotic changes as indicated by flow cytometry and DNA fragmentation assay. In addition, the loss of mitochondrial transmembrane potential was detected at 24 h in CEM cells after 2 μM 2-ME2 treatment and at this dose and time point, 2-ME2 resulted in cytochrome c release in cytosol and increased the expression of procaspase-9, -3, and PARP cleavage, whereas caspase-8 expression at 48 h, suggesting that both the intrinsic and extrinsic apoptotic pathways were involved in 2-ME2-induced apoptosis. This finding is consistent with previous reports [14,15].

Overexpression of the bcl-2 gene is found in a majority of human cancers. Thus, it is believed to promote tumorigenesis and chemoresistance, suggesting that inhibition of the Bcl-2 protein may be lethal to cancer cells [25]. The results from our study showed that 2-ME2 significantly inhibited Bcl-2 protein expression in a time-dependent manner. This may be the mechanism underlying 2-ME2-induced apoptosis in CEM cells. It is consistent with what has been previously reported [12].

p21 is a cyclin-dependent kinase (CDK) inhibitor, which was initially thought to function as a negative regulator of cell-cycle progression via its ability to bind CDKs [26]. However, in our study, p21 expression was not altered after 2 μM 2-ME2 treatment for 24 h (Figs. 6 and 7) and at this dose and time point, 2-ME2-induced G2/M growth arrest
(Fig. 2). Our explanation is that p21 is not involved in cell-cycle arrest, but may rather be involved in the induction of apoptosis [27]. Thus, we suggested that 2-ME2-induced p21 protein expression in CEM cells could contribute to 2-ME2-induced apoptosis. This is consistent with some previous reports that 2-ME2-induced apoptosis was accompanied by an increase in p21 protein level in hepatocellular carcinoma and in multiple osteosarcoma cell lines [8,10]. However, not all of the effects of 2-ME2 can be attributed to increased p21 levels. Zhou et al. reported that 2-ME2-induced apoptosis was not accompanied by changes in p21 expression in nasopharyngeal carcinoma CNE2 cells [12]. Whether p21 participates in regulating apoptotic function in response to 2-ME2 depends on the cellular context [27].

p21 was originally known as a p53-target gene because it is induced directly by a p53-dependent mechanism. Carothers et al. [28] reported that treatment of CRC cells with 2-ME2 increased the expressions of p53 and p21 proteins and induced apoptosis. However, previous reports have suggested that p21 is implicated as a downstream effector of various tumor suppressors, growth factors, and cytokines, including interferon, transforming growth factor-β, and NF-κB [29–32], suggesting that p21 can be induced by a p53-independent mechanism.

p53 protein is a transcription factor and tumor suppressor that has several anti-cancer mechanisms, including activation of DNA repair proteins, induction of cell-cycle block, and induction of apoptosis [8]. 2-ME2 treatment increases expression of functional and wild-type p53 in cancer cells, resulting in apoptosis. The requirement of p53 in 2-ME2-mediated induction of apoptosis was documented [10]. The results of our study showed that p53 was decreased following 2-ME2 treatment. In CEM cells, p53 has two heterozygous mutations located in the DNA-binding domain at codons 175 (Arg to His) and 248 (Arg to Gln). Thus, both alleles of p53 may have been functionally activated by two different point mutations [33,34]. p53 mutations occur in the majority of human tumors and are often associated with advanced tumor stage and poor prognosis [25]. This indicates that decreased dysfunctional p53 could contribute to 2-ME2-induced apoptosis in CEM cells and is consistent with the reports of Zhou et al. [12].

In conclusion, our results revealed that 2-ME2 induced apoptosis via the intrinsic and extrinsic apoptotic pathways and involvement of increased expression of p21 protein and decreased Bcl-2 and dysfunctional p53 proteins expressions, thus providing a molecular mechanism for understanding the anti-proliferative effect of 2-ME2, suggesting that 2-ME2 might be a potent natural candidate for chemotherapeutic treatment of human acute T lymphoblastic leukemia when the precise effect of 2-ME2 had been investigated further in other T leukemia cell lines and in primary T-cell leukemias.

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**References**