Cytotoxic Effects of Mansonone E and F Isolated from *Ulmus pumila*

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Two sesquiterpenoids, mansonone E (ME) and mansonone F (MF) were first isolated from the dried root bark of *Ulmus pumila* (shironire in Japanese), and their antiproliferative activities on human tumor cells were evaluated in vitro. ME had more potent cytotoxic effects on four tumor cell lines, human cervical cancer HeLa, human malignant melanoma A375-S2, human breast cancer MCF-7, and human histiocytic lymphoma U937, than those of MF. The results showed that ME induced oligonucleosomal fragmentation of DNA in HeLa cells and activated caspase-3, followed by the degradation of the inhibitor of caspase-activated DNase, decreased the expression of anti-apoptotic mitochondrial proteins Bel-2 and Bel-xL, and increased that of proapoptotic Bax.

Key words Ulmaceae; *Ulmus pumila*; mansonone; apoptosis; antitumor activity

*Ulmus pumila* L. is a deciduous tree that is widely distributed in East Asia. The stem and root bark of this species have been used in traditional Chinese medicine (TCM) for edema, mastitis, gastric cancer, and inflammation. 1, 2) To search for biologically active substances, we separated the 80% ethanol extract of the root bark of *U. pumila*, and two sesquiterpenes α-naphthoquinones, mansonone E (ME) and F (MF), were isolated from this species for the first time. It has been reported that ME and MF have antioxidant activities, 3) and they have been used in traditional Chinese medicine (TCM) for edema, mastitis, gastric cancer, and inflammation. 1, 2) Therefore in this study the cytotoxic effects of ME and MF on the human tumor cell lines A375-S2, HeLa, MCF-7, and U937 were investigated. Both ME and MF showed potent antiproliferative effects, and ME induced apoptosis of HeLa cells by downregulation of Bel-2 and Bel-xL, and upregulation of Bax, leading to the initiation of mitochondria-apoptosome pathways. The effects of ME on normal human cells, human peripheral blood mononuclear cells (PBMCs) and human embryonic lung (HEL) fibroblasts were also investigated.

MATERIALS AND METHODS

**General Experimental Procedures** The NMR spectra were recorded on a Bruker ARX-300 NMR spectrometer (1H, 300 MHz; 13C, 75 MHz), using TMS as an internal standard. EI-MS were recorded on a Bruker ARX-300 NMR spectrometer. Analytical TLC was performed using silica gel plates (Kieselgel 60 F 254 precoated plates, Merck, Darmstadt, Germany). Semi-preparative HPLC was performed on an ODS C-18 column (9.8 mm × 250 mm, 5 μm, Phenomenex, Torrance, CA, U.S.A.) using a water-methanol system as a mobile phase, monitored with an RID detector. Some solvents were of analytical reagent or chromatographic reagent grade.

**Plant Material** The root barks of *U. pumila* were collected from Fuxin, Liaoning Province, China, in May 2000 and identified by Prof. Zheng Cui of Shenyang Pharmaceutical University, China. Fresh root barks were dried in a dark, well-ventilated place, and a voucher specimen has been deposited in the Department of Traditional China Materia Medica, Shenyang Pharmaceutical University.

**Extraction and Isolation** The air-dried root barks of *U. pumila* (10 kg) were milled and extracted three times with 80% ethanol under reflux. The ethanol extract was filtered and concentrated under reduced pressure. The crude product (1195 g) was successively partitioned with EtOAc and n-butanol. The EtOAc layer was concentrated under reduced pressure, and the residue (111.8 g) was subjected to silica gel column chromatography eluted with c-hexane and a c-hexane-acetone mixture with increasing proportions of acetone. The fractions were collected and combined by monitoring with analytical TLC to afford seven fractions (fractions 1—7) in order of increasing polarity.

Fraction 5 was loaded on a silica gel column using a CHCl3—MeOH mixture with increasing polarity and four fractions (fractions 5-1—4) were separated. Fraction 5-2 was recrystallized on a Sephadex LH-20 column (MeOH), followed by HPLC (85% aqueous MeOH) to give 31.2 mg of compound (I). Fraction 6 was recrystallized on a silica gel column using a CHCl3—MeOH mixture with increasing polarity, and seven fractions were produced (6-1—7). Fraction 6-5 was recrystallized on a Sephadex LH-20 column (MeOH), followed by semipreparative HPLC (80% aqueous MeOH) to give 26.7 mg of compound (II). The purity of both compounds was assessed by HPLC. ME and MF, orange needles (MeOH), mp 146—148 °C, EI-MS: m/z 242 [M]+. 1H-NMR (300 MHz, CDCl3): δ 1.37 (3H, d, J=7.1 Hz, 3-Me), 1.96 (3H, s, 9-Me), 2.65 (3H, s, 6-Me), 3.10 (1H, m, 3-H), 4.23 (1H, dd, J=10.7, 5.1 Hz, 2-H), 4.41 (1H, dd, J=10.7, 4.0 Hz, 2-H), 7.26 (1H, d, J=6.7 Hz, 5-H), 7.35 (1H, d, J=8.0 Hz, 4-H). 13C-NMR (75 MHz, CDCl3): δ 7.8 (9-Me), 17.6 (3-Me), 22.5 (6-Me), 31.4 (C-3), 71.5 (C-2), 116.4 (C-9), 127.0 (C-9b), 127.5 (C-6a), 132.6 (C-3), 134.9 (C-2), 136.9 (C-3a), 142.9 (C-6), 162.4 (C-3), 22.5 (6-Me), 31.4 (C-3), 71.5 (C-2), 116.4 (C-9), 127.0 (C-9b), 127.5 (C-6a), 132.6 (C-3), 134.9 (C-2), 136.9 (C-3a), 142.9 (C-6), 162.4 (C-3), 22.5 (6-Me), 31.4 (C-3), 71.5 (C-2), 116.4 (C-9), 127.0 (C-9b), 127.5 (C-6a), 132.6 (C-3), 134.9 (C-2), 136.9 (C-3a), 142.9 (C-6), 162.4

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(C-9a), 180.3 (C-8), 182.3 (C-7).

ME, violet needles (MeOH), mp 213—215 °C, EI-MS: m/z 240 [M]⁺. ¹H-NMR (300 MHz, CDCl₃): δ 1.99 (3H, s, 9-Me), 2.13 (3H, s, 3-Me), 2.72 (3H, s, 6-Me), 7.10 (1H, s, 2-H), 7.42 (1H, d, J=8.1 Hz, 5-H), 7.49 (1H, d, J=8.2 Hz, 4-H). ¹³C-NMR (75 MHz, CDCl₃): δ 7.7 (9-Me), 12.9 (3-Me), 23.1 (6-Me), 112.2 (C-3), 113.5 (C-9), 124.0 (C-9b), 126.3 (C-6a), 128.4 (C-4), 129.5 (C-3a), 136.4 (C-5), 140.4 (C-2), 146.6 (C-6), 161.7 (C-19a), 178.0 (C-8), 182.0 (C-7). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as measured at different time points in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were incubated with or without ME for 48 h. Cell growth was inhibited using 3,3-diaminobenzidine tetrahydrochloride as the HRP substrate.

**Drug Solutions** ME and MF were dissolved in DMSO to make stock solutions, then diluted in cell culture medium at different concentrations, and used immediately. The positive control was 5-fluorouracil (5-FU) dissolved in the same solution. In all assays, the final concentrations of DMSO in the culture medium were less than 0.01%.

**Cell Culture** The cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% fetal bovine serum (FBS) (Dalian Biological Reagent Factory, Dalian, China) and 0.03% L-glutamine (Gibco) at 37 °C in 5% CO₂. HEL fibroblast cells were maintained in Dulbecco’s modified Eagle’s medium (Gibco) with 10% FBS and cultured at 37 °C in 5% CO₂.

**Growth Inhibition Test** A375-S2 cells were seeded at 5×10⁴ cells/well in 96-well plates (Nunc, Roskilde, Denmark), and the another three tumor cell lines and HEL cells were seeded at 1×10⁴ cells/well in 96-well plates. After overnight incubation, different concentrations of ME and MF were added and incubated for 0, 12, 24, and 48 h separately. After preincubation with the caspase family inhibitor z-VAD-fmk, caspase 3 inhibitor z-DEVD-fmk, and caspase 9 inhibitor z-LEHD-fmk at given concentrations for 1 h, the cells were incubated with or without ME for 48h. Cell growth was measured at different time points in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described with a plate reader (Tecan, Austria). The antiproliferative effects of ME on HeLa cells were confirmed using the trypan blue (0.4%) dye exclusion test, and the inhibition ratio (%) was calculated using the following formula:

\[
\text{inhibition ratio} \%(\%) = \left(1 - \frac{\text{mean survival of treated group}}{\text{mean survival of control}}\right) \times 100\%
\]

**Observation of Morphologic Changes** HeLa cells in RPMI-1640 containing 10% FBS were seeded into 6-well culture plates (Nunc) and cultured overnight. ME (8 µM) was added to the cell culture and the cellular morphology was observed using phase-contrast microscopy (Leica, Braunschweig, Germany) at 48 h.

**Nuclear Damage Observed by Hoechst 33258 Staining** Apoptotic nuclear morphology was assessed using Hoechst 33258 as described previously. Cells were fixed with 3.7% paraformaldehyde at room temperature for 2 h, then washed and stained with Hoechst 33258 167 µM at 37 °C for 10 min. At the end of incubation, the cells were washed and resuspended in PBS for observation of nuclear morphology using a fluorescence microscope (Leica).

**Separation of Human PBMCs** Blood was obtained from three healthy adult volunteers. PBMCs were separated by density centrifugation in cell separation solution (Shanghai Biological Reagent Factory, Shanghai, China) and suspended in RPMI-1640 medium containing 2% human AB-type serum. PBMCs (5×10⁵ cells/well) were seeded into 96-well culture plates. Cell viability was measured using the MTT method.

**DNA Extraction and Detection of DNA Fragments** Adherent and nonadherent HeLa cells (1×10⁶ cells) were harvested and centrifuged at 1000×g for 10 min, and washed once with Ca²⁺- and Mg²⁺-free PBS. Cell pellets were lysed in 100 µl of cell lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM EDTA, pH 8.0, 0.5% Triton X-100) at 4 °C for 15 min, and centrifuged at 15000×g for 20 min. The supernatants were incubated with 40 µg·ml⁻¹ proteinase K and 40 µg·ml⁻¹ of RNase A at 37 °C for 2 h. The lysate was extracted with 0.5 M NaCl and 50% 2-propanol, incubated overnight at −20 °C, and centrifuged at 15000×g for 20 min. The pellets were suspended in TE buffer (10 mM Tris–HCl, pH 7.4, and 1 mM EDTA, pH 8.0). DNA was separated by 2% agarose gel electrophoresis at 100 V for 40 min and stained with 0.1 mg·l⁻¹ of ethidium bromide.

**Western Blotting** Immunoblotting of cell lysates was performed as previously described. Both adherent and nonadherent cells were harvested, washed twice with ice-cold PBS, and then lysed in lysis buffer (50 mM HEPES, pH 7.4, 1% Triton X-100, 2 mM sodium orthovanadate, 100 mM sodium fluoride, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, aprotinin 10 µg/ml, and leupeptin 10 µg/ml) for 60 min at 4 °C. The lysate was centrifuged at 15000×g for 10 min and the supernatant was used for Western blotting. Equivalent amounts of protein were separated by SDS-PAGE and wet-electrotransferred onto a nitrocellulose membrane, and equivalent loading was confirmed using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, U.S.A.). Proteins were detected with antibodies to Bcl-2, Bax, ICAD, caspase-3, and Bcl-xL (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) and visualized using 3,3-diaminobenzidine tetrahydrochloride as the HRP substrate.

**Statistical Analysis** IC₅₀ values were calculated by regression analysis of the concentration response data. Data of the representatives were analyzed for statistical significance with Student’s t-test. p values of less than 0.01 were considered to be significant.

**RESULTS**

**Antiproliferative Activities of ME and MF** ME and MF are sesquiterpene ortho-naphthoquinones (Fig. 1). The antiproliferative effects of ME, MF, and the positive control 5-FU were detected in the four human cell lines A375-S2, HeLa, MCF-7, and U937 in the MTT assay (Table 1). The IC₅₀ values of ME against the four tumor cell lines were 2.2,
7.9, 3.1, and 0.9 \text{mM}, respectively while those of MF were 13.3, 30.5, 29.4, and 3.0 \text{mM}, respectively, with 48-h drug treatment. As shown in Fig. 2, ME and MF inhibited HeLa and U937 cell growth in a time- and dose-dependent manner. The antiproliferative effects of ME on HeLa cells were also confirmed in trypan blue assays. After treatment with ME 0.064, 0.32, 1.6, 8, and 40 \text{mM} for 48 h, the inhibition ratios were 11.30\%, 19.77\%, 50.28\%, 76.27\%, and 100\%, respectively (Fig. 3).

After treatment for 48 h, ME 8 \text{mM} showed at least 40\% inhibitory effects on the four human tumor cell lines while it had no cytotoxic effect on PBMCs (Fig. 4). ME showed antiproliferative effects in HEL fibroblast cells, with an inhibitory rate of 41.5\% after treatment with ME 8 \text{mM} for 48 h (Fig. 5).

**Apoptosis in HeLa Cells Induced by ME** The inhibitory ratio of ME on HeLa cell growth was effectively increased to 7.7\%, 37.8\%, 53.7\%, and 75.5\% by ME 8 \text{mM} at 12, 24, 48, and 72 h, respectively. Therefore this concentration was used for the apoptosis assessment assay and DNA fragmentation analysis. The cells underwent marked morphologic changes such as becoming round in shape and apoptotic bodies were observed after treatment with ME 8 \text{mM}, compared with the untreated control (Fig. 6A). Hoeschst 33258-stained HeLa cells showed that the apoptotic cells had characteristic condensed nuclei (Fig. 6B). Treatment with ME 8 \text{mM} for 36 and 48 h resulted in typical DNA fragmentation on agarose electrophoresis which is a hallmark of apoptosis (Fig. 6C). These results indicate that ME induces apoptosis in HeLa cells.

### Table 1. Antiproliferative Effects of ME, MF, and 5-FU on U937, MCF-7, HeLa, and A375-S2 Cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (\text{mM})</th>
<th>U937</th>
<th>MCF-7</th>
<th>HeLa</th>
<th>A375-S2</th>
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<tr>
<td>ME</td>
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<td>8.64</td>
<td>0.90</td>
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<td>54.60</td>
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<td></td>
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<td>101.22</td>
<td>98.05</td>
<td>94.23</td>
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<tr>
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<td>2.60</td>
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<td>100.06</td>
<td>82.16</td>
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<tr>
<td>S-FU</td>
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<td>3.72</td>
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<tr>
<td></td>
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<td>0.81</td>
<td>3.32</td>
<td>5.73</td>
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<tr>
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<td>40.000</td>
<td>91.65</td>
<td>59.59</td>
<td>51.49</td>
<td>88.81</td>
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</table>

Cells (U937, MCF-7, HeLa, and A375-S2) were treated with various concentrations of ME, MF, and 5-FU for 48 h. Each value represents the mean of three independent measurements.

7.9, 3.1, and 0.9 \text{mM}, respectively while those of MF were 13.3, 30.5, 29.4, and 3.0 \text{mM}, respectively, with 48-h drug treatment. As shown in Fig. 2, ME and MF inhibited HeLa and U937 cell growth in a time- and dose-dependent manner. The antiproliferative effects of ME on HeLa cells were also confirmed in trypan blue assays. After treatment with ME 0.064, 0.32, 1.6, 8, and 40 \text{mM} for 48 h, the inhibition ratios were 11.30\%, 19.77\%, 50.28\%, 76.27\%, and 100\%, respectively (Fig. 3).

After treatment for 48 h, ME 8 \text{mM} showed at least 40\% inhibitory effects on the four human tumor cell lines while it had no cytotoxic effect on PBMCs (Fig. 4). ME showed antiproliferative effects in HEL fibroblast cells, with an inhibitory rate of 41.5\% after treatment with ME 8 \text{mM} for 48 h (Fig. 5).

**Caspase Involvement in ME-Induced HeLa Cell Death** The pan-caspase inhibitor z-VAD-fmk, caspase-3 inhibitor z-DEVD-fmk, and caspase-9 inhibitor z-LEHD-fmk effectively inhibited ME 8 \text{mM}-induced HeLa cell death (Fig. 7). Activation of caspase 3 was measured by its conversion from a 32-kDa zymogen to active forms of 17 and 20 kDa.
using Western blotting. As shown in Fig. 8, procaspase-3 was proteolytically activated after treated with ME 8 μM for 36 h, and cleaved fragments were detected at the same time. ICAD, the inhibitor of a caspase-dependent DNase, is a caspase-3 substrate that is cleaved to the inactivated form and allows the DNase to execute the characteristic fragmentation of DNA. ICADs are expressed as two isoforms, 45 kDa (ICAD-L/DFF45) and 35 kDa (ICAD-S/DFF35), and only ICAD-L/DFF45 was reported to be functional. Thus the ICAD-L/DFF45 expression in HeLa cells was examined to confirm further the activation of caspase-3. After exposure to ME 8 μM for 24 h, ICAD-L expression began to decline (Fig. 8). Pretreatment with z-DEVD-fmk blocked the degradation of ICAD. Taken together, these results suggest that the activation of caspase cascades occurs in HeLa cells after exposure to ME 8 μM.

Involvement of Bcl-2, Bcl-XL, and Bax in ME-Induced Apoptosis Since Bcl-2 family members are critical regulators of the mitochondrial pathway, we assessed the expression of the Bcl-2 family proteins Bcl-2, Bcl-XL, and Bax. As shown in Fig. 9, after 24-h treatment with ME 8 μM, expression of Bcl-XL and Bcl-2 began to decrease, while that of Bax began to increase.

DISCUSSION

The inhibitory effects of ME and MF on the proliferation of human HeLa, A375-S2, U937, and MCF-7 cells were investigated in the MTT assay. ME and MF have cytotoxic activities against the four human tumor cell lines, and U937 is the most sensitive cell line to these compounds. MF has similar antiproliferative activity compared with 5-FU, but ME was more potent. MF has a \( \Delta^{2,3} \) structure, but C-2 and C-3 of ME are \( sp^3 \) carbons, and therefore MF has a plane structure and ME is a stereo molecule. This structural discrepancy may result in the difference in bioactivity. It is possible that the antiproliferative effect of the stereo structure was more potent than the plane structure in this type of compound. Johnson Inbaraj and colleagues used the method of counting cell colonies to calculate the cytotoxic effects of mansonone.
D (MD) and H (MH) on MCF-7 cells, and the LD$_{50}$ of MD and MH were 0.14 and more than 10 μM, respectively. The IC$_{50}$ values of ME and MF against MCF-7 cells are 3.1 and 29.4 μM, and these results suggest that MD has the strongest cytotoxic effect on MCF-7 cells among the four similar compounds. ME 8 μM had almost no cytotoxic effects on PBMCs, while it showed more than 40% antiproliferative activity on four human tumor cell lines and HEL fibroblast cells. These results indicate that the antiproliferative effect of ME has selectivity to different human tumor or normal cells.

Based on the morphologic changes in HeLa cells and DNA fragmentation, we conclude that ME induced HeLa cell death through apoptotic pathways.

Caspases are a family of proteins that play an essential role in the apoptotic process. They are activated either by death receptors such as the Fas and tumor necrosis factor α...
receptors or by molecules released from mitochondria. Caspase-8 and -10 are initiator caspases. Caspase-8 and -10 are activated by the death receptors, and caspase-9 is activated by the mitochondrial pathway. When they are activated, they initiate a cascade of executioner caspases, including caspase-3 and others. Unleashing of executioner caspases results in cleavage of a number of caspase substrates responsible for the apoptotic demise of cells. The results in this study showed that procaspase-3 was cleaved at 36 h, followed by the degradation of the caspase-3 substrate ICAD, and DNA was fragmented at a later stage. Pretreatment with z-VAD-fmk, z-DEVD-fmk, or z-LEHD-fmk effectively inhibited ME 8 µM-induced cell death, and the caspase-3 inhibitor attenuated the degradation of ICAD. All these suggest that the caspase-3 cascade plays a critical role in ME-mediated HeLa cell apoptosis, especially caspase-3 and -9 which are thought to take part in the mitochondrial pathway.

A balance between members of the Bcl-2 family is thought to determine whether mitochondria remain intact or become permeabilized and release proteins that promote cell death. Activation of proapoptotic proteins such as Bax or Bak results in their oligomerization at the outer mitochondrial membrane and causes the release of a variety of proapoptotic molecules, including cytochrome c, from the inter mitochondrial membrane space into the cytosol. Released cytochrome c then binds to Apaf-1 and forms the apoptosome, which proteolytically activates procaspase-9 and in turn activates the effector caspases and initiates terminal events that overlap with those initiated by death receptors. Bcl-xL binds to the CED-4-like portion of Apaf-1 and inhibits the association of Apaf-1 with procaspase-9 and thereby prevents caspase-9 activation.

In this study, we found that 24 h after ME treatment the expression of the antiapoptotic proteins Bcl-xL and Bel-2 began to decrease and that of the proapoptotic protein Bax began to increase at the same time, followed by the degradation of procaspase-3 and the caspase-3 substrate. The caspase-9 inhibitor attenuated ME-induced HeLa cell death. Thus it is possible that in ME-induced HeLa cell apoptosis occurs by the downregulation of Bcl-xL and Bel-2 and activation of Bax, initiating the mitochondria-apoptosome pathways.

ME, an active component of U. pumila promotes tumor cell death by modulating the balance of Bel-2 family proteins and signals to apoptotic effector molecules (caspases), which subsequently cleave key cellular proteins to generate the apoptotic morphology. But the antiproliferative activity of ME cannot be entirely blocked by caspase inhibitors; therefore other pathways might participate in inducing apoptosis in human tumor cells. The mechanism of tumor cell apoptosis induced by ME remains to be further elucidated.

REFERENCES


Fig. 8. Involvement of Caspase-3 in ME-Induced HeLa Cell Death
(A) Activation of caspase-3 and ICAD. The cells were treated with ME 8 µM for the indicated times. Caspase-3 and ICAD were analyzed by Western blotting. (B) The caspase-3 inhibitor z-DEVD-fmk attenuated degradation of ICAD. HeLa cells were cultured with or without caspase-3 inhibitor for 1 h prior to the addition of ME 8 µM, then further incubated for 48 h. Caspase-3 and ICAD were analyzed by Western blotting. β-Actin was used as an equal loading control.

Fig. 9. Expression of BCL-xL, Bax, and Bel-2 in ME-Treated HeLa Cells
The cells were treated with ME 8 µM for the indicated time periods. BCL-xL, Bax, and Bel-2 were analyzed by Western blotting. β-Actin was used as an equal loading control.