

Title

Kaurene diterpene induces apoptosis in human leukemia cells partly through a caspase-8-dependent pathway

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d) Abbreviations: KD, *Ent*-11 -hydroxy-16-kauren-15-one; PARP, poly(ADP-ribose) polymerase; FasL, Fas ligand; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor receptor 1; NF- κ B, nuclear factor- κ B; Apaf-1, apoptotic protease-activating factor-1; CDDP, Cis-Diammineplatinum-(II) dichloride; DISC, death-inducing signaling complex; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ECH, epoxy-cyclohexenone.

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Abstract

Defects in apoptosis signaling pathways contribute to tumorigenesis and drug resistance, and these defects are often a cause of failure of chemotherapy. Thus, a major goal in chemotherapy is to find cytotoxic agents that restore the ability of tumor cells to undergo apoptosis. We previously found that an *Ent*-kaurene diterpene, *Ent*-11 -hydroxy-16-kauren-15-one (KD), induced apoptosis in human promyelocytic leukemia HL-60 cells. Here we found that caspase-8, an apoptotic factor, is involved in KD-induced apoptosis. Although treatment of HL-60 cells with KD resulted in the activation of caspase-8 and -9, a caspase-8-specific inhibitor but not a caspase-9-specific inhibitor attenuated KD-induced apoptosis. Expression of a catalytically inactive caspase-8 partly attenuated KD-induced apoptosis. Treatment with KD led to a time-dependent cleavage of Bid, a substrate of caspase-8, as well as to the proteolytic processing of pro-caspase-8, indicating that KD treatment induces apoptosis through a caspase-8-dependent pathway. Moreover, overexpression of the drug resistance factor Bcl-2, which is frequently overexpressed in many tumors, failed to confer resistance to KD-induced cytotoxicity. Thus, KD may be a promising experimental cytotoxic agent that possibly points to new strategies to overcome a drug resistance.

Introduction

Most chemotherapeutic drugs have been shown to use apoptotic pathways to mediate their cytotoxic effect (Lowe and Lin, 2000). Apoptosis, or programmed cell death, is triggered by biochemical complex systems (Hengartner, 2000). Caspases, a family of cysteine proteases, play a pivotal role in apoptosis (Thornberry and Lazebnik, 1998). Caspases are synthesized in an inactive pro-form and are cleaved to activate their proteolytic activity. Caspases are classified into initiators of and effectors of apoptosis. The main initiator caspases are caspase-8 and caspase-9, which serve to activate the chief effector caspases such as caspase-3 followed by cleavage of DNA into internucleosomal fragments and cleavage of poly(ADP-ribose) polymerase (PARP). Caspase-8 is typically activated in response to receptor-mediated signals such as the binding of Fas to Fas ligand (FasL), or the binding of tumor necrosis factor receptor 1 (TNFR1) to TNF. These bindings cause the cleavage of pro-caspase-8 followed by the initiation of caspase cascade. Interestingly, an antiapoptotic factor, nuclear factor κ B (NF- κ B) is also activated after the binding of TNF to TNFR1 (Ashkenazi and Dixit, 1998). In contrast, caspase-9 is activated in response to cytochrome c release from mitochondria. Cytochrome c binds to apoptotic protease-activating factor-1 (Apaf-1) in an ATP-dependent manner followed by cleavage pro-caspase-9 to its active form (Ferri and Kroemer, 2001). Caspase-9 in turn induces the activation of caspase-3. In addition, caspase-8 can also lead to activation of caspase-9 via the release of cytochrome c, dependent on a cleavage of Bid, a Bcl-2 family protein (Ferri and Kroemer, 2001).

Many antiapoptotic pathways are observed in cancers, and defects in apoptosis causes both tumorigenesis and a drug resistance of cancer (Johnstone et al., 2002). For instance, Bcl-2 is frequently overexpressed, and activity of NF- κ B is deregulated in many tumors. Bcl-2 family proteins are classified into two groups: an antiapoptotic group including Bcl-2 and Bcl-xL and a proapoptotic group, including Bax and Bik (Gross et al., 1999a). Bcl-2 has been shown to prevent mitochondrial cytochrome c release, and subsequent caspase activation and cell death are suppressed (Reed, 1999). In many cases of human neoplasia, antiapoptotic Bcl-2 proteins were found to be overexpressed or proapoptotic Bcl-2 homologues appeared to be reduced or functionally

inactive (Reed, 1999). These alterations in expression or functionality of Bcl-2 family proteins can render tumor cells more resistant to chemotherapeutic drugs (Johnstone et al., 2002; Reed, 1999). NF- κ B is a transcriptional factor and its activity is required for the induction of more than 150 genes involved in cell growth, differentiation and apoptosis. NF- κ B targets genes that may provide antiapoptotic function, including a family of endogenous direct inhibitors of caspases, TNFR-associated factor 1 and TNFR-associated factor-2, which are thought to suppress caspase-8 activation, and the antiapoptotic Bcl-2 homolog protein Bcl-xL (Herr and Debatin, 2001).

Whole plant extracts of *Isodon japonicus* have been used in folk medicine in China, Korea and Japan for treating tumors and inflammatory diseases (Fujita et al., 1988; Tang and Eisenbrand, 1992). Kaurane diterpenes are rich in the genus *Isodon*. Therefore, much interest has recently been shown in the biological effects of kaurane diterpenes (Hwang et al., 2001; Bruno et al., 2002; Yang et al., 2002; Niu et al., 2002; Nagashima et al., 2002; Lee et al., 2002). Some kauranes also exhibit not only cytotoxic activity against various cancer cell lines but also inhibitory activity of the NF- κ B pathway in macrophages (Hwang et al., 2001; Nagashima et al., 2002; Lee et al., 2002). The inhibitory mechanism of the NF- κ B pathway by kaurane diterpenes has been well characterized and is thought to be attributable to the ability of α -methylene cyclopentanone in the molecules to react with sulfhydryl groups of cysteine residues within NF- κ B protein by Michael-type addition (Lee et al., 2002; Fujita et al., 1976). Based on the antiapoptotic properties of NF- κ B pathway, these reports suggested that kaurane diterpenes are useful candidates for antitumor agents.

We previously found cytotoxic compounds of kaurane diterpenes isolated from the Japanese liverwort *Jungermannia truncata* NEES, and we found that *ent*-11 β -hydroxy-16-kauren-15-one (KD) (Fig. 1) exhibited a cytotoxic effect via induction of apoptosis in a human leukemia cell line (HL-60 cells) (Nagashima et al., 2002; Nagashima et al., 2003). In the present study, we characterized the contribution of the caspase -8 and -9 pathways to apoptosis induced by KD in human leukemia cell lines. We presented evidence that the apoptosis induced by KD is largely dependent on the activation of caspase-8, and we also found that the cytotoxicity of KD failed to be attenuated by the overexpression of an antiapoptotic factor, Bcl-2.

Materials and methods

Reagents

Ent-11 -hydroxy-16-kauren-15-one (KD) (Fig. 1), *ent*-11, 15 -dihydroxy-16-kaurene (H-KD), (16*R*)-*ent*-11 -hydroxykauran-15-one (M-KD) (Fig. 6A) were isolated from the Japanese liverwort *Jungermannia truncata* NEES as described previously (Nagashima et al., 2002). All reagents were of analytical grade. Z-IETD-FMK (IETD), a caspase-8 inhibitor, and Z-LEHD-FMK (LEHD), a caspase-9 inhibitor, were obtained from Calbiochem (San Diego, CA). Epoxycyclohexenone (ECH) was isolated from the culture broth of a producing fungal strain as described previously (Miyake et al., 2003). KD and the above inhibitors were dissolved in dimethyl sulfoxide (DMSO). In the experimental conditions, the final concentration of DMSO did not exceed 0.5% (v/v) in the culture medium. Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Co. (Bedford, MA). Antibodies for caspase-8 and caspase-9 were purchased from Cell Signaling Technology (Beverly, MA). Anti-Bid antibody and anti- β -actin antibody were obtained from BioSource International (Camarillo, CA) and Sigma (St. Louis, MO), respectively. Dominant negative caspase-8 plasmid was kindly provided by Dr. T.C. Wu (Johns Hopkins University). Effectene reagent was obtained from Qiagen (Valencia, CA). Anti-Bcl-2 antibody and α -tubulin antibody were obtained from DAKO (Glostrup, Denmark) and Sigma, respectively. Cis-Diammineplatinum-(II) dichloride (CDDP) was purchased from Sigma.

Cell culture

HL-60 cells (human promyelocytic leukemia cells) and K562 cells (human chronic myelogenous leukemia cells) were cultured in RPMI 1640 containing 10% fetal bovine serum. HeLa cells (human cervical carcinoma) was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. All cells were maintained in a 5% CO₂ humidified atmosphere at 37 °C.

Chromatin condensation

To analyze changes in chromatin structure, cells were collected by centrifugation,

fixed with 1% glutaraldehyde, stained for 15 min at room temperature with Hoechst33342 and mounted on glass slides. Chromatin structures were examined by fluorescence microscopy, and at least 500 nuclei were observed in each sample.

DNA fragmentation assay

DNA ladder formation was assayed as described previously (Watabe et al., 2000). Briefly, cells were harvested and incubated in the lysis buffer [10 mM Tris-HCl (pH 8.0), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% (w/v) SDS, and 0.1% (w/v) RNase A] for 60 min at 50 °C. Phenol/chloroform-extracted DNA was subjected to a 1.8% agarose electrophoresis and stained with ethidium bromide.

Western immunoblots

Cells were lysed in the lysis buffer consisting of 1% Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonylfluoride and 1 mM EDTA by sonication. Equal amounts of samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to PVDF membranes. The membranes were blocked with 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.05% Tween-20 containing 5% (w/v) nonfat milk, washed, and probed with the appropriate concentration of antibody. A horseradish peroxidase-labeled antibody was used as a secondary antibody. The antibody-reactive bands were revealed by ECL-based detection (Amersham Pharmacia Biotech).

Caspase assays

Activities of caspase-8 and caspase-9 were quantified using caspase colorimetric assay kit according to the manufacturer's protocol (Promega, Madison, WI).

Establishment of Bcl-2-overexpressing stable cell lines

The *bcl-2* gene was cloned into the pGEM-T Easy vector (Promega, Madison, WI). An *EcoR* I fragment of pGEM-T Easy vector-inserted human wild-type *bcl-2* was subcloned into the pCI-neo vector (Promega). A permanent cell line expressing Bcl-2 was established by transfecting pCI-*bcl-2* into K562 cells followed by G418 selection. The cells transfected with the pCI-neo vector were designated K562/Neo cells, and the

clones expressing high levels of Bcl-2 protein were designated K562/Bcl-2 cells.

Cytotoxicity

Cytotoxicity of KD in cells was determined by the colorimetric 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt using a cell counting kit-8 according to the manufacturer's instructions (WAKO Pure Chemicals, Ltd, Osaka, Japan).

Statistical analysis

Differences were statistically evaluated by one-way ANOVA. The acceptable level of significance was set at $p < 0.05$.

Results

Involvement of caspase-8 activation in KD-induced apoptosis

There are various biochemical complex systems that induce apoptosis (Hengartner, 2000). Among them, caspases, a family of proteases, play central roles in the initiation and execution of the apoptotic pathway (Thornberry and Lazebnik, 1998). Caspase-8 and -9 are representative of the initiator caspases, which activate the executor caspases, including caspase-3. We previously found that treatment of human leukemia HL-60 cells with KD induced proteolysis of PARP, a hallmark of caspase-3 activation (Nagashima et al., 2003; Lippke et al., 1996; Tewari et al., 1995). So, we firstly determined whether treatment with KD induces activation of initiator caspases. As shown in Fig. 2A, treatment with KD elevated the activity levels of both caspase-8 and caspase-9. Caspase-8 and -9 exist as a pro-form and is processed into an active form by apoptotic stimuli (Thornberry and Lazebnik, 1998). Bid, a Bcl-2 family member, is cleaved by the active form of caspase-8 but not by caspase-9 (Li et al., 1998). We next examined by immunoblotting the time course of caspase-8 and -9 activation in response to KD (2 μ M) treatment using processing of the caspases and cleavage of Bid as a sign of activation of caspase-8. After KD treatment, caspase-8 was processed within 3 hr, and cleaved Bid also appeared at 3 hr (Fig. 2B, 4A). Processing of caspase-9 was also observed at 6 hr of treatment (Fig. 2B). To determine the involvement of caspase-8 and caspase-9 in KD-induced apoptosis, we investigated the induction of a ladder pattern typical of internucleosomal fragmentation, which is considered to be an early event in apoptosis, using each specific inhibitor against caspase-8 and caspase-9. IETD, a caspase-8-specific inhibitor, but not LEHD, a caspase-9-specific inhibitor, attenuated the appearance of DNA fragmentation and nuclear condensation, suggesting that caspase-8 is involved in apoptosis induced by KD (Fig. 3A, B). IETD inhibited the proteolysis of Bid but not the activation of caspase-9 induced by KD (Fig. 4B, data not shown). While pre-treatment with LEHD did not inhibit both the cleavage of Bid (Fig. 4C). Epoxycyclohexenone (ECH) is a novel type of inhibitor of caspase-8, and ECH inhibited caspase-8 via directly binding to caspase-8 (Miyake et al., 2003). Indeed, treatment with ECH partly attenuated cytotoxicity and induction of apoptosis induced by KD, whereas ECH did not affect cytotoxicity and induction of apoptosis

induced by CDDP, an inducer of apoptosis via caspase-9-dependent pathway (Fig. 5A). Next, we confirmed the involvement of caspase-8 in KD-induced apoptosis by expression of dominant negative caspase-8 (dncaspase-8). HL-60 cells are difficult to introduce plasmid, and so we investigated the effects of dncaspase-8 on KD-induced apoptosis in HeLa cells, a well-used cell line in apoptotic studies. Expression of dncaspase-8 resulted in partial attenuation of apoptosis (Fig. 5B). Taken together, these results suggest that caspase-8 activation is partly involved in KD-induced apoptosis.

Involvement of the enone group in induction of apoptosis by KD

The structure of α -methylene cyclopentanone in diterpenes reacted with sulfhydryl groups of cysteine residues in a Michael-type addition (Hwang et al., 2001; Lee et al., 2002; Fujita et al., 1976). Next, we examined the structure-activity relationship of KD. KD-related compounds without the enone group exhibited weaker cytotoxicity than that of KD, and IC₅₀ values against HL-60 cells after 12 hr of treatment were 0.56 μ M (KD), 50 μ M (hydroxy-KD) and 70 μ M (methyl-KD) (Fig. 6A and B). In fact, treatment with KD but not 1 or 2 at 2 μ M resulted in appearance of DNA ladder and processing of caspase-8 (Fig. 6C and D), suggesting that the enone group in the molecule is important functional group for induction of apoptosis.

Effect of antiapoptotic Bcl-2 proteins on KD-induced apoptosis

Bcl-2 is an integral membrane protein located mainly at mitochondria and is known to prevent apoptosis in response to a variety of stimuli, including treatment with chemotherapeutic agents (Gross et al., 1999a; Reed, 1999). Overexpression of Bcl-2 is frequently observed in many tumors and is thought to be a major factor in resistance to anticancer drugs in chemotherapy (Johnstone et al., 2002; Reed, 1999). To evaluate the effect of Bcl-2 overexpression on KD-induced cytotoxicity, we introduced *bcl-2* gene into K562, a human leukemia cell line (Fig. 7A). As expected, the cytotoxicity of CDDP was significantly inhibited by overexpression of Bcl-2, whereas KD-induced cytotoxicity was not inhibited by Bcl-2 (Fig. 7B). Interestingly, K562/Bcl-2 cells were more sensitive to KD than K562/Neo cells. To evaluate this phenomenon, we examined the induction of apoptosis in KD-treated K562/Neo and K562/Bcl-2 cells.

Overexpression of Bcl-2 inhibited apoptosis induced by CDDP, but the percentage of apoptotic cells was increased by overexpression of Bcl-2 (Fig. 7C). Processing of caspase-8 and proteolysis of Bid appeared earlier in K562/Bcl-2 cells than in K562/Neo cells (Fig. 7D). Thus, it is likely that overexpression of Bcl-2 made cells sensitive against KD-induced apoptosis in the case of K562 cells.

Discussion

Chemotherapeutic agents have been shown to activate apoptotic pathways (Lowe and Lin, 2000), but tumor cells confer resistance to apoptotic stimuli in tumorigenesis through a variety of antiapoptotic factors, including Bcl-2 and NF- κ B (Lowe and Lin, 2000; Johnstone et al., 2002). Therefore, effective strategies for overcoming the resistance to anticancer drugs are important in chemotherapy. Indeed, inhibition of NF- κ B was shown to potentiate the efficacy of antitumor activities by ionizing and some chemotherapeutic compounds (Wang et al., 1996; Wang et al., 1999). In this respect, various terpenoids are attractive natural compounds as therapeutic agents for the treatment of cancer. Some terpenoids, including kaurane diterpenes, kaurene diterpenes and sesquiterpenes inhibited the activity of the NF- κ B pathway (Lee et al., 2002; Castrillo et al., 2001; Lyss et al., 1998). Therefore, terpenoids that activate proapoptotic signals are promising candidates for cancer chemotherapy.

We previously found that *ent*-11-hydroxy-16-kauren-15-one (KD) (Fig. 1), isolated from the Japanese liverwort *Jungermannia truncata* NEES, exhibited a cytotoxic effect via induction of apoptosis in human leukemia cell lines (HL-60 cells) (Nagashima et al., 2002; Nagashima et al., 2003). In the present study, we investigated the involvement of the proapoptotic signal in KD-induced apoptosis. Treatment of HL-60 cells with KD elevated the activity level of caspase-8, and a specific inhibitor of caspase-8 attenuated KD-induced apoptosis. Proteolysis of Bid, a substrate for activated caspase-8, was significantly reduced by pretreatment with a caspase-8 inhibitor. Moreover, expression of dominant negative caspase-8 also attenuated KD-induced apoptosis. These results suggest that KD induces apoptosis through a caspase-8 dependent pathway. Apoptotic pathways are classified into two routes: death-receptor pathways and mitochondrial pathways (Hengartner, 2000). The apoptotic signal is transmitted for activation of caspase-8 in the death-receptor route or activation of caspase-9 in the mitochondrial route. Cross-talk and integration between the death-receptor and mitochondrial pathways is provided by Bid, a member of the proapoptotic Bcl-2 family of proteins. Caspase-8-mediated cleavage of Bid greatly increases its pro-death activity and results in its translocation to mitochondria, where it promotes cytochrome *c* release from mitochondria with the result that cytochrome *c* and ATP-

dependent oligomerization of Apaf-1 allow recruitment of procaspase-9 into the apoptosome complex followed by the activation of caspase-9 (Hengartner, 2000). Indeed, KD elevated the activity level of caspase-9 and increased the proteolysis of caspase-9 (6 hr after KD treatment) posterior to cytochrome *c* release from mitochondria (3 hr) (data not shown); however, a specific caspase-9 inhibitor did not attenuate KD-induced apoptosis (Fig. 3A, B). Previous reports indicated that cross-talk between the death-receptor and mitochondrial pathways in apoptosis was minimal and that the two pathways operated largely independently of each other (Gross et al., 1999b). KD-induced apoptosis and activation of caspases were not inhibited by overexpression of Bcl-2 (Fig. 7). Taken together, the results suggest that KD causes apoptosis partly via caspase-8. More recently, the mechanism of activation of caspase-8 has been investigated in detail. The mechanism of activation of caspase-8 by KD is considered to involve three pathways: a death receptor-dependent pathway, a p38^{MAPK}-dependent pathway and a caspases-dependent pathway. Schrantz et al. reported that p38^{MAPK} activated caspase-8 during transforming growth factor α -induced apoptosis (Schrantz et al., 2001), and Castrillo and colleagues reported that kaurene diterpenes modulated p38^{MAPK} activity (Castrillo et al., 2001). In our preliminary experiment, treatment with SB203580, a specific inhibitor of p38^{MAPK}, resulted in attenuation of KD-induced apoptosis accompanied by inhibition of caspase-8 processing (data not shown). Further experiment for determination of involvement of p38^{MAPK} activation in KD-induced caspase-8 activation will be useful for understanding the apoptotic machinery during KD-induced apoptosis. Fas is a cell surface death receptor belonging to the tumor necrosis factor receptor (TNFR) family (Peter et al., 1998). Binding of the Fas ligand to Fas causes the formation of a death-induced signaling complex (DISC), in which Fas recruits FADD/MORT1 and procaspase-8, followed by the activation of caspase-8 (Medema et al., 1997). There is also evidence supporting the idea that KD-induced apoptosis involves pathways dependent on death receptors, including Fas and TNFR. Fas-mediated or TNFR-mediated apoptosis was not inhibited by overexpression of antiapoptotic Bcl-2 protein (Moreno et al., 1996). In line with this, we found that overexpression of Bcl-2 reduced the cytotoxicity of the anticancer drug cisplatin but did not affect that of KD, suggesting that KD activates a death-receptor pathway in its apoptotic pathway. It would be interesting to investigate the

involvement of DISC in KD-mediated activation of caspase-8.

The most interesting finding is that overexpression of Bcl-2, which is a chemoresistance factor in many tumors, was unable to prevent KD-induced apoptosis. An additional putative mechanism of the outcome of antiapoptotic Bcl-2 seems to be the binding to a cysteine residue of procaspase-8 or an activator of caspase-8. The selective reactions of α -methylene-cyclopentanone system with only SH-group among many nucleophilic groups (-OH, -NH₃, -COO-) in the protein can be rationalized by the “Hard Soft Acids Base” principle., and kaurane diterpenes can react covalently with a thiol group via Michael-type addition (Lee et al., 2002; Fujita et al., 1976). Indeed, Voehringer et al. found that overexpression of Bcl-2 caused redistribution of glutathione to the nucleus (Voehringer et al., 1998), and glutathione depletion enhanced cell death via mitochondrial dysfunction in Bcl-2-overexpressing cells (Armstrong and Tones, 2002). In fact, pretreatment with glutathione or N-acetyl cysteine, but not N-acetyl serine, inhibited induction of apoptosis by KD treatment (data not shown).

In summary, we found that a kaurene diterpene, *ent*-11-hydroxy-16-kauren-15-one (KD), possesses interesting properties with regard to its apoptosis-inducing mechanism. Most chemotherapeutic agents exhibit only pro-apoptotic effects. However, kaurene diterpenes also inhibited the NF- κ B pathway, which is also induced by antiapoptotic factors (Castrillo et al., 2001). KD activated the proapoptotic protease caspase-8. Taken together, the results suggest that KD has dual effects on the induction of apoptosis: induction of a proapoptotic pathway and inhibition of an antiapoptotic pathway. In fact, the apoptotic effect of TNF- α was enhanced by co-treatment with KD (data not shown). Thus, KD may serve as a leading compound for the development of new chemotherapeutic drugs to overcome resistance of tumors to chemotherapy.

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Figure Legends

Figure 1. Structure of KD.

Figure 2. Activation of caspase-8 and caspase-9 in KD-treated HL-60 cells.

A) Caspase activity. HL-60 cells were treated with KD (2 μ M) for 6 hr. Then, the activities of caspase-8 and -9 were evaluated by a colorimetric assay using a commercially available kit. Data are means \pm SD (n=3). *Significantly different from the vehicle-treated cells ($p < 0.05$). The data are representative of three independent experiments. B) Time-course changes in activation of caspases during KD-induced apoptosis. Cells were treated with KD (2 μ M) for the indicated periods, and then processing of caspase-8 and -9 were examined by Western blotting. Data are representative of three independent experiments.

Figure 3. Suppression of KD-induced apoptosis by caspase-8 inhibitor. After treatment with an inhibitor of caspase-8 (IETD) or caspase-9 (LEHD) at 200 μ M for 2 hr, HL-60 cells were treated with KD (2 μ M) for 6 hr or etoposide (2 μ M) for 3 hr. DNA ladder formation (A) was then evaluated. Nuclear condensation (B) was also examined. The cells were then fixed with glutaraldehyde and stained with Hoechst33342. Nuclear condensations were observed under a fluorescence microscope. At least 500 nuclei in each sample were evaluated. Data are means \pm SD (n=4). Data without superscript letters are significantly different at $p < 0.05$. The data are representative of three independent experiments.

Figure 4. Effect of IETD on cleavage of Bid induced by KD.

A) Time-course changes in cleavage of Bid during KD-induced apoptosis. Cells were treated with KD (2 μ M) for the indicated periods, and then cleavage of Bid was examined by Western blotting. B) Effect of IETD and LEHD on cleavage of Bid. After 2 hr of pretreatment with IETD or LEHD at 200 μ M, HL-60 cells were treated with KD (2 μ M) for 6 hr. Then lysates were prepared. Cleavage of Bid was assayed by Western blotting. The data are representative of three independent experiments.

Figure 5. Involvement of caspase-8 in apoptosis induced by KD.

A) Effects of ECH on apoptosis induced by KD. After 1 hr of pretreatment with ECH (25 μ M), HL-60 cells were treated with KD (2 μ M) or CDDP (50 μ M) for 6 hr. The cells were then fixed with glutaraldehyde and stained with Hoechst33342. Nuclear condensations were observed under a fluorescence microscope. At least 500 nuclei in each sample were evaluated. Data are means \pm SD (n=4). Data without superscript letters are significantly different at $p < 0.05$. B) Effects of dominant negative caspase-8 on KD-induced apoptosis. HeLa cells were transfected mock plasmid or dominant negative caspase-8 (dncasp-8) plasmid using effectene reagent according to the manufacture's protocol. After 24 hr, the cells were treated with KD or vehicle for 18 hr. Then nuclear condensation was observed as described above. Data are means \pm SD (n=4). *Significant difference between the indicated group ($p < 0.05$). The data are representative of three independent experiments.

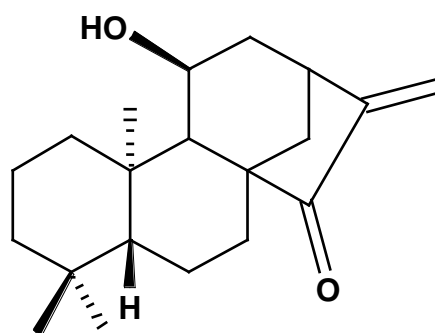
Figure 6. Involvement of the enone group at C₁₅₋₁₆ in apoptosis induced by KD.

A) Structures of KD-related compounds. B) Cytotoxicity of KD-related compounds. HL-60 cells were treated with the compounds for 12 hr at the indicated concentration. Viability of the cells was determined using a commercial available kit as described in Materials and methods. Data are means \pm SD (n=4). C) Induction of apoptosis by KD-related compounds. The cells were treated with the compounds at 2 μ M for 12 hr, and then DNA ladder formation was assayed. D) Activation of caspase-8 by KD-related compounds. The cells were treated with the compounds at 2 μ M for 12 hr, and then processing of caspase-8 and β -actin were assayed by Western blotting. Data are representative of three independent experiments.

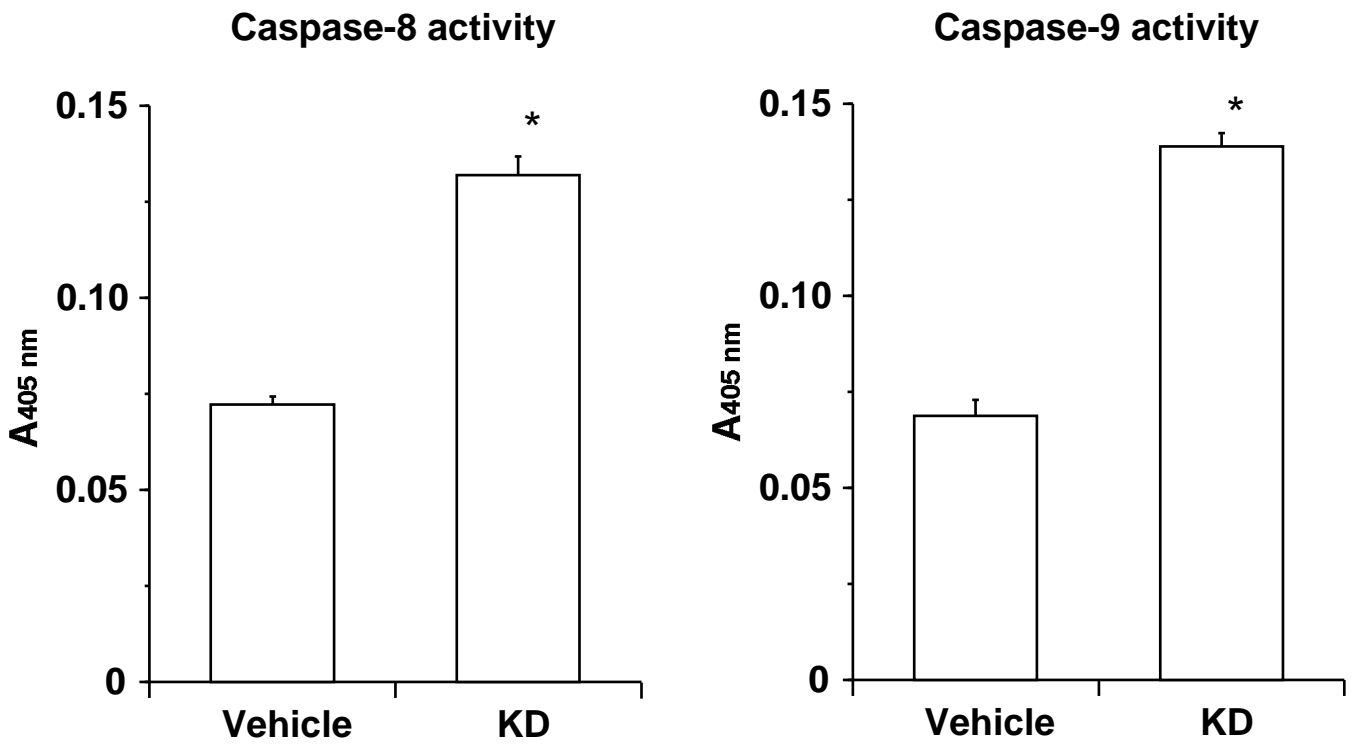
Figure 7. Effect of Bcl-2 overexpression on KD-induced cytotoxicity.

A) Expression of Bcl-2 in K562/Bcl-2. Bcl-2 expression was assayed by Western blotting in K562/Bcl-2 and K562/Neo. B) Effect of Bcl-2 expression on KD-induced cytotoxicity. Mock-transfected (open column) or Bcl-2-overexpressing (scattered column) K562 cells were treated with CDDP or KD at the indicated concentrations for 18 hr. Then the viability was determined using a cell counting kit as described in Materials and methods. Data are means \pm SD (n=4). *Significantly different from

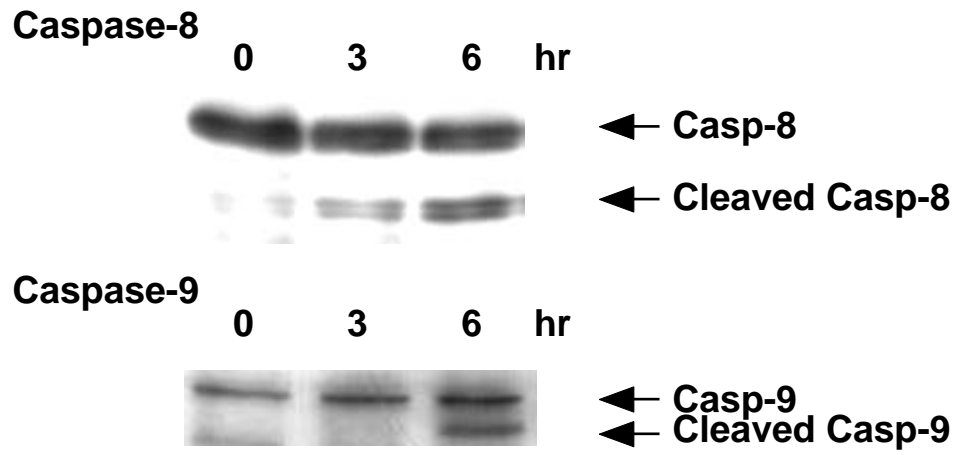
the Neo vector-transfected cells treated similarly. Data are representative of three independent experiments. C) Effect of Bcl-2 on CDDP- or KD-induced apoptosis. K562/Neo or K562/Bcl-2 cells were treated with CDDP (100 μ M) or KD (5 μ M) for 12 hr. The cells were then fixed with glutaraldehyde and stained with Hoechst33342. Nuclear condensations were observed under a fluorescence microscope. At least 500 nuclei in each sample were evaluated. Data are means \pm SD (n=4). *Significantly different from the Neo vector-transfected cells treated similarly. Data are representative of three independent experiments. D) Time-course changes in activation of caspase-8 by the treatment with KD in Bcl-2-overexpressing K562 cells. K562/Neo and K562/Bcl-2 cells were treated with KD (5 μ M) for the indicated periods. Then lysates were subjected to SDS-PAGE, and processing of caspase-8 and proteolysis of Bid were assayed by Western blotting. The results are representative of four independent experiments.



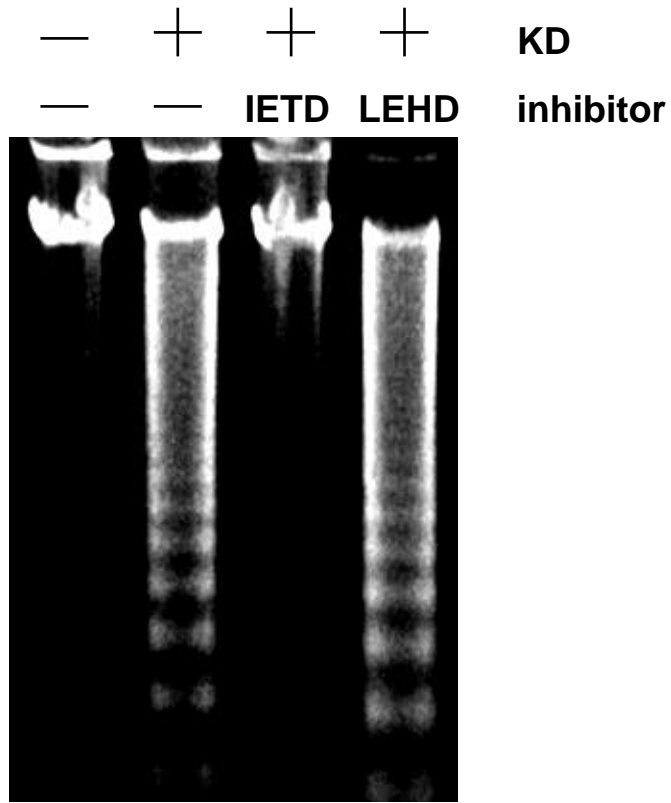
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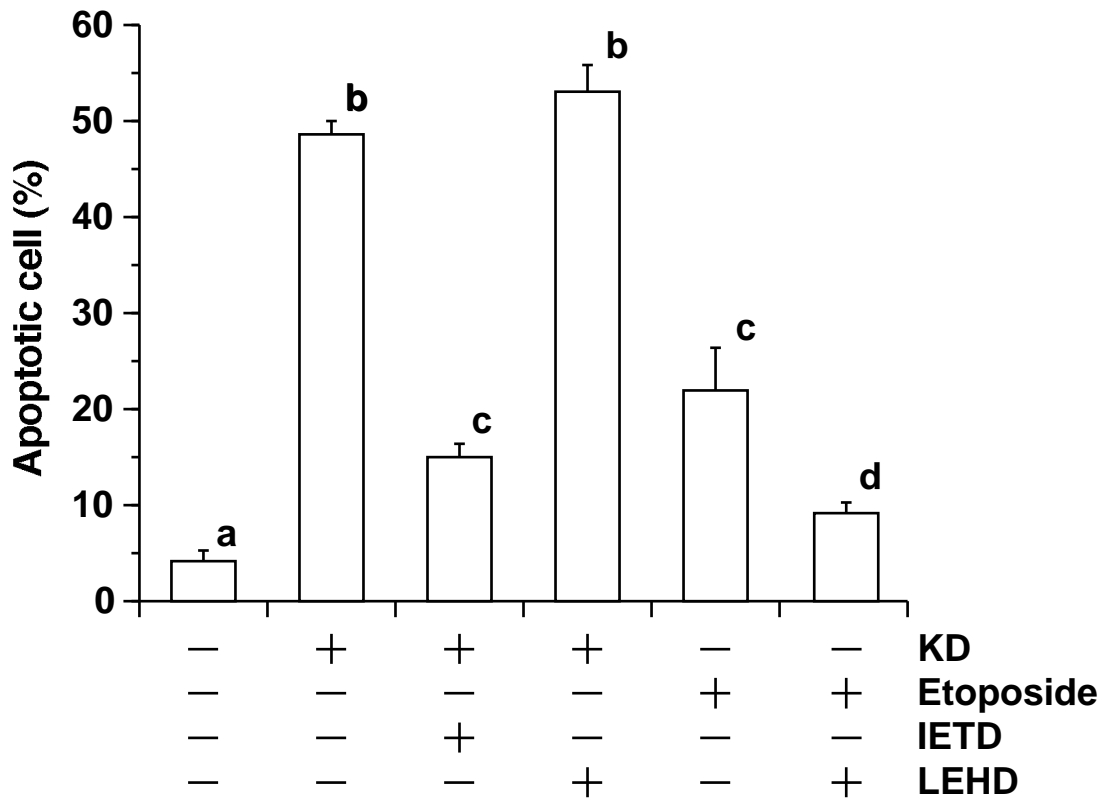
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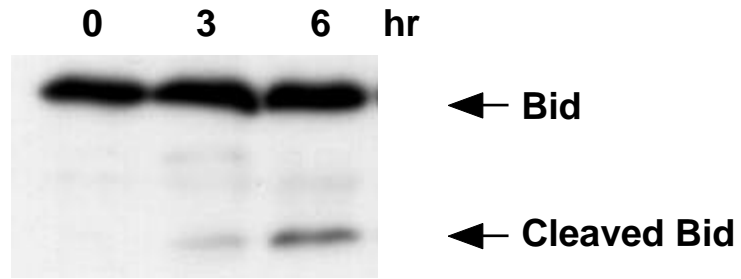


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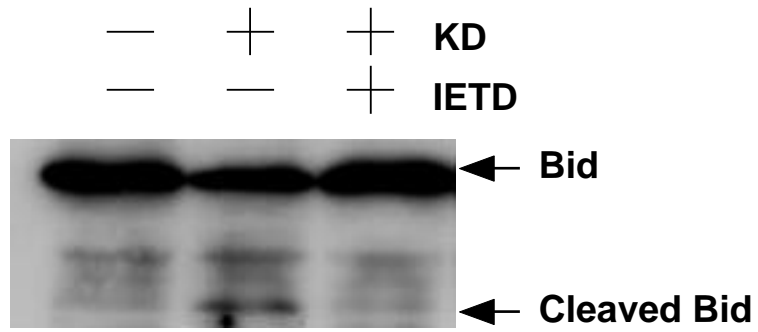
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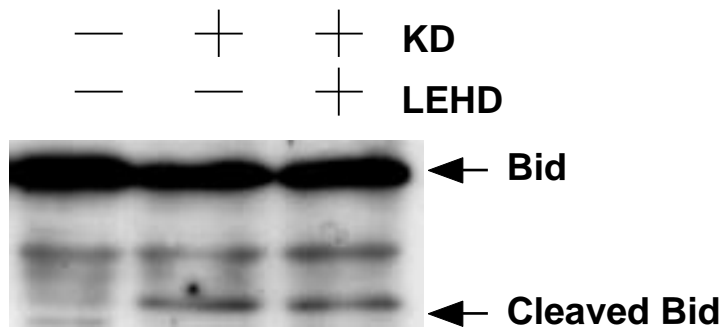
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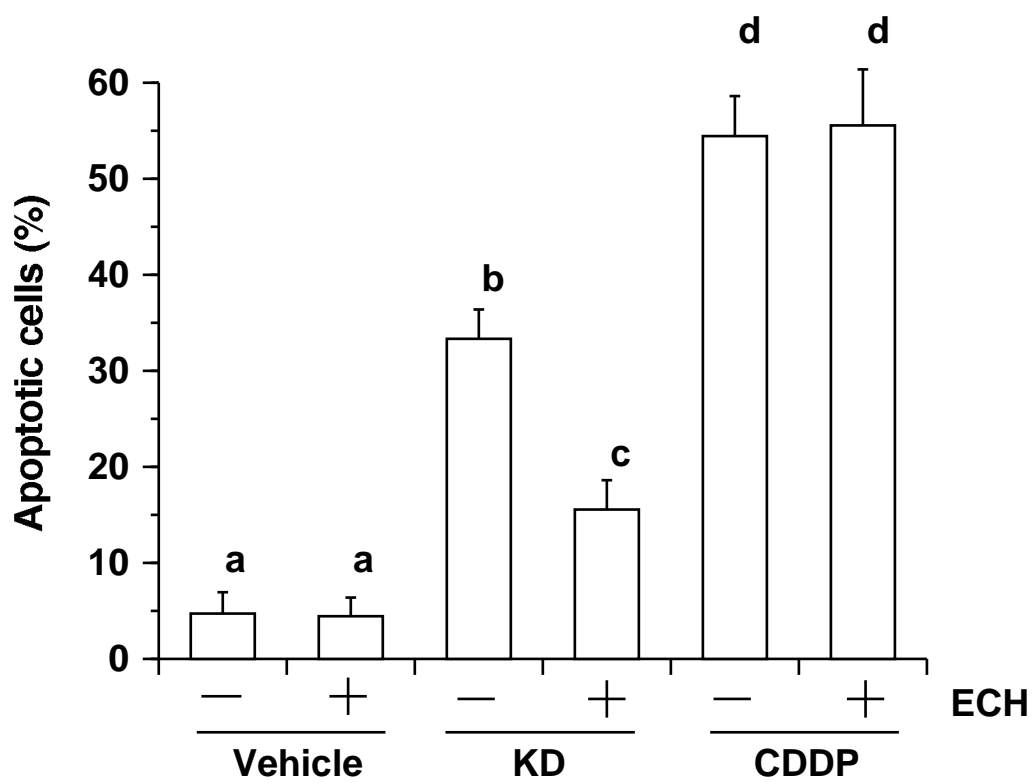


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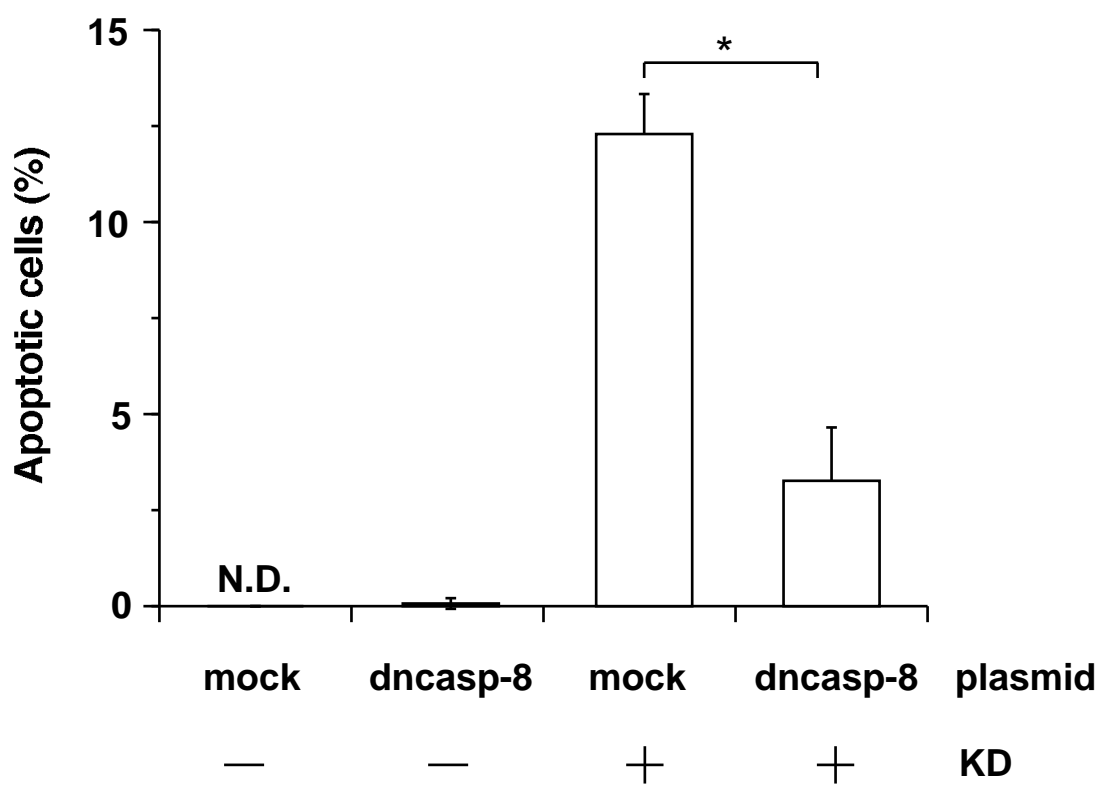
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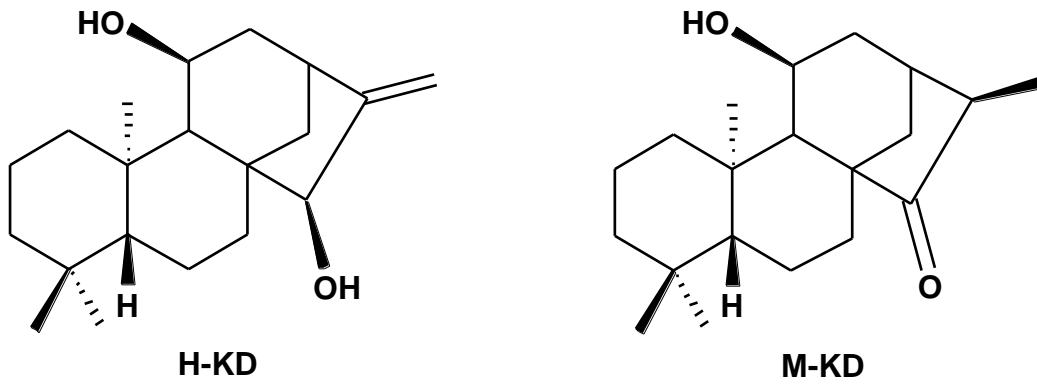
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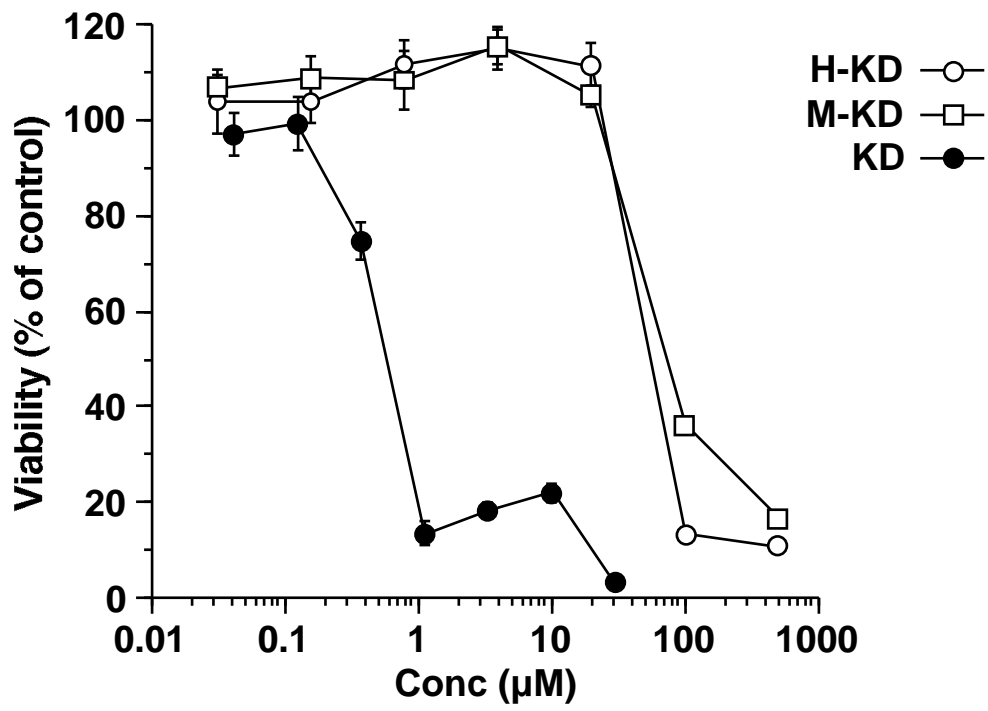
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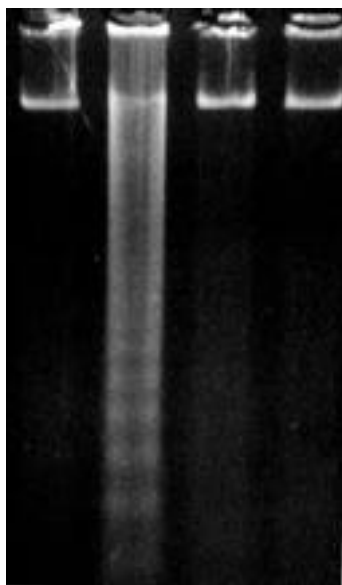


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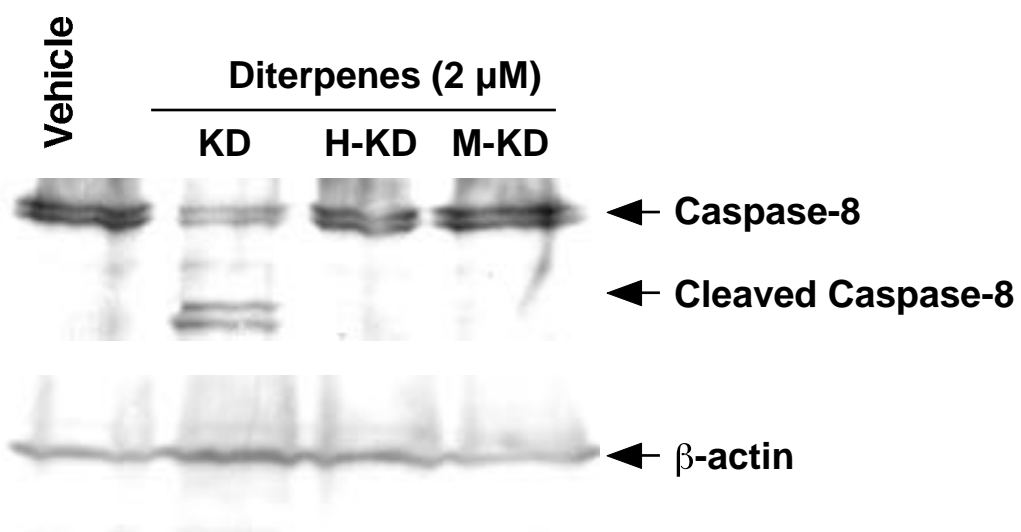


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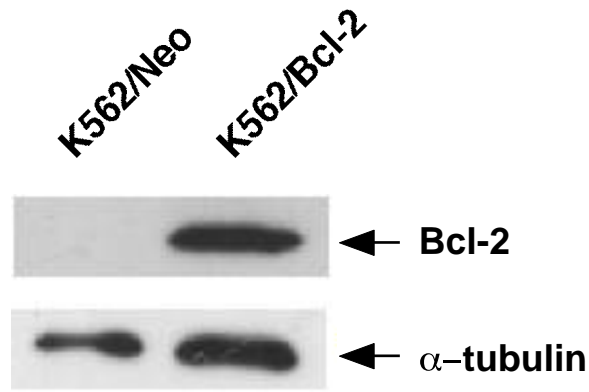
Vehicle Diterpenes (2 μ M)
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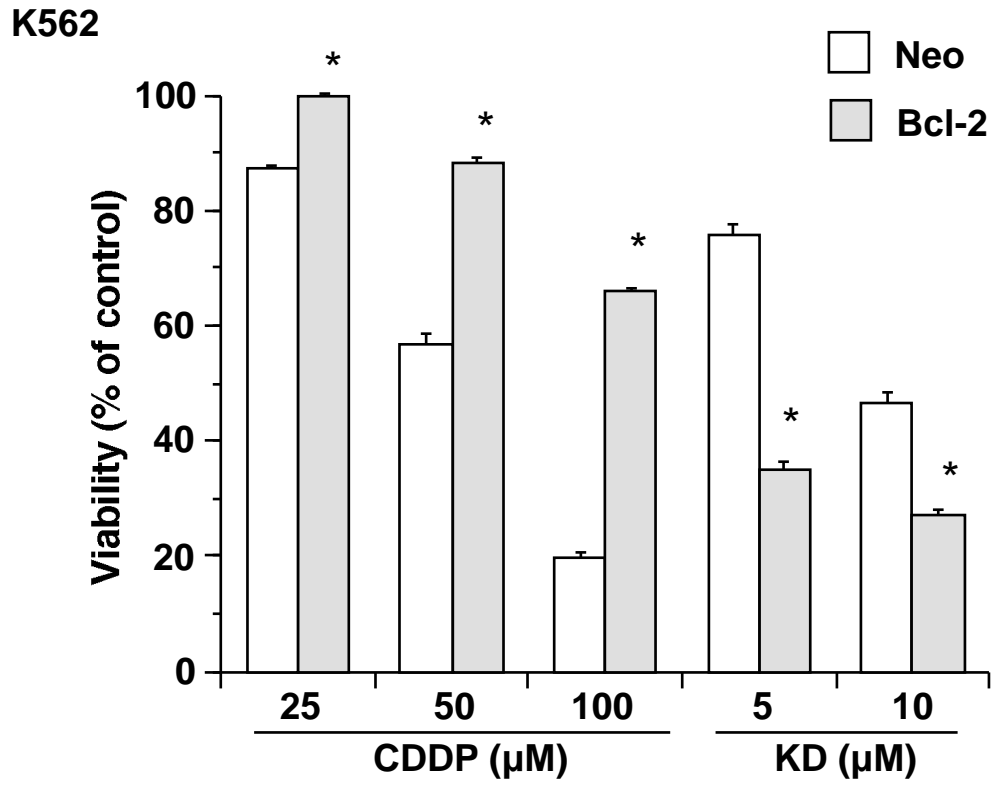
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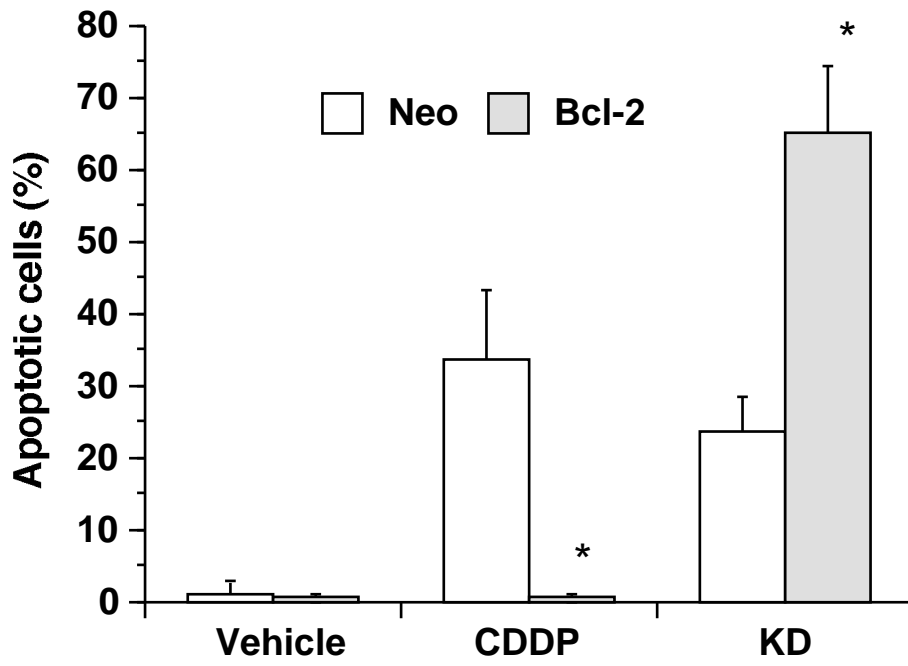
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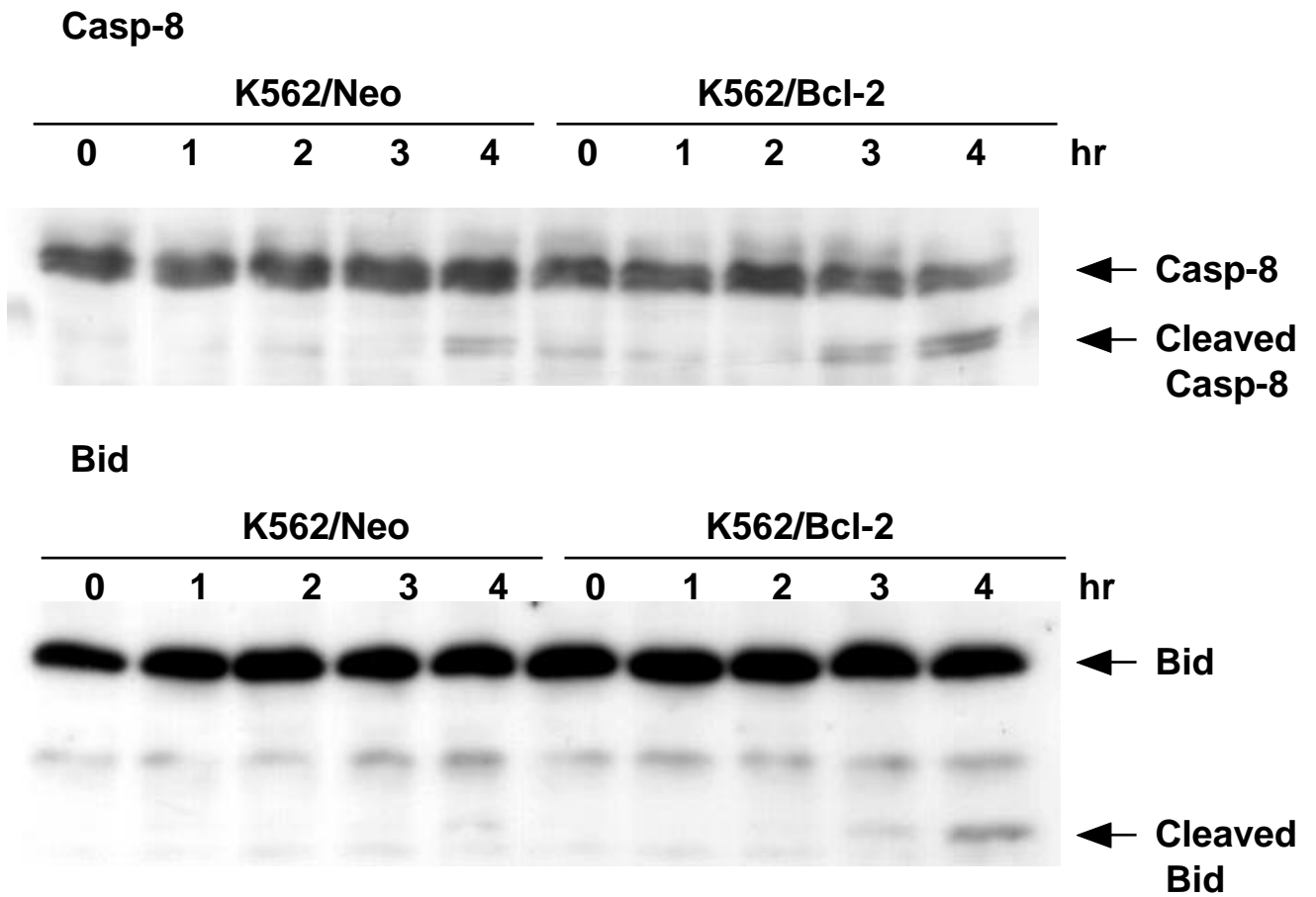
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