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Acetoxycycloheximide (E-73) rapidly induces apoptosis mediated by the release of cytochrome *c* via activation of c-Jun N-terminal kinase

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Abstract

Cycloheximide (CHX) is an inhibitor of protein synthesis and commonly used to modulate death receptor-mediated apoptosis or to induce apoptosis in a number of normal and transformed cells. In this study we show that a close structural derivative of CHX, acetoxycycloheximide (E-73) induced rapid processing of procaspases and subsequent apoptosis with much higher efficacy than CHX in human leukemia Jurkat T cells. E-73 induced the release of cytochrome *c* from mitochondria even in the presence of the caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone. The Bcl-2 family protein Bcl-x_L suppressed cytochrome *c* release as well as processing of procaspases-3, -8, and -9 in E-73-treated cells. In Jurkat T cells transfected with the caspase-8 modulator $FLIP_L$, E-73 still induced activation of procaspase-3 and subsequent apoptosis, suggesting that the caspase-8 activity is dispensable for apoptosis. In contrast to CHX, E-73 drastically induced activation of extracellular signal-regulated kinase, c-Jun N-terminal kinase (JNK), and p38 MAP kinase. Inhibitory profiles of small-molecular kinase inhibitors revealed that JNK activation was critical for induction of cytochrome *c* release in E-73-induced apoptosis. Thus, our present results demonstrate that E-73, unlike CHX, induces strong activation of the JNK pathway and triggers rapid apoptosis mediated by the release of cytochrome *c*.

Keywords: Acetoxycycloheximide; Apoptosis; c-Jun N-terminal kinase; Cycloheximide; Cytochrome *c*; E-73

1. Introduction

Apoptosis is a physiological process that plays an important role during development and in the homeostasis of mature tissues. Caspases are a family of cysteine proteinases essential for apoptosis by cleaving selected substrates [1]. The initiator procaspase-8 is activated by self-processing in the death-inducing signaling complex containing the adaptor protein FADD upon stimulation with death receptors [2,3], whereas the initiator procaspase-9 is activated by self-processing in the apoptosome which is formed following release of cytochrome *c* from mitochondria [4]. Cytochrome *c* is normally confined to the intermembrane space between inner and outer membranes, and released into the cytosol upon multiple apoptotic signals [4,5]. Active caspases-8 and -9 cleave and activate executioner caspases such as procaspase-3.

In mammals, three major groups of MAP kinases, i.e. extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase, are activated by protein kinase cascades in response to growth factors, cytokines, and environmental stresses, and play an important role in cellular responses including proliferation, differentiation and cell death [6]. The JNK pathway is essential

Abbreviations: CHX, cycloheximide; ERK, extracellular signal-regulated kinase; FasL, Fas ligand; JNK, c-Jun N-terminal kinase; MCA, 4-methyl-coumaryl-7-amide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluromethylketone

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to regulate apoptosis in response to various chemical and physical stresses [6]. JNK is required for the release of cytochrome *c* from mitochondria in stress-induced apoptosis [7–9]. However, it has been also shown that prolonged JNK activation induces Fas ligand (FasL) upregulation and FADD-dependent apoptosis [10–12]. Thus, JNK activation can induce both the cytochrome *c*/caspase-9-mediated pathway and the FADD/caspase-8-mediated pathway.

Acetoxycycloheximide (initially termed as E-73; see Fig. 1A) was originally isolated from the culture filtrates of *Streptomyces albulus* as an antitumor substance [13,14] and its stereochemistry was determined later [15,16]. The initial report showed that E-73 exhibits in vivo antitumor activity 200–400 times effectively than cycloheximide (CHX) [13]. However, the molecular basis of the antitumor activity of E-73 has remained unclear. CHX has been frequently used to sensitize cells to death receptor-mediated apoptosis largely by preventing constitutive expression or NF-κB-inducible expression of the caspase-8 modulator c-FLIP [17–19]. Moreover, CHX has been reported to induce apoptosis in a number of cell types including neutrophils and leukemia cell lines [20–22].

In our screening program for small-molecular compounds that target the NF-κB signaling pathway induced by proinflammatory cytokines, we found an inhibitory activity in the culture broth of an unidentified actinomycete strain, and isolated E-73 as an active compound [23]. In contrast to CHX, E-73 blocked signaling events upstream of IκB degradation in the NF-κB signaling pathway induced by TNF-α [23]. Unexpectedly, when E-73 was tested for its biological activity on various in vitro cultures of human tumor cell lines, we noticed that human leukemia cell lines such as Jurkat T cells were extremely sensitive to E-73. Jurkat T cells underwent apoptosis shortly after exposure to E-73 at concentrations 100-fold lower than CHX. In the present paper, we have studied the molecular mechanism of E-73-induced apoptosis in Jurkat T cells, and demonstrate that E-73 strongly induces activation of the JNK pathway and thereby triggers apoptosis via the release of cytochrome *c*.

2. Materials and methods

2.1. Cells

Human leukemia Jurkat T cells and the $FLIP_L$-transfected Jurkat T cell clone (JFL2) [24] were maintained in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (JRH Bioscience, Lenexa, KS, USA) and penicillin/streptomycin/neomycin antibiotic mixture (Invitrogen).

2.2. Reagents

E-73 was purified from the culture broth of an unidentified actinomycete strain designated ML44-113F2 as pre-

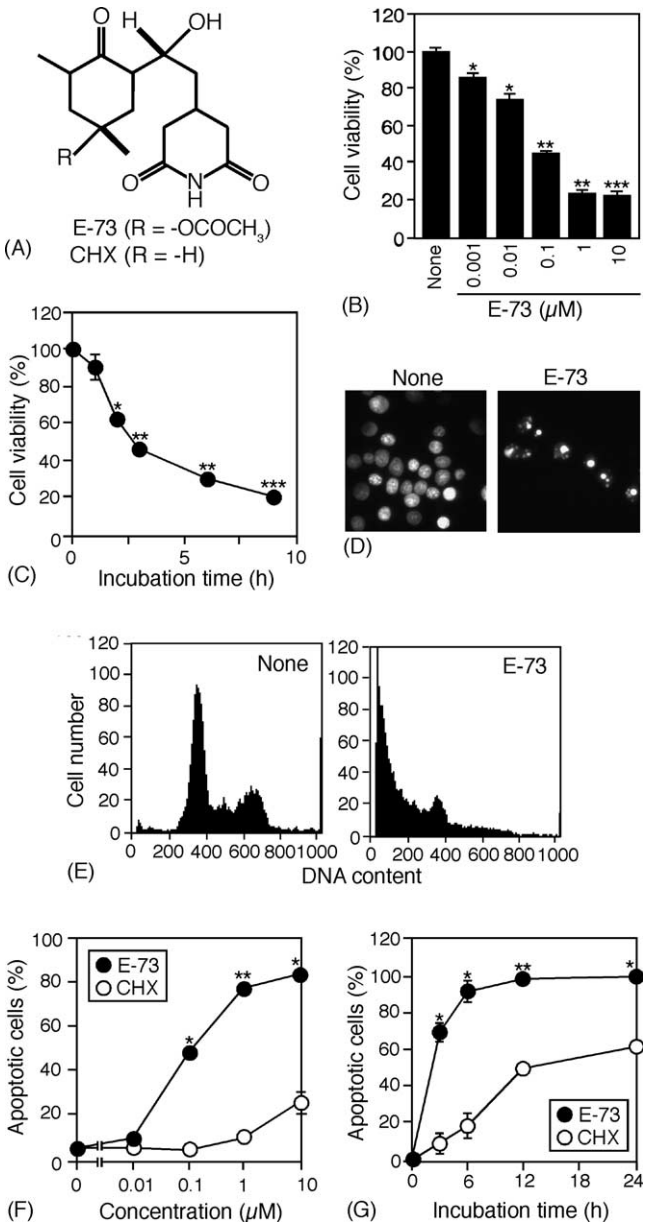

Fig. 1. E-73 rapidly induces nuclear condensation and DNA fragmentation characteristic of apoptosis. (A) Structures of E-73 and CHX. (B) Jurkat T cells were incubated with serial dilutions of E-73 for 24 h. Cell viability (%) was measured by MTT assay. The result represents the mean ± S.D. of triplicate cultures. $^{*}P < 0.01$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ relative to non-treated cells. (C) Jurkat T cells were incubated with 1 μM E-73 for the indicated times. Cell viability (%) was measured by MTT assay. Data points represent the mean ± S.D. of triplicate cultures. $^{*}P < 0.01$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ relative to non-treated cells. (D and E) Jurkat T cells were incubated with or without 1 μM E-73 for 3 h. Nuclei were visualized with Hoechst 33342 staining and observed under fluorescent microscopy (D). Nuclei were stained with propidium iodide, and DNA content was analyzed under flow cytometry (E). (F) Jurkat T cells were treated with serial dilutions of E-73 (filled circles) or CHX (open circles) for 3 h. Apoptotic cells (%) were measured by Hoechst 33342 staining. Data points represent the mean ± S.D. of triplicate determinations. $^{*}P < 0.01$ and $^{**}P < 0.001$ relative to cells treated with the same concentrations of CHX. (G) Jurkat T cells were treated with 1 μM E-73 (filled circles) or 1 μM CHX (open circles) for the indicated times. Apoptotic cells (%) were measured by Hoechst 33342 staining. Data points represent the mean ± S.D. of triplicate determinations. $^{*}P < 0.01$ and $^{**}P < 0.001$ relative to cells treated with CHX for the same incubation times.

viously described [23]. Benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVAD-fmk) was purchased from Peptide Institute Inc. (Osaka, Japan). Puromycin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Curcumin, CHX, etoposide, and PD98059 were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). SB203580 was purchased from Alexis Co. (San Diego, CA, USA). SP600125 was purchased from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA).

2.3. Cell transfection

Jurkat T cells were diluted one day before transfection. Cells were washed three times with fetal calf serum-free RPMI 1640 medium, and suspended in the same medium. To establish stable transfectants, Jurkat T cells were transfected with either pEFpGKpuro expression vector [25] or FLAG-Bcl-x_L-pEFpGKpuro [25] by the Gene Pulser Xcell electroporation system (Bio-Rad Laboratories, Hercules, CA, USA). The cells were cultured for 48 h without selection and then cultured in 96-well microtiter plates in the presence of puromycin (5 μg/ml). Puromycin-resistant clones expressing FLAG-Bcl-x_L were selected by Western blotting using anti-FLAG antibody.

2.4. Assays for cell viability and apoptosis

To measure cell viability, cells were pulsed with 500 μg/ml of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) for 4 h in 96-well microtiter plates, and MTT-formazan was then solubilized with 5% SDS overnight. Absorbance at 595 nm was measured by the Model 680 microplate reader (Bio-Rad). Cell viability (%) was calculated as (experimental absorbance − background absorbance)/(control absorbance − background absorbance) × 100. To measure nuclear condensation, cells were fixed with 4% paraformaldehyde/PBS, and then stained with 300 μM Hoechst 33342 (Calbiochem, San Diego, CA, USA). Nuclear morphology was observed under fluorescent microscopy (Axiovert 200 M; Carl Zeiss, Jena, Germany). Apoptotic cells (%) were calculated as (condensed nuclei/total nuclei) × 100. To measure DNA fragmentation, cells were treated with propidium iodide (50 μg/ml) in 0.1% Triton X-100/PBS. DNA content was then analyzed by the FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.5. Western blotting

Cells were washed with PBS and lysed in the lysis buffer containing 50 mM Tris–HCl (pH 7.4), 1% Triton X-100, 2 mM DTT, 2 mM sodium orthovanadate, and the protease inhibitor mixture Complete (Roche Diagnostics, Mannheim, Germany). Equal amounts of cell lysates (30 μg/lane) were separated by SDS-PAGE and analyzed by Western blotting using ECL detection reagents (Amersham Biosciences, Piscataway, NJ, USA). Antibodies to caspase-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), caspase-8 (Medical and Biological Laboratories (MBL), Co. Ltd., Nagoya, Japan), caspase-9 (MBL), cytochrome *c* (BD Biosciences), FLAG (Sigma), and c-FLIP (Alexis) were commercially obtained. Antibodies to ERK, phospho-ERK, HSP27, phospho-HSP27, JNK, phospho-JNK, c-Jun, phospho-c-Jun (Ser-63), p38 MAP kinase and phospho-p38 MAP kinase were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.6. Cytochrome c release

Cells were washed with PBS, and lyzed in the lysis buffer containing 10 mM HEPES-KOH (pH 7.2), 100 μM digitonin, 250 mM sucrose, 1 mM DTT, 5 mM EGTA, 2 mM $MgCl_2$, 50 mM NaCl, and the protease inhibitor mixture. Cytosolic fractions were separated by centrifugation (10,000 × *g*, 10 min) and detected for cytochrome *c* by Western blotting.

2.7. Assay for caspase activity

Cell lysates (30 μg) were mixed with caspase substrates in the reaction buffer (50 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 10% sucrose, 1 mM DTT) for 1 h. Ac-DEVD-4-methylcoumaryl-7-amide (MCA) for the caspase-3 substrate, Ac-IETD-MCA for the caspase-8 substrate, and Ac-LEHD-MCA for the caspase-9 substrate were obtained from the Peptide Institute Inc. To block caspase-8-independent cleavage of Ac-IETD-MCA, the proteasome inhibitor MG-132 (Peptide Institute Inc.) was included in the reaction mixture at the final concentration of 2.5 μM [26]. Fluorescent intensity of 7-amino-4-methylcourmarin with an excitation at 360 nm and an emission at 460 nm was measured by the Cytofluor 4000 series multi-well plate reader (Applied Biosystems, Foster City, CA, USA).

2.8. Assay for protein synthesis

Cells were pulsed with [4,5-^{3}H] leucine (Moravek Biochemical Corp., Brea, CA, USA) for 2 h in 96-well microtiter plates (9.25 kBq/well, 100 μl) and harvested on grass filters. Radioactivity incorporated into the cell was measured by the LS6500 liquid scintillation counter (Beckman Coulter, Inc., Palo Alto, CA, USA).

2.9. Statistical analysis

Student's *t* test was used to analyze statistical difference. *P* values less than 0.05 were considered significant.

3. Results

3.1. E-73 rapidly induces nuclear condensation and DNA fragmentation characteristic of apoptosis

Treatment of Jurkat T cells with E-73 resulted in a dose-dependent reduction of cell viability as judged by the MTT assay (Fig. 1B). Jurkat T cell viability decreased shortly after exposure to E-73 (Fig. 1C), indicating that the reduction in cell viability was not due to cell cycle arrest. To examine whether E-73 treatment induces apoptotic or necrotic cell death, nuclear morphological changes were assayed via fluorescent microscopy using Hoechst 33342 staining. In contrast to normal nuclear morphology in non-treated Jurkat T cells, E-73-treated cells displayed obvious characteristics of apoptosis including nuclear condensation (Fig. 1D) and DNA fragmentation (Fig. 1E). Furthermore, apoptosis was measured by the quantitative analysis of cells harboring normal and condensed nuclei after Hoechst 33342 staining. E-73 induced apoptosis in Jurkat T cells in a dose-dependent manner with higher efficiency (over 100-fold) than CHX and at early time points (Fig. 1F). Moreover, E-73 induced apoptosis much faster than CHX when Jurkat T cells were exposed to the same dose of E-73 or CHX (Fig. 1G).

It has been shown that E-73 inhibits protein synthesis 10–20 times more effectively than CHX in intact cells and a cell-free system [27,28]. We similarly observed that E-73 blocked protein synthesis in Jurkat T cells with 10-fold greater efficiency than CHX (Fig. 2A). In the presence of the broad-spectrum caspase inhibitor zVAD-fmk, E-73 was still able to inhibit protein synthesis 10-fold stronger than CHX (Fig. 2B), suggesting that apoptosis does not induce inhibition of protein synthesis. As shown in Figs. 1F and 2A, CHX induced only 5–20% apoptosis in Jurkat T cells under concentrations (1–10 μM) where CHX reduced protein synthesis by more than 80–90%. These observations also suggest that inhibiting protein synthesis does not induce rapid apoptosis in Jurkat T cells.

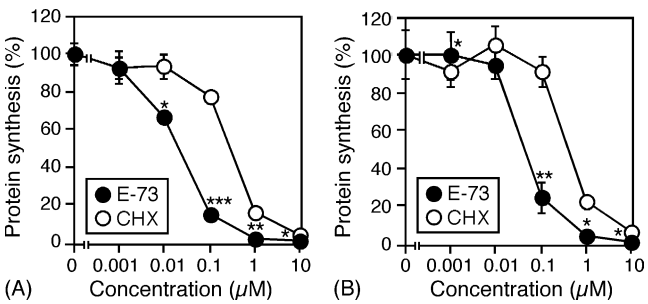

Fig. 2. E-73 inhibits de novo protein synthesis with 10-fold greater efficacy with CHX. (A) Jurkat T cells were incubated with serial dilutions of E-73 (filled circles) or CHX (open circles) for 1 h, and pulsed with [^{3}H] leucine for 2 h. Radioactivity incorporated into the cell was measured. Data points represent the mean ± S.D. of triplicate cultures. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ relative to cells treated with the same concentrations of CHX. (B) Jurkat T cells were preincubated with 50 μM zVAD-fmk for 1 h. Cells were then incubated with serial dilutions of E-73 (filled circles) or CHX (open circles) for 1 h, and pulsed with [^{3}H] leucine for 2 h. Radioactivity incorporated into the cell was measured. Data points represent the mean ± S.D. of triplicate cultures. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ relative to cells treated with the same concentrations of CHX.

3.2. E-73 rapidly induces activation of caspases essential for apoptosis

Activation of the caspase cascade is central for execution of apoptosis. Two initiator caspases (procaspases-8 and -9) are activated via autoprocessing and cleave downstream executioner caspases such as procaspase-3 [1]. To investigate whether E-73 induces activation of these caspases, Jurkat T cells were treated with different doses of E-73 or CHX, and processing of procaspases-3, -8, and -9 was monitored by Western blotting. At concentrations more than 0.1 μM, E-73 induced processing of procaspase-3 (32 kDa) into active forms (17 and 12 kDa) (Fig. 3A), processing of procaspase-9 (43 kDa) into active forms (37 and 35 kDa) (Fig. 3B) and processing of procaspase-8 (55 and 53 kDa) into caspase-8(p43) (43 and 41 kDa) (Fig. 3C) within 2 h. In contrast, CHX only marginally induced processing of these caspases even at high concentrations (10 μM) (Fig. 3A–C). Pretreatment of Jurkat T cells with zVAD-fmk prevented apoptosis induced by E-73 in a dose-dependent manner (Fig. 3D). The enzymatic activity of caspases-3, -8, and -9 induced by E-73 was almost completely suppressed by zVAD-fmk (Fig. 3E). Moreover, E-73-induced processing of procaspase-3 into active forms was markedly blocked when Jurkat T cells were pretreated with zVAD-fmk (Fig. 3F). These data clearly indicate that rapid activation of caspases is essential for E-73-induced apoptosis.

3.3. E-73 induces the release of cytochrome c into the cytosol independently of caspase activation

The release of cytochrome *c* from mitochondria into the cytosol is central for induction of the intrinsic apoptotic pathway [4,5]. Treatment with E-73, but not CHX, induced redistribution of cytochrome *c* from mitochondria to the cytosol at more than 0.1 μM (Fig. 4A) and within 1 h (Fig. 4B). The release of cytochrome *c* in E-73-treated cells still proceeded in the presence of zVAD-fmk (Fig. 4B), although the enzymatic activity of caspases-3, -8, and -9 was almost completely abrogated by zVAD-fmk (Fig. 3E). By contrast, zVAD-fmk markedly inhibited cytochrome *c* release induced by cross-linked FasL in Jurkat T cells (Fig. 4C). Thus, these data suggest that E-73 induces the release of cytochrome *c* independently of caspase activation.

3.4. Increased expression of Bcl-x_L prevents cytochrome c release and activation of caspases-3, -8, and -9 induced by E-73

The Bcl-2 family protein Bcl-x_L is able to block the release of cytochrome *c* from mitochondria in response to

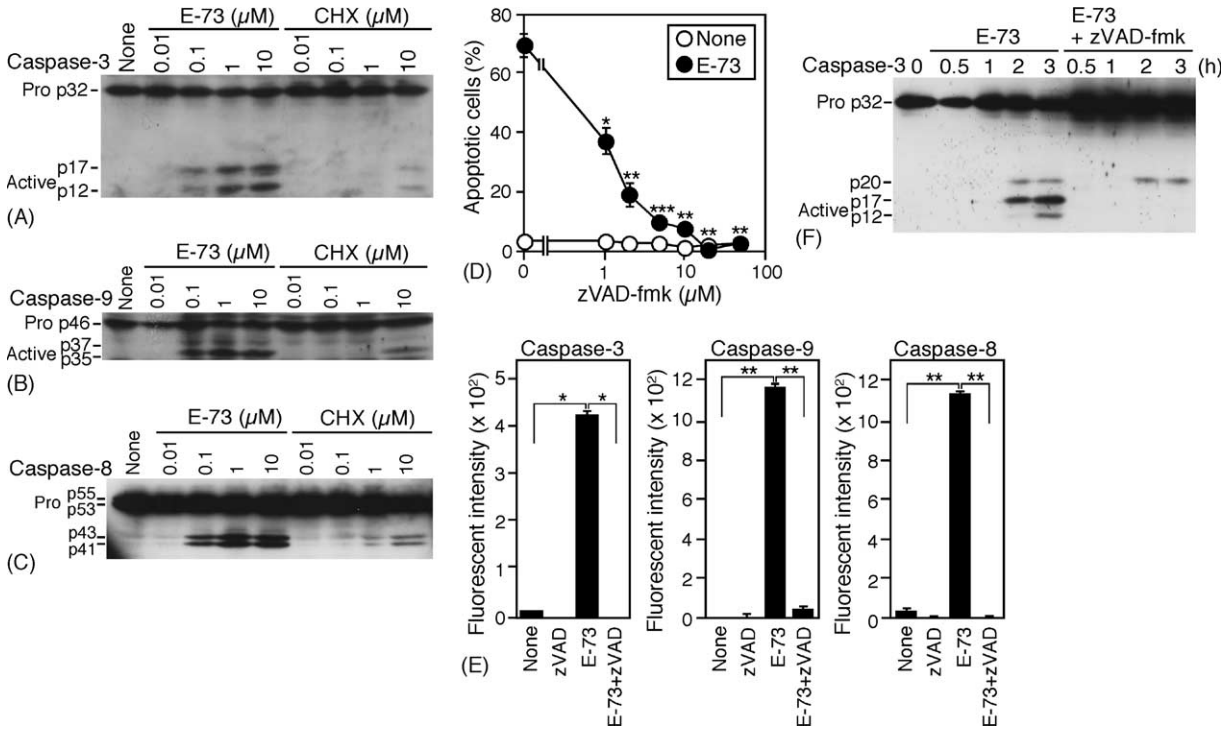

Fig. 3. Caspase activation is essential for apoptosis induced by E-73. (A–C) Jurkat T cells were treated with various concentrations of E-73 and CHX for 2 h. Processing of procaspase-3 (A), procaspase-9 (B), and procaspase-8 (C) was analyzed by Western blotting. (D) Jurkat T cells were pretreated with serial dilutions of zVAD-fmk for 1 h, and then treated with (filled circles) or without (open circles) 1 μM E-73 for 3 h. Apoptotic cells (%) were measured by Hoechst 33342 staining. Data points represent the mean ± S.D. of triplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ relative to non-treated cells. (E) Jurkat T cells were pretreated with or without 50 μM zVAD-fmk for 1 h, and then incubated for 2 h in the presence or the absence of 1 μM E-73. Cell lysates were measured for the proteolytic activity using specific caspase substrates. Fluorescent intensity (arbitrary units) is shown as the mean ± S.D. of triplicate determinations. $^{*}P < 0.001$ and $^{**}P < 0.0001$. (F) Jurkat T cells were pretreated with or without 50 μM zVAD-fmk for 1 h, and then treated with 1 μM E-73 for the indicated times. Processing of procaspase-3 was analyzed by Western blotting.

various stimuli [29,30]. To address whether the mitochondrial apoptotic machinery is required for apoptosis induction in E-73-treated cells, Jurkat T cells expressing increased levels of Bcl-x_L were established. Two independent transfectants expressing different levels of Bcl-x_L were selected and used for further experiments (Fig. 5A). Treatment with etoposide (10–100 μM) led to extensive apoptosis in wild-type and control vector-transfected Jurkat T cells, whereas these Bcl-x_L transfectants were resistant to etoposide (Fig. 5B). E-73 induced apoptosis at more than 0.1 μM in wild-type and control vector-transfected cells, whereas it barely induced apoptosis in Bcl-x_L-transfected cells even at 10 μM (Fig. 5C and D). Consistent with these observations, enhanced expression of Bcl-x_L completely prevented processing of procaspase-3 (Fig. 5E), procaspase-9 (Fig. 5F), and procaspase-8 (Fig. 5G). Moreover, the cytochrome *c* release into the cytosol induced by E-73 completely diminished in the Bcl-x_L-transfected Jurkat T cells (Fig. 5H).

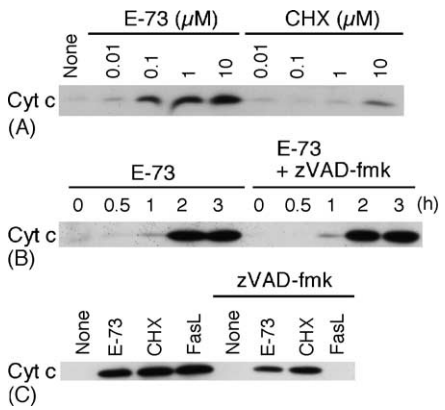

Fig. 4. E-73 induces the release of cytochrome *c* from mitochondria independently of caspase activation. (A–C) Jurkat T cells were incubated with various concentrations of E-73 or CHX for 2 h (A). Jurkat T cells were pretreated with or without 50 μM zVAD-fmk for 1 h, and then treated with 1 μM E-73 for the indicated times (B). Jurkat T cells were pretreated with or without 50 μM zVAD-fmk for 1 h, and then treated with E-73 (1 μM), CHX (50 μM) or cross-linked FasL (200 ng/ml) or left untreated (C). Cytoplasmic fractions were analyzed by Western blotting using anti-cytochrome *c* antibody.

3.5. Increased expression of $FLIP_L$ does not prevent apoptosis in E-73-treated Jurkat T cells

The caspase-8 modulator $FLIP_L$ can bind to procaspase-8 and inhibit conversion of procaspase-8 into its active form [24,31]. Overexpression of $FLIP_L$ protects Jurkat T cells from FasL-induced apoptosis [24]. Although the Jurkat T cell clone transfected with $FLIP_L$ (JFL2) was more resistant to apoptosis induced by FasL (Fig. 6A), JFL2 underwent apoptosis upon treatment with E-73 as

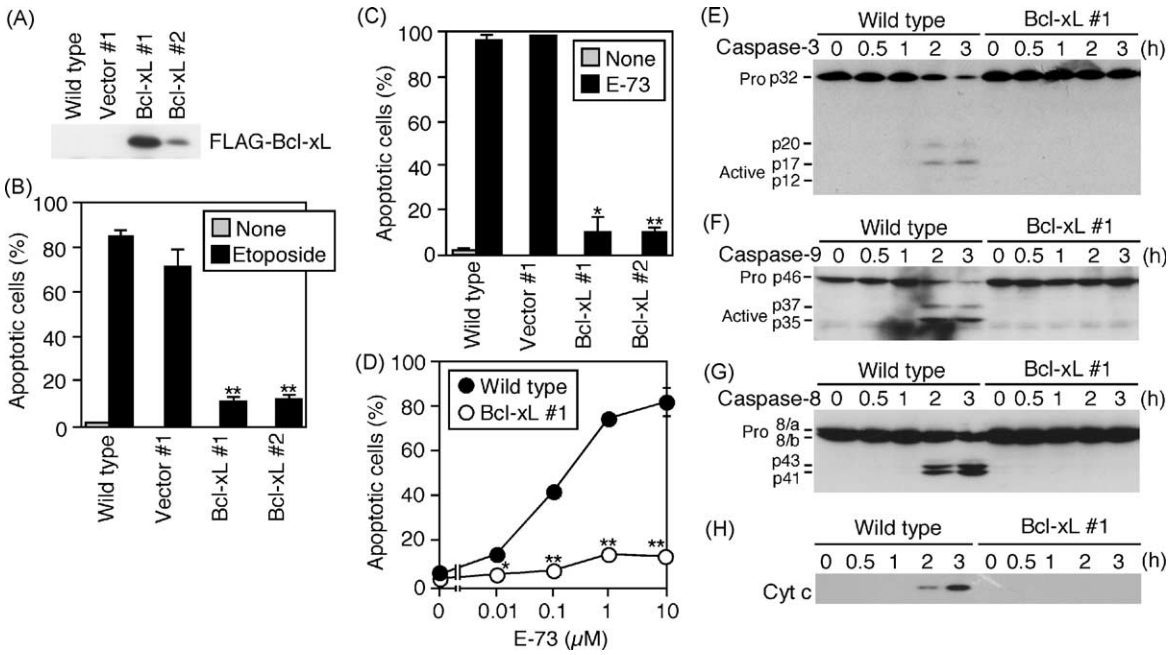

Fig. 5. Increased expression of Bcl-x_L prevents cytochrome *c* release as well as caspase activation upon exposure to E-73. (A) Expression of FLAG-Bcl-x_L in wild-type, control vector-transfected (Vector #1), and two independently derived Bcl-x_L-transfected (Bcl-x_L #1 and #2) Jurkat T cells was detected by Western blotting using anti-FLAG antibody. (B and C) Wild-type, control vector-transfected (Vector #1), and the Bcl-x_L-transfected (Bcl-x_L #1 and Bcl-x_L #2) Jurkat T cells were treated with (black bars) or without (gray bars) 10 μM etoposide (B) or 1 μM E-73 (C) for 12 h. Apoptotic cells (%) were measured by Hoechst 33342 staining. The result represents the mean ± S.D. of triplicate determinations. $^{*}P < 0.01$ and $^{**}P < 0.001$ relative to wild-type cells treated with etoposide or E-73. (D) Wild-type Jurkat T cells (open circles) and the Bcl-x_L #1 transfectant (filled circles) were treated with various concentrations of 1 μM E-73 for 3 h. Apoptotic cells (%) were measured by Hoechst 33342 staining. Data points represent the mean ± S.D. of triplicate determinations. $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to wild-type cells with the same concentrations of E-73. (E–G) Wild-type Jurkat T cells and the Bcl-x_L #1 transfectant were treated with 1 μM E-73 for the indicated times. Processing of procaspase-3 (E), procaspase-9 (F), and procaspase-8 (G) was analyzed by Western blotting. (H) Jurkat T cells and the Bcl-x_L #1 transfectant were incubated with 1 μM E-73 for the indicated times. Cytoplasmic fractions were analyzed by Western blotting using anti-cytochrome *c* antibody.

efficiently as wild-type Jurkat T cells (Fig. 6B). Consistent with this observation, procaspase-3 was processed into its active forms in the $FLIP_L$ transfectant JFL2 and wild-type Jurkat T cells (Fig. 6C). The cellular level of transfected $FLIP_L$ was only marginally decreased by E-73 within 3 h in the presence of zVAD-fmk (Fig. 6D), excluding the possibility that transfected $FLIP_L$ is downregulated and therefore at levels insufficient to block activation of procaspase-8. Although FasL-induced processing of procaspase-8 into active caspase-8(p18) was profoundly reduced in the $FLIP_L$ transfectant JFL2, active caspase-8(p18) was barely detectable in E-73-treated cells regardless of the presence of transfected $FLIP_L$ (Fig. 6E). Consistent with this observation, the enzymatic activity of caspase-8 induced by E-73 was significantly lower than that induced by FasL (Fig. 6F). Thus, in contrast to Fas stimulation, procaspase-8 might be processed into caspase-8(p43) but inefficiently converted into fully active forms in E-73-treated cells.

3.6. Activation of the JNK signaling pathway is essential for cytochrome c release in E-73-treated Jurkat T cells

The JNK pathway plays a pivotal role in stress responses and induces apoptosis in response to various stimuli [6–12]. Jurkat T cells were treated with E-73 or CHX, and activation of the MAP kinase superfamily was analyzed by Western blotting using antibodies specific to phosphorylated MAP kinases. Even at low concentrations (10–100 nM), E-73 dramatically induced activation of ERK, JNK and p38 MAP kinase (Fig. 7A). Phosphorylation of JNK was detected at 15 min and was sustained for at least 3 h in E-73-treated cells in a similar fashion to JNK activation induced by the protein synthesis inhibitor anisomycin (Fig. 7B). In contrast, CHX-induced JNK activation was weak and occurred only transiently (Fig. 7A and B). E-73 treatment also induced sustained phosphorylation (15 min–3 h) of ERK and p38 MAPK (Fig. 7C).

To clarify which kinase pathway is essential for E-73-induced apoptosis, Jurkat T cells were pretreated with membrane-permeable small-molecular inhibitors for ERK, JNK, and p38 MAP kinase. E-73-induced apoptosis was markedly suppressed by the JNK inhibitor SP600125 [32] and curcumin that inhibits the JNK pathway [33], whereas the MEK inhibitor PD98059 and the p38 MAP kinase inhibitor SB203580 had no effect (Fig. 8A). SP600125 blocked apoptosis induced by E-73 at a concentration of 25 μM, although this compound formed visible crystals at more than 50 μM in the culture medium (data not shown). The release of cytochrome *c* induced by

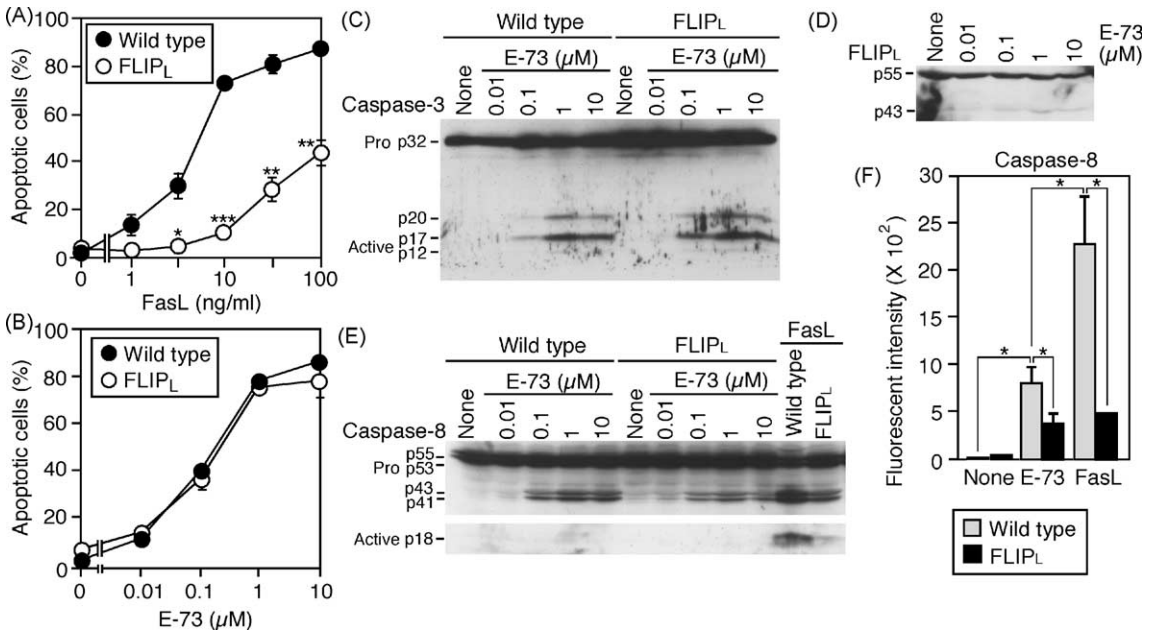

Fig. 6. Increased expression of $FLIP_L$ neither prevents procaspase-3 activation nor apoptosis upon exposure to E-73. (A and B) Wild-type Jurkat T cells (filled circles) and the $FLIP_L$-transfected Jurkat T cell clone JFL2 (open circles) were treated with various concentrations of FasL for 6 h (A) and with various concentrations of E-73 for 3 h (B). Apoptotic cells (%) were measured by Hoechst 33342 staining. Data points represent the mean ± S.D. of triplicate determinations. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ relative to wild-type cells treated with the same concentrations of FasL or E-73. (C) Wild-type Jurkat T cells and the $FLIP_L$ transfectant JFL2 were incubated with 1 μM E-73 for the indicated times. Processing of procaspase-3 was analyzed by Western blotting. (D) The $FLIP_L$ transfectant JFL2 was preincubated with 50 μM zVAD-fmk for 1 h, and then treated with various concentrations of E-73 for 3 h. The amount of transfected $FLIP_L$ was analyzed by Western blotting using anti-FLIP antibody. (E) Wild-type Jurkat T cells and the $FLIP_L$ transfectant JFL2 were treated with various concentrations of E-73 or cross-linked FasL (200 ng/ml) for 2 h. Processing of procaspase-8 was analyzed by Western blotting. (F) Wild-type Jurkat T cells (gray bars) and the $FLIP_L$ transfectant JFL2 (black bars) were treated with or without 1 μM E-73 or cross-linked FasL (200 ng/ml) for 2 h. The enzymatic activity of caspase-8 was measured. The result represents the mean ± S.D. of triplicate determinations. $^{*}P < 0.05$.

E-73 was prevented by SP600125 and curcumin, whereas it was insensitive to PD98059 and SB203580 (Fig. 8B). Phosphorylation of c-Jun at the serine-63 by JNK was strongly induced by E-73 within 30 min, and it was inhibited profoundly by SP600125 and curcumin at concentrations that blocked cytochrome *c* release (Fig. 8B and C). By contrast, SB203580 and PD98059 profoundly inhibited phosphorylation of HSP27 by p38 MAP kinase and phosphorylation of ERK1/2 by MEK1/2, respectively (Fig. 8D and E). Thus, the inhibitory profiles of these kinase inhibitors suggest that JNK activation is essential for cytochrome *c* release induced by E-73.

4. Discussion

E-73 was originally identified in the culture broth of *S. albulus* as a highly active antitumor compound [13,14]. However, the molecular mechanism of the antitumor activity of E-73 has remained unclear, although it has been shown that E-73 inhibits protein synthesis greater than CHX [27,28]. In this study we have shown that E-73 induces rapid cell death in human leukemia Jurkat T cells. E-73-induced cell death was accompanied by nuclear condensation and DNA fragmentation characteristic of apoptosis. E-73 triggered apoptosis with faster kinetics and at concentrations 100-fold lower than CHX. Therefore, E-73 appears to be one of the most potent inducers of apoptosis, and our present study might account for the strong in vivo antitumor activity of E-73 [13].

The JNK signaling pathway plays an essential role in apoptosis in response to various physical and chemical stresses [6]. It has been shown that CHX is a weaker activator of the JNK signaling pathway compared with the protein synthesis inhibitor anisomycin [34–36]. In contrast to CHX, E-73 induced JNK activation as efficiently as anisomycin in Jurkat T cells. Anisomycin can induce apoptosis either in the JNK-dependent pathway [7] or the JNK-independent pathway [37]. The cellular context might determine which pathway is dominantly activated by anisomycin. Since anisomycin-induced apoptosis was insensitive to SP600125 (data not shown), anisomycin seems to mainly induce JNK-independent apoptosis in Jurkat T cells. E-73 activated JNK at a concentration of 0.01 μM, whereas it induced apoptosis more than 0.1 μM. E-73 profoundly inhibited protein synthesis at concentrations at more than 0.1 μM. These data suggest the possibility that inhibition of protein synthesis might increase cell susceptibility to JNK-dependent apoptosis by down-regulation of short-lived anti-apoptotic proteins.

The release of cytochrome *c* from mitochondria is involved in apoptosis induced by JNK activation [7–9].

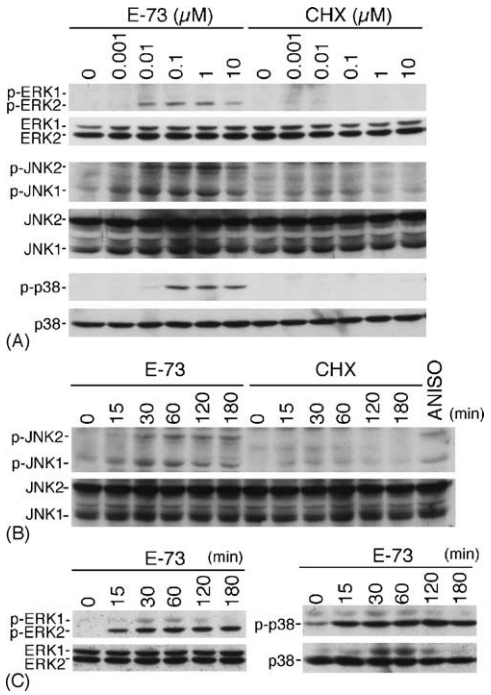

Fig. 7. E-73 induces rapid activation of ERK, JNK, and p38 MAP kinase. (A–C) Jurkat T cells were treated with various concentrations of E-73 or CHX for 30 min (A). Jurkat T cells were treated with 1 μM E-73 or 1 μM CHX for the indicated times, or 1 μM anisomycin for 15 min (B). Jurkat T cells were incubated with 1 μM E-73 for the indicated times (C). Phosphorylation of ERK, JNK, and p38 MAPK was analyzed by Western blotting.

The Bax subfamily (Bax and Bak) is essential for apoptotic signal transduction by JNK [38]. Moreover, JNK phosphorylates the BH3-only proteins (Bim and Bmf) that are normally sequestered in the motor complex interacting with the cytoskeleton, and phosphorylation by JNK causes their release from the motor complex which might be essential for Bax-dependent apoptosis [39]. Recently, it has been also reported that JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 protein [9]. Bcl-2 and Bcl-x_L are phosphorylated by JNK and might play a role in regulation of the mitochondrial apoptotic machinery [6]. Thus, JNK activation appears to induce cytochrome *c* release via cooperative regulation of the Bcl-2 family members. E-73 induced cytochrome *c* release from mitochondria within 1–2 h, and SP600125 prevented cytochrome *c* release as well as apoptosis in E-73-treated cells. Enforced expression of Bcl-x_L completely blocked cytochrome *c* release as well as processing of procaspases-3 and -9 in Jurkat T cells treated with E-73. Therefore, these results suggest that E-73-induced JNK activation is essential for cytochrome *c* release and subsequent apoptosis.

In addition to rapid activation of the initiator procaspase-9, the initiator procaspse-8 was processed into caspase-8(p43) within 2 h upon treatment with E-73 as observed with Fas stimulation. Procaspase-8 is mainly activated via autoprocessing by death receptors such as Fas [2,3] but may be processed by other caspases in a feedback amplification loop [40]. Active caspase-8 is able to convert procaspase-3 into active forms as well as to cleave Bid into a truncated form that is translocated to the mitochondria and induces cytochrome *c* release [41,42]. However, the Bid pathway does not seem to be involved in cytochrome *c* release during E-73-induced apoptosis, since zVAD-fmk failed to block cytochrome *c* release in E-73-treated cells. Bcl-2 inhibits activation of procaspase-8 in type II cells such as Jurkat T cells treated with anti-Fas antibody [43,44]. In contrast, physiological FasL induces apoptosis by a mechanism insensitive to Bcl-2 or Bcl-x_L [45]. Consistent with this notion, Bcl-x_L-transfected Jurkat T cells used in this study underwent apoptosis by FasL as efficiently as wild-type cells (data not shown). However, overexpression of Bcl-x_L blocked E-73-induced processing of procaspases-8. Thus, it is possible that procaspase-8 is primarily cleaved by downstream caspases during E-73-induced apoptosis, although we can not rule out the possibility that E-73 triggers a Bcl-2/Bcl-x_L-regulated early signaling event that is proximal or upstream of procaspase-8 autoprocessing in Jurkat T cells.

It has been also shown that activation of the JNK pathway by dominant-active MEKK1 and DNA-damaging agents induces FasL upregulation and Fas-dependent apoptosis in Jurkat T cells [10,11]. In contrast, it was also reported that activation of the JNK pathway by dominant-active MKK7 induces FasL upregulation but FADD-dependent apoptosis without Fas–FasL interaction in Jurkat T cells [12]. These findings suggest that FADD and caspase-8 are at least common components essential for JNK-dependent apoptosis in Jurkat T cells. Due to the ability to induce a strong JNK activation, E-73 might directly induce activation of the FADD-caspase-8 pathway. However, unlike Fas stimulation, overexpression of $FLIP_L$ did not have a protective effect on E-73-induced procaspase-3 activation and subsequent apoptosis. Moreover, active caspase-8(p18) was barely detectable in E-73-treated cells despite the efficient processing of procaspase-8 into caspase-8(p43). These data suggest that active caspase-8 is not essential for E-73-induced apoptosis. Nevertheless, the enzymatic activity of caspase-8 was induced by E-73 at lower levels than that induced by FasL. It has been recently shown that the caspase-8-$FLIP_L$ heterodimers have catalytic activity and substrate specificity similar to active caspase-8 [46–48]. Thus, the enzymatic activity of caspase-8 induced by E-73 might be mainly due to the caspase-8-$FLIP_L$ heterodimers that do not induce apoptosis.

Protein synthesis inhibitors such as anisomycin and the trichothecene family block the peptidyltransferase reaction and thereby trigger the ribotoxic stress response that induces JNK activation [35,49]. CHX interferes with the translocation reaction by preventing the release of the deacylated tRNA after the peptidyltransferase reaction [50]. The structure–activity relationship of CHX and its

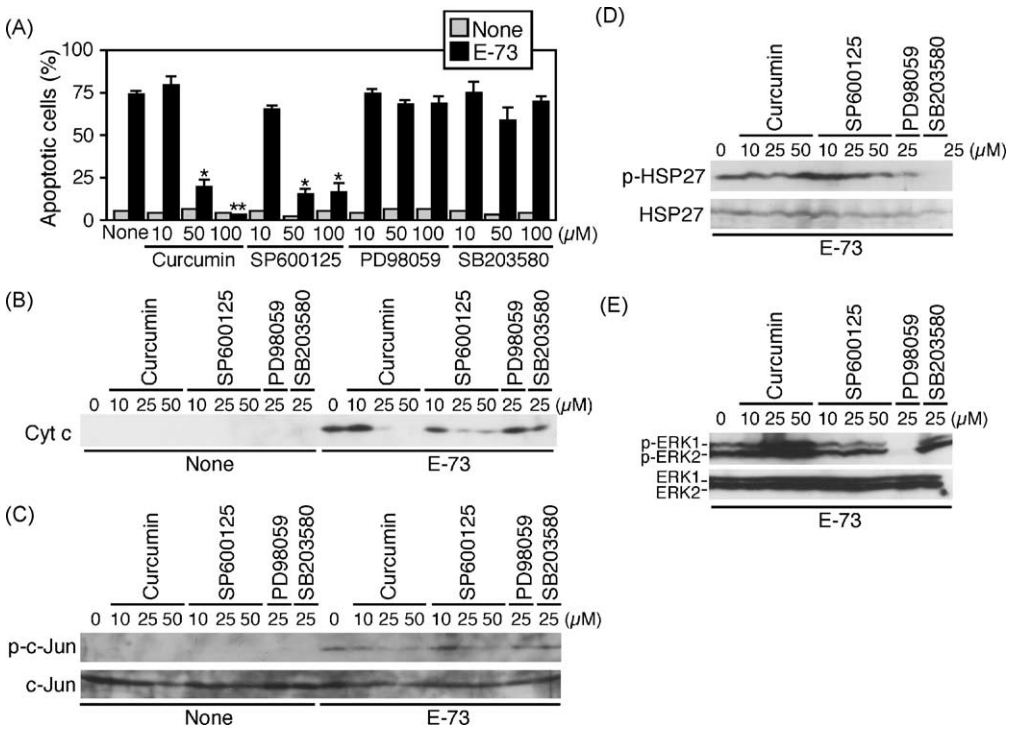

Fig. 8. Activation of the JNK pathway is essential for cytochrome *c* release upon exposure to E-73. (A) Jurkat T cells were pretreated with the indicated kinase inhibitors for 1 h, and then treated with (black bars) or without (gray bars) 1 μM E-73 for 3 h. Apoptotic cells (%) were measured by Hoechst 33342 staining. The result represents the mean ± S.D. of triplicate determinations. $^{*}P < 0.01$ and $^{**}P < 0.001$ relative to E-73-treated cells. (B) Jurkat T cells were pretreated with the indicated kinase inhibitors for 1 h, and then treated with or without 1 μM E-73 for 2 h. Cytoplasmic fractions were analyzed by Western blotting using anti-cytochrome *c* antibody. (C) Jurkat T cells were pretreated with the indicated kinase inhibitors for 1 h, and then treated with or without 1 μM E-73 for 30 min. Phosphorylation of c-Jun was analyzed by Western blotting. (D and E) Jurkat T cells were pretreated with the indicated kinase inhibitors for 1 h, and then treated with 1 μM E-73 for 30 min. Phosphorylation of HSP27 (D) and ERK1/2 (E) was analyzed by Western blotting.

congeners revealed that a substitution by an acetoxyl or hydroxyl group of the hydrogen attached to C-4 of the cyclohexenone ring (as in E-73 and streptovitacin A, respectively) increases its inhibitory activity on protein synthesis [27,28]. Moreover, we have shown that an additional acetoxyl group of the cyclohexenone ring is required for an efficient induction of JNK activation. In support of this finding, deacetylanisomycin inhibits protein synthesis with a 10,000-fold lower potency and is unable to activate JNK at a concentration where protein synthesis is inhibited by 65% [49]. Thus, it seems likely that E-73 interacts with the same binding site as CHX on the 60S ribosome and triggers the ribotoxic stress response leading to JNK activation. Further investigation will clarify the molecular mechanism how E-73 induces the ribosome-dependent JNK activation and how E-73 induces the release of cytochrome *c* from mitochondria.

Acknowledgments

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