The induction of G_2/M cell-cycle arrest and apoptosis by cucurbitacin E is associated with increased phosphorylation of eIF2α in leukemia cells

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The antiproliferative and apoptotic effects of cucurbitacin E, a natural product isolated from *Cucurbitaceae*, were determined in human leukemia HL-60 cells. Cucurbitacin E at low concentrations (3–50 nmol/l) inhibited the growth of HL-60 cells, which was associated with G_2/M cell-cycle arrest, decrease in the levels of cyclin-dependent kinase1, and increase in the levels of $p21^{waf1}$. Cucurbitacin E at high concentrations (1–10 μmol/l) induced apoptosis of HL-60 cells and activation of caspase-3, caspase-8, and caspase-9. Jurkat leukemia cells with or without caspase-8 expression were nearly equally sensitive to cucurbitacin E-induced apoptosis. Cucurbitacin E did not increase the levels of reactive oxygen species and antioxidants, *N*-acetylcysteine and catalase, did not block cucurbitacin E-induced apoptosis. Cucurbitacin E decreased the levels of the antiapoptotic proteins XIAP, survivin, and Mcl-1, but increased the level of the proapoptotic protein, Bax. The levels of phosphorylated eukaryotic translation initiation factor 2 subunit α (eIF2α) were induced in cells undergoing both apoptosis and cell-cycle arrest. As phosphorylated eIF2α is an inhibitor of protein translation initiation, our data suggest that cucurbitacin E induces cell growth arrest and apoptosis through the induction of eIF2α phosphorylation, which leads to the inhibition of cyclin-dependent kinase 1, Mcl-1, survivin, and/or XIAP protein synthesis and that cucurbitacin E induces apoptosis mainly through a mitochondrial-mediated pathway. *Anti-Cancer Drugs* 21:389–400 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Cucurbitacins are tetracyclic triterpenoids that exist in the species *Cucurbitaceae* and *Wilbrandia ebracteata* [1,2]. Cucurbitacins have been found to inhibit cell growth in several types of cancer [2]. Among these cucurbitacins, cucurbitacin B was reported to inhibit the growth of breast cancer, glioblastoma multiforme, hepatocellular carcinoma, and laryngeal cancer cells and to induce differentiation in leukemia cells [3–8]. Cucurbitacin I was reported to inhibit the growth and/or induce apoptosis in colon cancer, glioblastoma multiforme, breast cancer, and anaplastic large cell lymphoma cells [9–12]. Cucurbitacin E was reported to inhibit the growth of leukemia, lung cancer, colon cancer, prostate cancer, and breast cancer cells [13] and to disrupt the cytoskeleton of actin and vimentin in prostate cancer cells [14]. Although antiproliferative effects are consistently observed in cancer cells after treatment with these cucurbitacins, the mechanism(s) of their actions are unclear.

In this communication, we studied the antiproliferative and apoptotic abilities of cucurbitacin E in human HL-60 leukemia cells and explored its mechanism(s) of action. The levels of cell growth inhibition, cell-cycle arrest as well as cell cycle regulatory proteins were determined in HL-60 cells after treatment with cucurbitacin E at low concentrations, which did not induce cytotoxicity. The apoptosis induction ability of cucurbitacin E at high concentrations was investigated by measuring DNA fragmentation and annexin V staining. The intrinsic and extrinsic apoptotic pathways were explored by measuring the cleavage of caspase-8 and caspase-9. The role of the extrinsic apoptotic pathway was explored using Jurkat cells with defective expression of caspase-8 or FADD. The intrinsic apoptotic pathway was explored by measuring changes in the levels of antiapoptotic proteins and reactive oxygen species (ROS) production. Our data indicated that cucurbitacin E at low concentrations induced G_2/M cell-cycle arrest, decreased the level of cyclin-dependent kinase (Cdk) 1 protein and increased the level of $p21^{waf1}$ protein. Cucurbitacin E at high concentrations decreased the levels of the antiapoptotic proteins Mcl-1, survivin, and XIAP, but increased the levels of Bax. Cucurbitacin E at both low and high concentrations induced phosphorylation of eukaryotic translation initiation factor 2 subunit α (eIF2α), which

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inhibits protein translation initiation [15]. Our studies reveal a novel mechanism of cucurbitacin E action in inhibiting cell growth and in inducing apoptosis through regulating protein synthesis because of increased levels of phosphorylated eIF2α.

Materials and methods

Chemicals

Cucurbitacin E was isolated from the calyx melo of *Cucumis melo* L. with a purity of 98%. Fetal bovine serum (FBS), trypan blue, penicillin G, and streptomycin were obtained from GIBCO BRL (Gaithersburg, Maryland, USA). RPMI 1640 medium, dimethylsulfoxide (DMSO), ribonuclease (RNase), ethidium bromide, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), *N*-acetylcysteine (NAC), catalase (CAT), and propidium iodide (PI) were purchased from Sigma Chemical (St. Louis, Missouri, USA). Antibodies against Bax, Bcl-2, Bid, Fas, Mcl-1, survivin, XIAP, and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Antibodies against peroxidase-conjugated anti-mouse and anti-rabbit IgG, cyclin A, cyclin B1, Cdk1, cdc25C, $p21^{waf1}$, caspase-3, caspase-8, caspase-9, eIF2α, and p-eIF2α were purchased from Cell Signaling Technology (Beverly, Massachusetts, USA). Antibodies against DR4 and DR5 were purchased from ProSci Inc. (San Diego, California, USA). The general caspase inhibitor, Z-VAD-FMK, caspase-8 inhibitor, Z-IETD-FMK, and caspase-9 inhibitor, Z-LEHD-FMK, were obtained from Calbiochem (La Jolla, California, USA). An antibody to poly-(ADP-ribose)-polymerase (PARP) was obtained from Boehringer Mannheim (Indianapolis, Indiana, USA); an antibody to FasL was obtained from BD Biosciences (San Diego, California, USA).

Cell culture

HL-60 cells were cultured in a RPMI-1640 medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mmol/l L-glutamine, and 10% (v/v) heat-inactivated FBS. Jurkat subclones including A3 cells, FADD-deficient I 9.1 cells, and caspase-8-deficient I 9.2 cells were obtained from ATCC (Rockville, Maryland, USA) and were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS [16].

Proliferation assay

Cells were seeded at 2.0×10^4 cells/ml in 24-well plates and incubated with various concentrations (3–50 nmol/l) of cucurbitacin E for 3 days. Cells treated with DMSO (0.1%) were used as a negative control. Total cell number and trypan blue staining positive and negative cells in each group were determined with the aid of a hemocytometer. The antiproliferative ability was expressed as the ratio of the cell number in the treated group to that of the DMSO-treated group. The concentration that inhibited half of the cell proliferation was calculated (GI_{50}). Cell viability was determined by trypan blue exclusion and expressed as a percentage of viable cells in the total cell population.

Cell-cycle analysis

Cells treated with different concentrations of cucurbitacin E at various times were harvested by centrifugation, washed with phosphate-buffered saline (PBS), then fixed in 70% ethanol at −20°C overnight. Fixed cells were resuspended in 1 ml PBS, treated with RNase (100 μg/ml) for 30 min at 37°C, then stained with PI (50 μg/ml) at 4°C for 15 min. DNA distribution was measured with a flow cytometer (Becton Dickinson, San Jose, California, USA) and the results were analyzed using the CellQuest program.

Quantitation of apoptotic cells

Levels of apoptotic cells were determined by PI staining, annexin V staining and DNA fragmentation. Cells in the $subG_1$ phase were described earlier [17]. Cells were fixed with ice-cold 70% ethanol at a density of 1×10^5 cells/ml and treated with 200 μg/ml RNase for 30 min at 37°C. PI was then added to a final concentration of 50 μg/ml and the cells in the $subG_1$ phase were quantitated by flow cytometry with an excitation wavelength of 488 nm and an emission wavelength of 625 nm. Data were analyzed using the CELLQuest software (Becton Dickinson, San Jose, California, USA). For the annexin V staining assay, 10^5 cells were washed twice with PBS, then labeled by annexin V–FITC and PI in a binding buffer according to the instructions provided by the manufacturer in the Annexin V–FITC Apoptosis Detection Kit (Oncogene, Cambridge, Massachusetts, USA). The fluorescent signals of FITC and PI were detected at 518 and at 620 nm, respectively, on a flow cytometer [18]. DNA fragmentation analysis was done as described earlier [19]. Briefly, the cells treated with different concentrations of cucurbitacin E for 12 h were harvested by centrifugation and washed twice in ice-cold PBS. The pellets were resuspended in lysis buffer (10 mmol/l Tris pH 7.4, 5 mmol/l EDTA, 1% Triton X-100) for 20 min on ice and centrifuged. Supernatants were collected and treated with 20 mg/ml RNase A at 37°C for 1 h and then treated with 0.1 mg/ml proteinase K for additional 1 h. DNA was extracted and separated by electrophoresis in 2% agarose and then digitally imaged after staining with ethidium bromide.

Determination of H_2O_2 production

Intracellular H_2O_2 production was monitored by flow cytometry after staining with 5,6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) [18]. Briefly, cells in logarithmic growth (1×10^5 cells/ml) were labeled with 5 μmol/l DCFH-DA for 1 h, followed by treatment with or without cucurbitacin E for the indicated periods of time. After washing with PBS, the cells were analyzed by flow cytometry with excitation and emission

wavelengths of 495 and 525 nm, respectively. The cells treated with 100 µmol/l H_2O_2 for 1 h were used as a positive control.

Western blot analysis

Protein extracts (50 µg) prepared with RIPA lysis buffer (50 mmol/l Tris–HCl, 150 mmol/l NaCl, 0.1% sodium dodecyl sulfate, 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/l phenylmethyl sulfonyl fluoride, 100 µmol/l leupeptin, and 2 µg/ml aprotinin, pH 8.0) were separated on 8–12% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were stained with 0.2% Ponceau S red to assure equal protein loading and transfer. After blocking with 5% nonfat milk, the membranes were incubated with a specific antibody overnight at 4°C. Immunocomplexes were visualized using ECL Western Blotting Detection reagents (Amersham Biosciences, Little Chalfont, UK).

Results

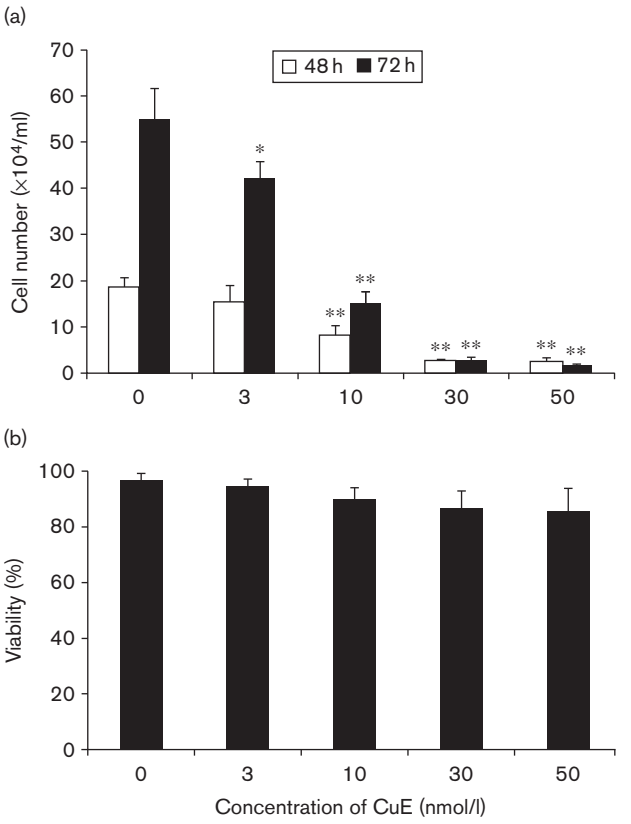
Cucurbitacin E at low concentrations inhibits cell growth and arrests cells in G_2/M phase

HL-60 cells were treated with various concentrations of cucurbitacin E or DMSO for 48 and 72 h. The total cell number in each group was counted. Viable cells were assessed by trypan blue exclusion. Cucurbitacin E at concentrations of 3–50 nmol/l inhibited cell growth in a time- and concentration-dependent manner (Fig. 1a) with a GI_{50} of 9.2 nmol/l after 72 h of treatment. HL-60 cells did not lose their viability after treatment with cucurbitacin E at concentrations below 50 nmol/l (Fig. 1b). Cell-cycle distribution after treatment with cucurbitacin E was investigated by flow cytometry. The number of cells in G_2/M phase was increased after treatment with cucurbitacin E at concentrations of 10–50 nmol/l (Fig. 2). The number of cells in G_2/M phase was increased from 21.1 to 61.6% after 12 h of treatment with 50 nmol/l cucurbitacin E (Fig. 2). These data suggest that the induction of G_2/M phase arrest accounts for the growth inhibitory effects of cucurbitacin E at low concentrations.

Cucurbitacin E decreases the levels of Cdk1 protein, but increases the levels of $p21^{waf1}$ and phosphorylated eIF2α protein

The levels of cyclin A, cyclin B1, Cdk1, cdc25C, and $p21^{waf1}$ protein were examined in HL-60 cells after treatment with cucurbitacin E to determine which protein might be involved in the induction of G_2/M cell-cycle arrest. Our data revealed that the levels of Cdk1 protein were decreased, but the levels of $p21^{waf1}$ protein were increased after cucurbitacin E treatment at concentrations of 30 and 50 nmol/l (Fig. 3). The levels of cyclin A, cyclin B1 and cdc25C protein were not altered after treatment with cucurbitacin E at those concentrations (Fig. 3). To investigate whether upregulation of $p21^{waf1}$ plays a role in the G_2/M cell-cycle arrest, $p21^{waf1}$ siRNA was used. There was no difference in the levels of cell cycle arrest after treatment with cucurbitacin E between the cells treated with $p21^{waf1}$ siRNA and control siRNA (data not shown). It should be noted that the upregulated levels of $p21^{waf1}$ protein shown in Fig. 3 were only detected after longer time exposures in western blot analysis. Therefore, the increased levels of $p21^{waf1}$ protein because of cucurbitacin E treatment, most likely, do not play a role in the cell-cycle arrest of these cells. After cucurbitacin E treatment the levels of Cdk1 were decreased and the levels of phosphorylated eIF2α were increased. It is possible that phosphorylated eIF2α is responsible for the decrease in the levels of Cdk1 and that this may mediate cell-cycle arrest because of cucurbitacin E treatment.

Fig. 1

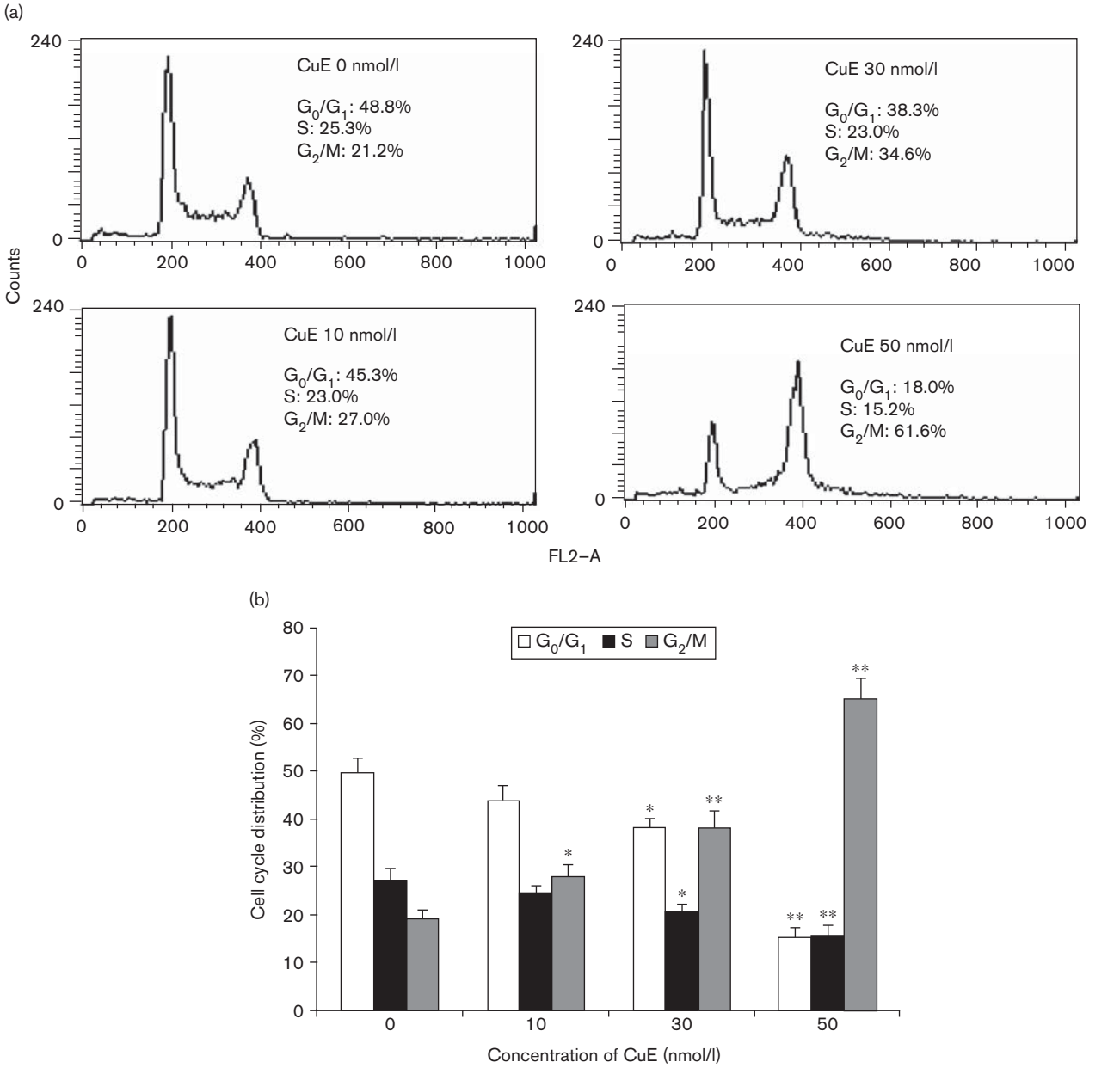


Cucurbitacin E (CuE) inhibited cell growth at low concentrations without causing cytotoxicity in HL-60 cells. (a) Growth rates of HL-60 cells. HL-60 cells were cultured with or without CuE at the indicated concentrations for 48 and 72 h. Total cell number was determined with the aid of a hemocytometer. (b) Viability of HL-60 cells. HL-60 was treated with or without CuE at the indicated concentrations for 72 h. Viable cells were determined by trypan blue exclusion. Data shown are mean ± SE of three independent experiments. *$P<0.05$, **$P<0.01$, compared with HL-60 cells without treatment.

Cucurbitacin E induces apoptosis at high concentrations

Cucurbitacin E at higher concentrations induced cytotoxicity. To determine whether the cytotoxic effects of cucurbitacin E are mediated through apoptosis induction,

Fig. 2



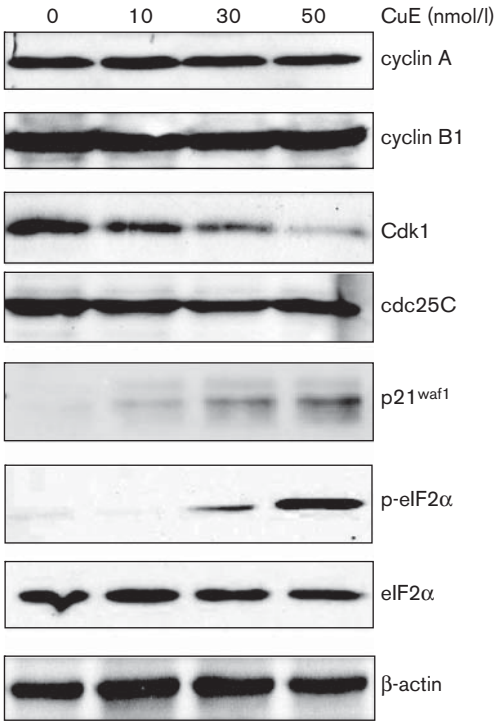
CuE induced G_2/M phase arrest in HL-60 cells at low concentrations. HL-60 cells were cultured with or without CuE at the indicated concentrations for 12 h. Cells were stained with propidium iodide. The DNA distribution was determined by flow cytometric analysis. (a) One representative experiment is depicted; (b) The mean ± SE of three independent experiments of cell numbers in G_0/G_1, S, and G_2/M phase. *$P<0.05$, **$P<0.01$, compared with control cells in G_0/G_1, S, and G_2/M phase, respectively. CuE, cucurbitacin E.

HL-60 cells were treated at concentrations ranging from 0.3 to 10 μmol/l for 12 h. Apoptotic cells were identified using a flow cytometer after staining with PI. On the basis of the cell number in the subG_1 phase determined by flow cytometric analysis, apoptotic cells were obtained after treatment with cucurbitacin E at 0.3–10 μmol/l (Fig. 4a and b). The apoptosis induction ability of cucurbitacin E was further confirmed based on DNA fragmentation using agarose electrophoretic analysis (Fig. 4c). These data suggest that the cytotoxic effect of cucurbitacin E at high concentrations is probably through induction of apoptosis in HL-60 cells.

Cucurbitacin E activates caspase-3, 8, and 9 in HL-60 cells

To determine the mechanism(s) of cucurbitacin E-induced apoptosis, the levels of procaspase-3, procaspase-8, and procaspase-9 and their cleaved fragments

Fig. 3



Western blot analysis of cell cycle-related proteins in HL-60 cells after treatment with CuE. HL-60 cells were treated with CuE at the indicated concentrations for 12 h. Total cell lysates were prepared. The levels of cyclin A, cyclin B1, Cdk1, cdc25C, eIF2α, p-eIF2α, $p21^{waf1}$ or β-actin were determined by western blot analysis using specific antibodies for each protein. CuE, cucurbitacin E; eIF2α, eukaryotic translation initiation factor 2 subunit α.

were determined by western blotting analyses. Treatments with cucurbitacin E (1–10 μmol/l) resulted in decreases in the levels of procaspase-3, procaspase-8, and procaspase-9 and the formation of their cleaved fragments (Fig. 5). These data suggest that all those three caspases are activated by cucurbitacin E treatment in HL-60 cells. To determine whether caspase activation plays an important role in cucurbitacin E-induced apoptosis, the general caspase inhibitor, Z-VAD-FMK, the caspase-8 inhibitor, Z-IETD-FMK, and the caspase-9 inhibitor, Z-LEHD-FMK were used to test whether they can block cucurbitacin E-induced apoptosis. All of these caspase inhibitors attenuated cucurbitacin E-induced apoptosis. The caspase-8 inhibitor Z-IETD-FMK was less effective than caspase-9 inhibitor Z-LEHD-FMK and the general caspase inhibitor Z-VAD-FMK in blocking cucurbitacin E-induced apoptosis (Fig. 6).

Cucurbitacin E decreases the levels of antiapoptotic proteins c-FLIP, Mcl-1, XIAP, and survivin

Mitochondria-mediated activation of caspase-9 and caspase-3 also can be regulated by the proapoptotic and antiapoptotic proteins of the Bcl-2 family. Therefore, the levels of Bax, Bcl-2, and Mcl-1 protein were determined. Treatments with cucurbitacin E increased the levels of the proapoptotic protein Bax, but decreased the levels of the antiapoptotic protein Mcl-1 (Fig. 5a). The levels of Bcl-2 protein were unchanged during cucurbitacin E treatment. The activities of caspase-3 and caspase-9 are inhibited by the inhibitor of apoptosis protein XIAP and survivin. The levels of XIAP and survivin were determined after cucurbitacin E treatment. As shown in Fig. 5a, the levels of both XIAP and survivin were decreased. These data suggest that the increased levels of Bax and the decreased levels of Mcl-1 lead to the activation of caspase-9 and caspase-3, which is amplified by decreases in the levels of XIAP and survivin.

To investigate the mechanism of caspase-8 activation by cucurbitacin E, the levels of DR4, DR5, Fas, FasL, TRAIL, and c-FLIP protein were investigated in HL-60 cells. Although cucurbitacin E treatment led to the activation of caspase-8, which was reflected in the detection of cleaved caspase-8 and down-regulation of Bid, the levels of DR4, DR5, Fas and their ligands, TRAIL and FasL, were not increased. However, decreases were observed in the levels of most of these moieties. The levels of c-FLIP, a caspase-8 inhibitor, were decreased by cucurbitacin E treatment. These data suggest that cucurbitacin E induced activation of caspase-8 by removing this inhibitor of caspase-8 activity, c-FLIP, rather than by activating death-receptor ligation or aggregation.

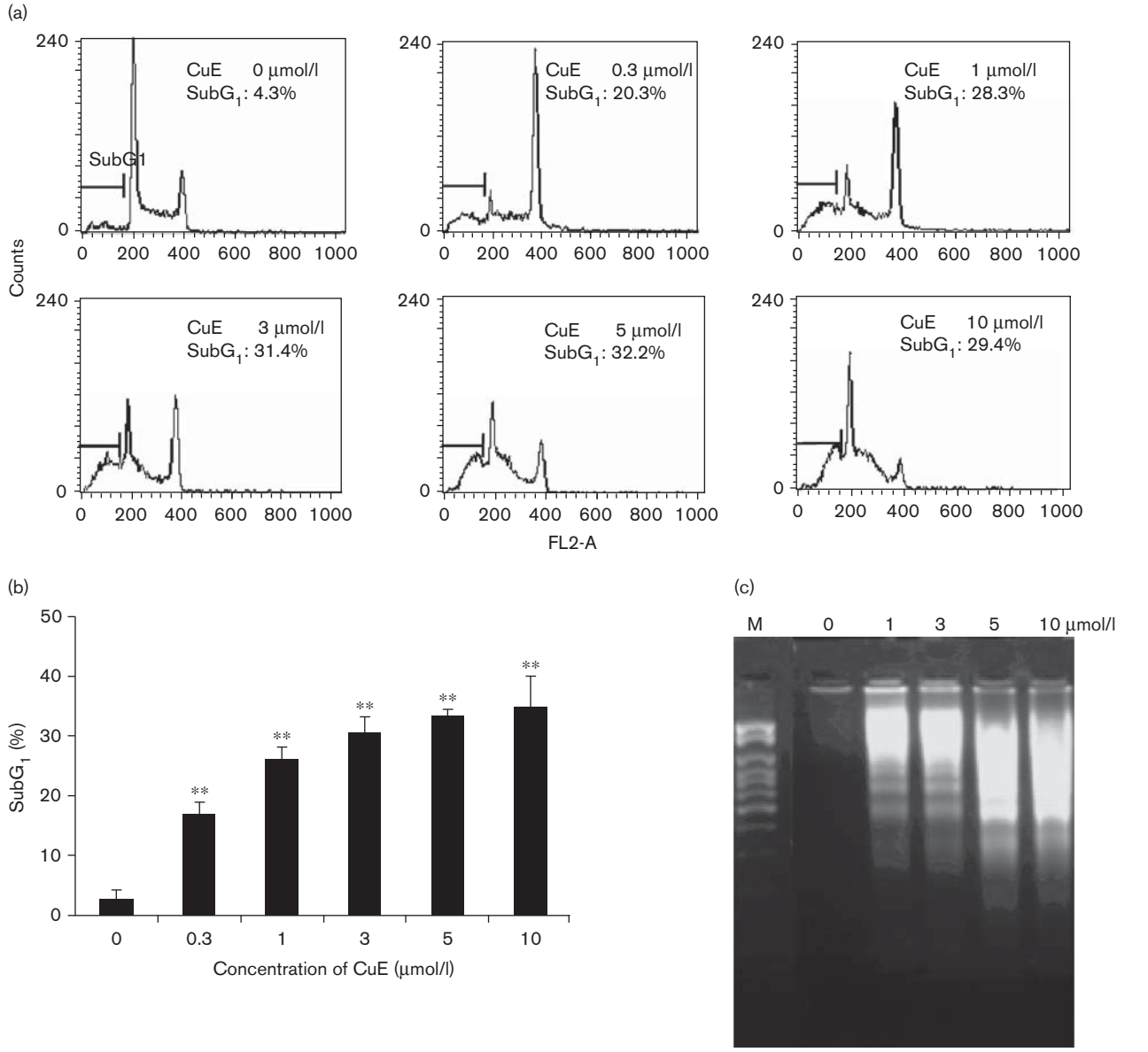
The decrease in the levels of XIAP, Mcl-1, and survivin is correlated with the induction of eIF2α phosphorylation

XIAP, Mcl-1, and survivin are short-lived proteins. The decreases in their levels may be because of the inhibition of their translation. It has been shown that Mcl-1 is repressed by increased phosphorylation of eIF2α [20]. We compared the relationship of the levels of phosphorylated eIF2α with the levels of XIAP, Mcl-1, and survivin proteins after cucurbitacin E treatment. The decrease in the levels of XIAP, Mcl-1, and survivin was correlated with increases in phosphorylated eIF2α levels (Fig. 7). Our data suggest that phosphorylated eIF2α might lead to the repression of the synthesis of XIAP, Mcl-1, and survivin proteins, which may play important roles in cucurbitacin E-induced apoptosis.

Production of reactive oxygen species is not involved in cucurbitacin E-induced apoptosis

ROS has been found to participate in apoptosis induction by many chemotherapeutic agents. To investigate whether ROS production is involved in cucurbitacin E-induced apoptosis, the amount of H_2O_2 and the protective effects of antioxidants NAC and CAT on cucurbitacin E-induced apoptosis were determined. Cucurbitacin E did not increase the levels of H_2O_2 (Fig. 8a). Neither NAC nor CAT prevented cucurbitacin

Fig. 4



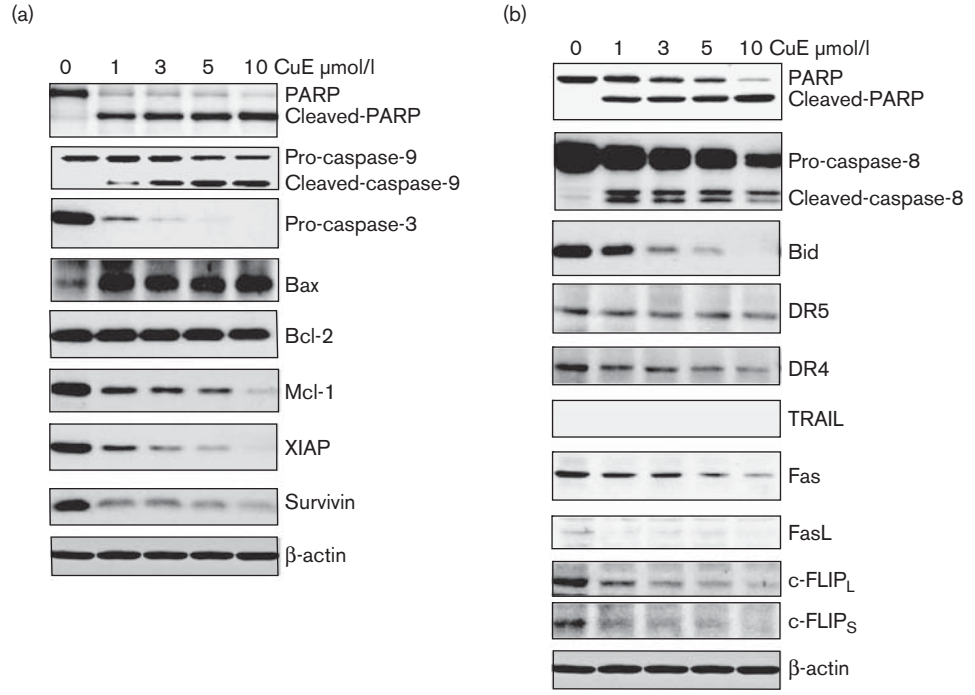
CuE induced apoptosis at high concentrations in HL-60 cells. (a) The subG$_1$ DNA content of HL-60 cells in one representative experiment is shown. (b) The mean ± SE of three independent experiments of cells in subG$_1$ phase. $^{**}P<0.01$, compared with cells without treatment. (c) Fragmented DNA. HL-60 cells were treated with CuE at the indicated concentrations for 12 h. The subG$_1$ content and DNA fragmentation were determined by flow cytometry and electrophoretic analysis as described in Materials and methods section. M, DNA size markers; CuE, cucurbitacin E.

E-induced apoptosis in HL-60 cells (Fig. 8b). These data suggest that cucurbitacin E-induced apoptosis in HL-60 cells is through a ROS-independent pathway.

Jurkat cells with defective of caspase-8 and FADD are sensitive to cucurbitacin E-induced apoptosis

To determine whether caspase-8 activation plays an important role in the apoptosis induction of cucurbitacin E treatment, we used two subclones of Jurkat cells with the lack of either caspase-8 (I 9.2) or FADD (I 9.1). Both I 9.1 and I 9.2 cells were sensitive to cucurbitacin-E induced apoptosis and there was no significant difference in the cells expressing or not expressing caspase-8 or FADD (Fig. 9). To further determine the mechanism of apoptosis induction of cucurbitacin E among these cells, the levels of XIAP, Mcl-1, and survivin as well as that of phosphorylated eIF2α were determined. As observed in HL-60 cells (Fig. 7), cucurbitacin E decreased the levels

Fig. 5



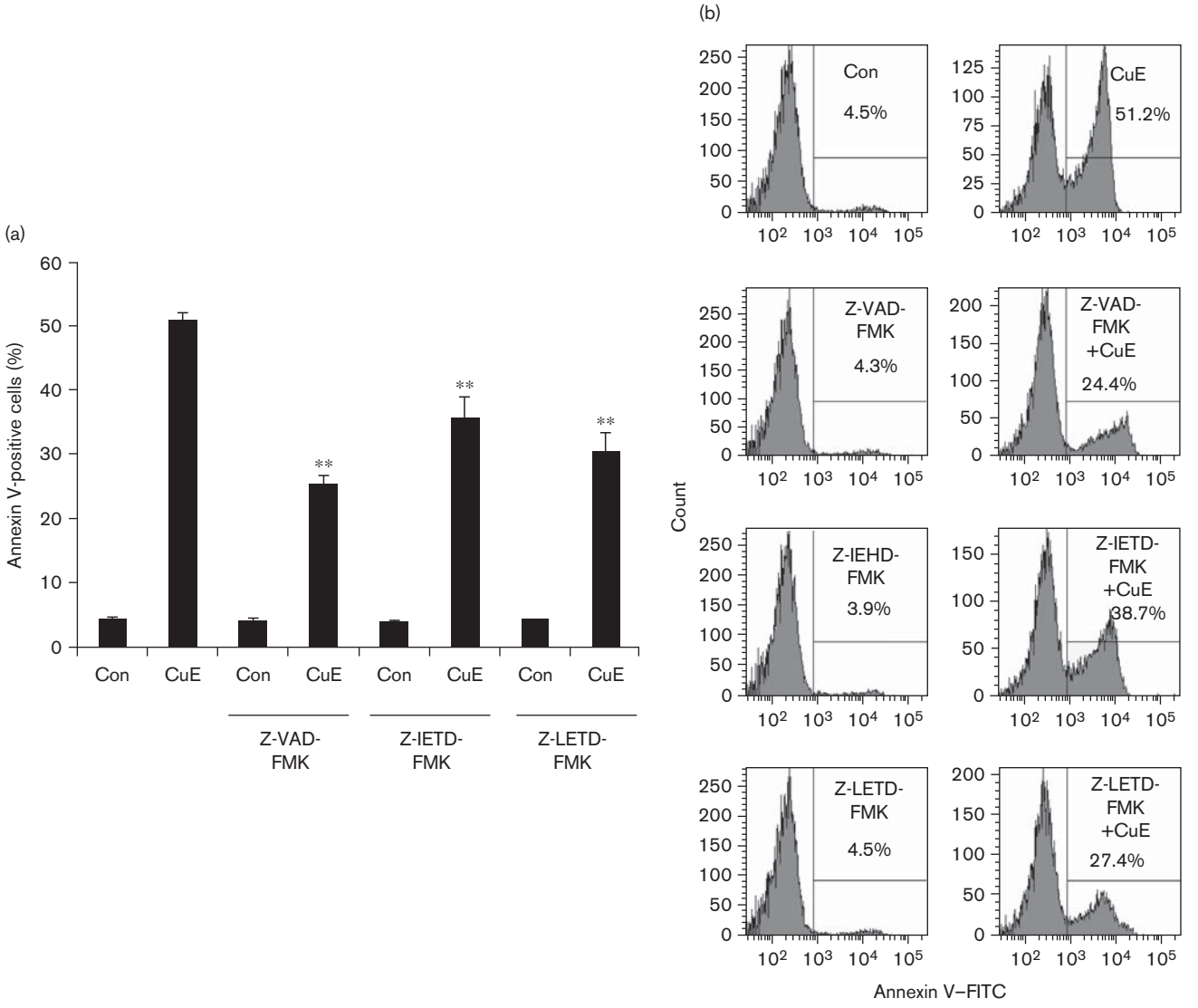
The levels of some proteins involved in mitochondrial and death receptor-mediated apoptosis pathways in HL-60 cells treated with CuE. HL-60 cells were treated with CuE at the indicated concentrations for 12 h. The protein levels of caspase-3, caspase-8, caspase-9, Bid, Bcl-2, Bax, DR4, DR5, Fas, FasL, TRAIL, c-FLIP, XIAP, and survivin were determined by western blot analyses. CuE, cucurbitacin E.

of XIAP, Mcl-1, and survivin and increased the levels of phosphorylated eIF2α (Fig. 9c). These data support the idea that the mitochondria-mediated apoptotic pathway, but not death receptor-mediated pathway, plays an important role in cucurbitacin E-induced apoptosis.

Discussion

Cucurbitacin E at concentrations less than 50 nmol/l inhibited the growth of HL-60 cells without causing cytotoxicity (Fig. 1). This is associated with cell-cycle arrest. Cucurbitacin E increased the number of cells in G_2/M phase of HL-60 cells (Fig. 2), which is consistent both with the observations obtained in MCF-7 cells treated with cucurbitacin B/E glucosides and in those noted in glioblastoma multiforme cells treated with cucurbitacin I [9,14]. Cdks have been shown to play an important role in controlling cell cycle progress and have been considered as drug targets [21]. Among these Cdks, Cdk1 interacts with cyclin B1 to form an active heterodimer. The progression of the cell cycle from G_2 to M-phase is driven by the activation of the Cdk1/cyclin B1 complex, whose activity must be sustained from the prophase to the metaphase [22]. $p21^{waf1}$ has been shown to inhibit Cdk1 activity and has been thought to contribute to cell-cycle arrest in the G_2 phase after treatment with DNA-damaging agents [23,24]. Correlated with the G_2/M phase arrest, the levels of $p21^{waf1}$ protein were upregulated and the levels of Cdk1 protein were downregulated (Fig. 3). Although it has been shown that cucurbitacin B/E glucosides decreased the protein levels of both Cdk1 and cyclin B1 in MCF-7 and MDA-MB-231 cells [14], we only observed a decrease in the Cdk1 levels, but not in cyclin B1 levels (Fig. 3). The difference between these results might be because of the cell types tested. $p21^{waf1}$ is known as a p53 target gene [23]. As HL-60 cells are p53-deficient [25,26], it seems that the $p21^{waf1}$ induction by cucurbitacin E is p53-independent. As the detection of $p21^{waf1}$ needs a longer time exposure and $p21^{waf1}$ siRNA did not block cell-cycle arrest (data not shown), it seems that upregulation of $p21^{waf1}$ does not play an important role in cucurbitacin E-induced G_2/M phase arrest. The decrease in Cdk1 levels might have a role in cell-cycle regulation. The eIF2 plays a significant role in the responses to environmental stresses [27]. When eIF2α is phosphorylated at the key serine residue (Ser51), the exchange of eIF2-GDP for eIF2-GTP required to bind and deliver the initiator, met-tRNA, to the translation machinery is reduced and, therefore, global protein synthesis is reduced [28]. As phosphorylated eIF2α is induced after cucurbitacin E treatment (Fig. 3), the decrease in the levels of

Fig. 6

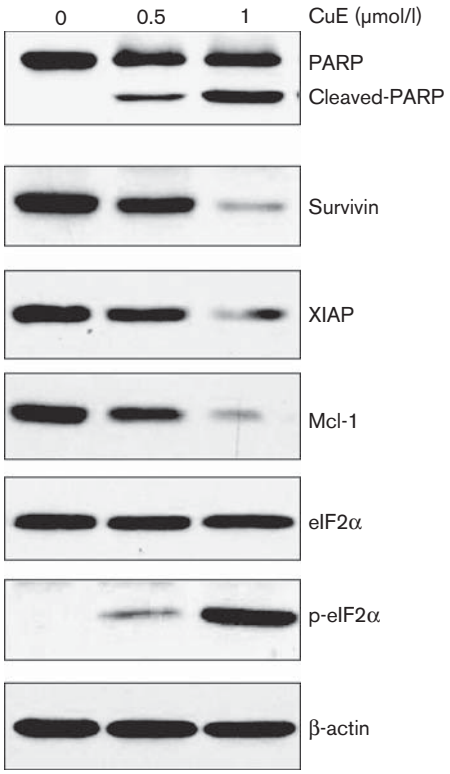


Caspase inhibitors attenuated CuE-induced apoptosis in HL-60 cells. (a) The mean ± SE of annexin V-positive cells of three independent experiments. $^{**}P<0.01$, compared with cells without treatment. (b) One representative experiment of the annexin V staining assay. HL-60 cells were treated with 3 μmol/l CuE, 50 μmol/l Z-VAD-FMK (a general caspase inhibitor), 50 μmol/l Z-IETD-FMK (a caspase-8 inhibitor), 50 μmol/l Z-LEHD-FMK (a caspase-9 inhibitor) alone and at the indicated combinations for 12 h. Apoptotic cells were determined by flow cytometry after staining with annexin V-FITC. Con, control; CuE, cucurbitacin E.

Cdk1 protein after cucurbitacin E treatment may be because of the increase in phosphorylated eIF2α. It has been shown that phosphorylated eIF2α is increased at G_2/M and phosphorylated eIF2α influences the efficiency of the PITSLRE protein kinase $p58^{PTTSLRE}$. This kinase is specifically expressed during the G_2/M stage of the cell cycle and has a role in the regulation of G_2/M cell cycling [29]. Overexpression of $p58^{PITSLRE}$ in the Chinese hamster ovary fibroblasts leads to mitotic delay [30]. Our data suggest that the cell-cycle arrest by cucurbitacin E treatment might be caused by increased levels of phosphorylated eIF2α, which then inhibits the translation of Cdk1 or regulates the expression and activity of $p58^{PITSLRE}$. These mechanisms are worthy of further study.

It has been reported that cucurbitacin I induces apoptosis in ALK-positive anaplastic large-cell lymphoma cells [12] and that cucurbitacin B induces apoptosis in several solid tumor cell lines [9,31]. The apoptotic effects of these cucurbitacins in myeloid leukemia cells have not been reported. As cucurbitacin E did not induce cytotoxicity in HL-60 cells at concentrations lower than

Fig. 7



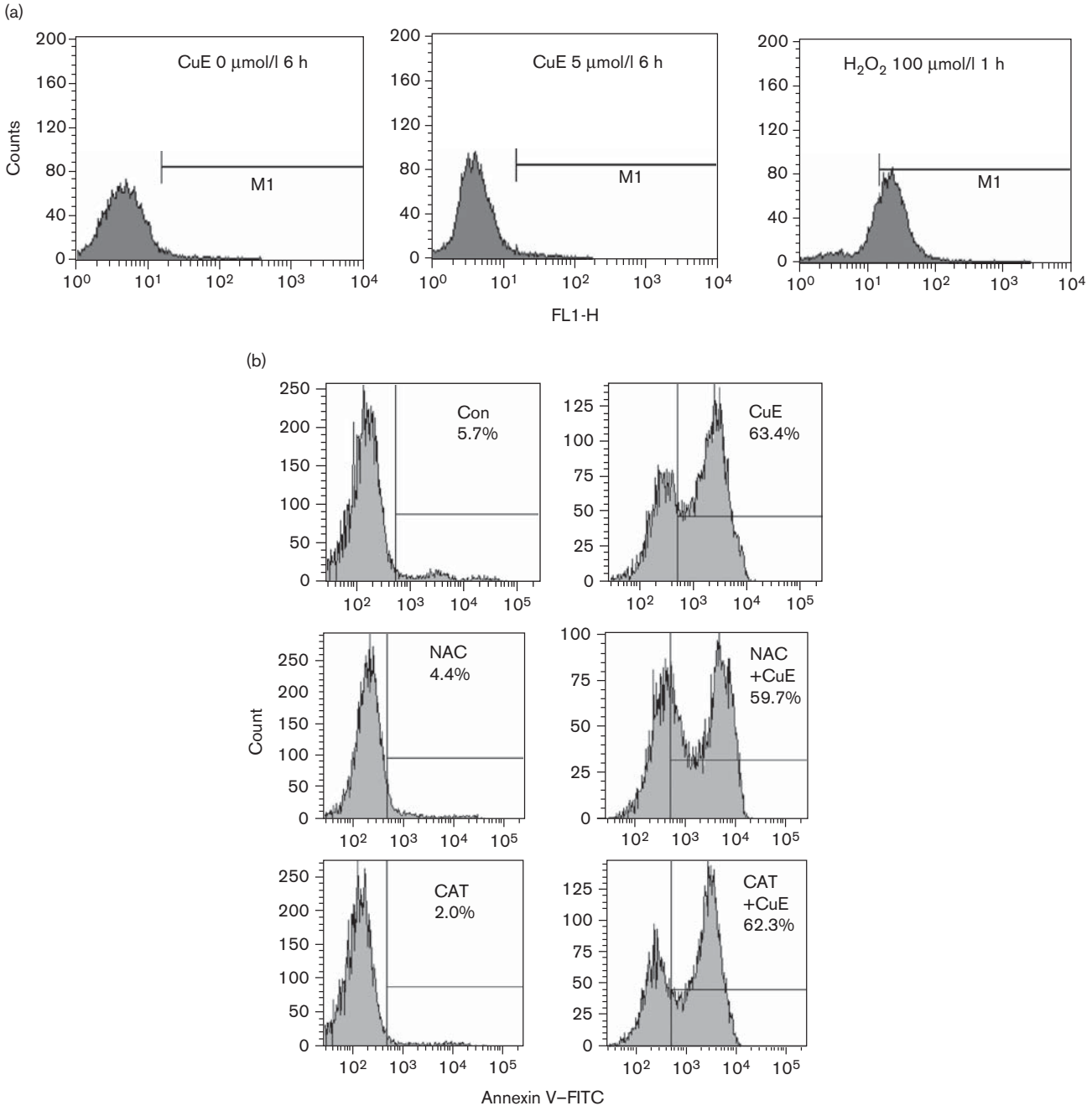
Decreases in the levels of Mcl-1, XIAP, and survivin are correlated with increases in the levels of phosphorylated eIF2α in HL-60 cells after treatment with CuE. HL-60 cells were treated with or without CuE at the indicated concentrations for 12 h. The protein levels of PARP, survivin, Mcl-1, and XIAP were determined by western blot analyses. CuE, cucurbitacin E; eIF2α, eukaryotic translation initiation factor 2 subunit α.

50 nmol/l (Fig. 1), concentrations of cucurbitacin E were tested at higher concentrations (0.3–10 μmol/l), which did induce cytotoxicity. On the basis of the results of flow cytometric analysis, annexin V-staining and agarose gel electrophoresis studies, apoptotic cells were observed in HL-60 cells after cucurbitacin E treatment at these concentrations (Fig. 4).

The mitochondria-mediated (intrinsic pathway) and death receptor (extrinsic pathway)-mediated apoptotic pathways have been well characterized [32,33]. Caspases have been shown to play crucial roles in the initiation and execution of apoptosis of both pathways [34]. Cucurbitacin E (1–10 μmol/l) treatment induced activation of caspase-3, caspase-8, and caspase-9 as determined by the formation of the cleaved fragments and/or decrease in the procaspase forms (Fig. 5). As neither the caspase-8 inhibitor nor the caspase-9 inhibitor attenuated the apoptosis induction ability of cucurbitacin E competely (Fig. 6), both mitochondria-mediated and death receptor-mediated pathways may be involved in cucurbitacin E-induced apoptosis in HL-60 cells. To explore the mechanism of csapase-8 activation in cucurbitacin E-treated cells, the levels of the death receptors and their ligands were determined. Decreased levels were observed for most of these moieties, which suggests global protein synthesis inhibition (Fig. 5b). These data do not support the conclusion that the activation of caspase-8 is because of increased levels of death receptors or their ligands. The activity of caspase-8 is inhibited by c-FLIP [35]. The levels of long and short forms of c-FLIP were decreased after cucurbitacin E treatment (Fig. 5b), which suggests that cucurbitacin E-induced activation of caspase 8 in HL-60 cells is, at least in part, because of the reduction in c-FLIP levels. To further explore the role of caspase-8 activation in cucurbitacin E-mediated apoptosis, Jurkat cells with a lack of expression of caspase-8 were used and they were almost equally responsive to cucurbitacin E treatment comparing with the caspase-8 expressing parental subclone A3 (Fig. 9). Therefore, apoptosis induction because of cucurbitacin E treatment is mainly executed through a mitochondria-mediated pathway.

ROS generation has been found to be involved in apoptosis induction by chemotherapeutic agents through the activation of the mitochondria-mediated apoptotic pathway [36,37]. Cucurbitacin E did not increase the levels of ROS in HL-60 cells (Fig. 8a) and neither NAC nor CAT blocked cucurbitacin E-induced apoptosis (Fig. 8b). Therefore, cucurbitacin E-induced apoptosis is independent of ROS. The mitochondrial membrane potential is controlled by the levels of proapoptotic and antiapoptotic proteins. By comparing the levels of Bax, Bcl-2, and Mcl-1, we found that cucurbitacin E increased the levels of Bax, but decreased the levels of Mcl-1. XIAP and survivin are inhibitors of apoptosis proteins and the decreases in their levels have been found to result in apoptosis [38,39]. Cucurbitacin E decreased the levels of XIAP and survivin in HL-60 cells (Fig. 5) as well as in Jurkat cells with the lack of caspase-8 expression (Fig. 9). It seems that decreases in the levels of Mcl-1, XIAP, and survivin play important roles in cucurbitacin E-mediated apoptosis. As these proteins are regulated by STAT3, the decrease in their levels by cucurbitacin E treatment might be because of the inhibition of STAT3. Cucurbitacin E has been reported to inhibit STAT3 phosphorylation in A549 cells [40]. Phosphorylated-STAT3 was not detectable in HL-60 cells and cucurbitacin E treatment did not alter the levels of phosphorylated-STAT3 (data not shown). Therefore, other mechanisms may be involved in the downregulation of these antiapoptotic proteins. Interestingly, these proteins have short half-lives and it has been shown that the levels of Mcl-1 were repressed by eIF2α phosphorylation in mitochondrial apoptosis [20]. As there is a relationship between increases in the levels of phosphorylated eIF2α and decreases in the levels of Mcl-1, XIAP and survivin proteins (Figs 7 and 9), our data suggest that

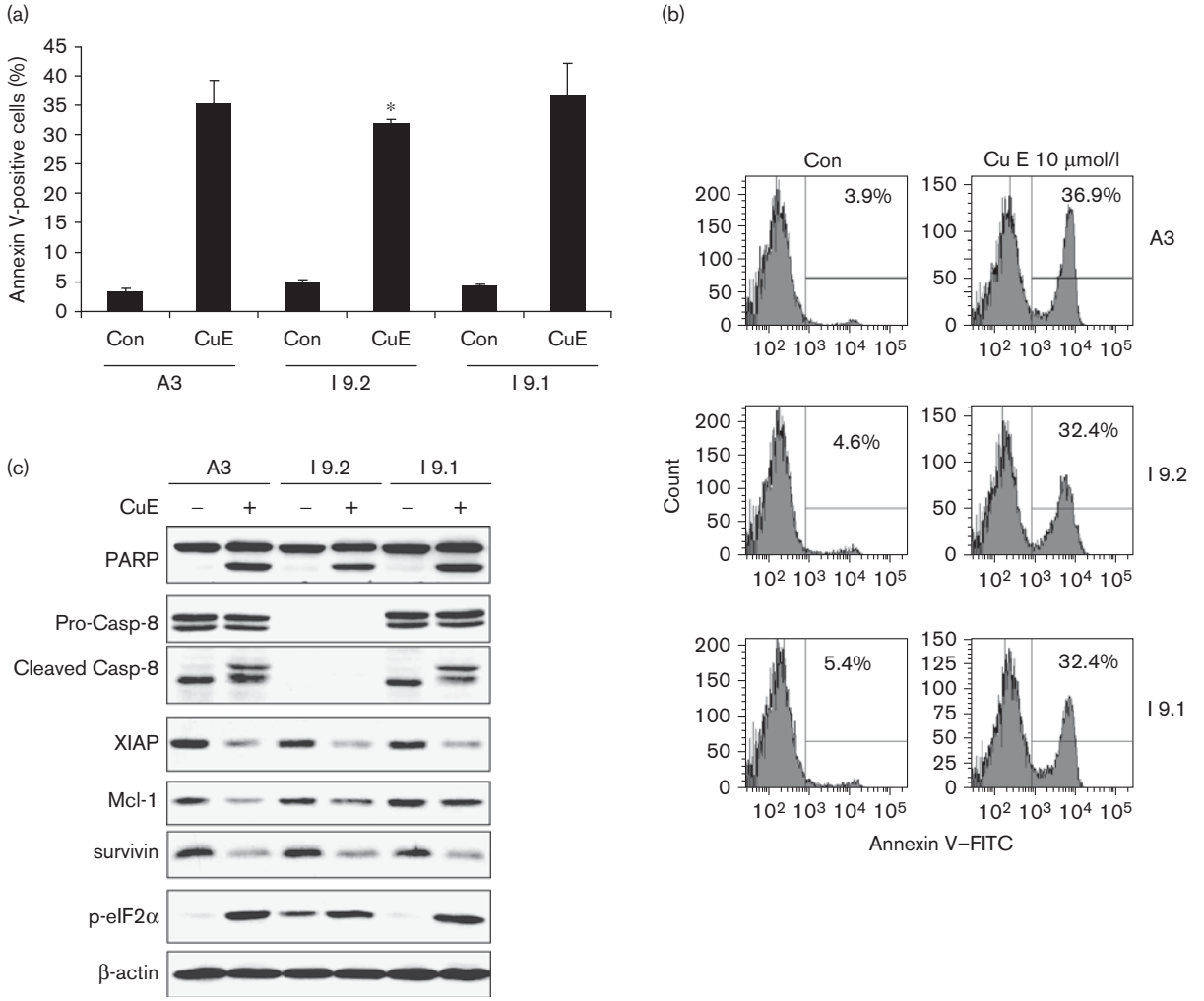
Fig. 8



Reactive oxygen species are not involved in cucurbitacin E (CuE)-induced apoptosis. (a) H_2O_2 content. HL-60 cells were treated with CuE (5 μmol/l) or H_2O_2 (100 μmol/l) for the indicated times, then the intracellular H_2O_2 content was determined as described in materials and methods. (b) The effects of antioxidants in inhibiting CuE-induced apoptosis**.** HL-60 cells were incubated with or without 10 mmol/l NAC or 500 U/ml catalase for 4 h and then treated with or without 3 μmol/l CuE for 12 h. Apoptotic cells were determined by flow cytometry after staining with annexin V–FITC. NAC, *N*-acetylcysteine; CAT, catalase.

phosphorylated eIF2α plays an important role in cucurbitacin E-induced apoptosis. In general, our data reveal a novel mechanism of cucurbitacin E action in inhibiting cell growth and in inducing apoptosis. The mechanism of cucurbitacin E-induced eIF2α phosphorylation is worthy of further study.

Fig. 9



Apoptosis induction of cucurbitacin E (CuE) in Jurkat subclones lacking FADD or caspase-8. (a) The mean ± SE of annexin V-positive cells of three independent experiments. (b) One representative experiment of annexin V detection is shown. Apoptotic cells were determined by flow cytometry after staining with annexin V–FITC. Cells were treated with CuE at 10 μmol/l for 24 h. (c) Western blot analysis of protein levels. FADD-deficient clone I 9.1, caspase-8-deficient clone I 9.2, and FADD/caspase-8 containing parental clone A3 all were treated with CuE at 10 μmol/l for 24 h. Con, control; eIF2α, eukaryotic translation initiation factor 2 subunit α. * $P<0.05$, compared with cells without treatment.

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