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Unspecific Activation of Caspases During the Induction of Apoptosis by Didemnin B in Human Cell Lines

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Abstract Caspases have been implicated in the induction of apoptosis in most systems studied. The importance of caspases for apoptosis was further investigated using the system of didemnin B-induced apoptosis. We found that benzyloxycarbonyl-VAD-fluoromethylketone, a general caspase inhibitor, inhibits didemnin B-induced apoptosis in HL-60 and Daudi cells. Acetyl-YVAD-chloromethylketone, a caspase-1-like activity inhibitor, inhibits didemnin B-induced apoptosis in Daudi cells, whereas the caspase-3-like activity inhibitor, acetyl-DEVD-aldehyde, has no effect. Using immunoblots to investigate cleavage of caspases-1 and -3, we found that both caspases are activated in both cell lines. We showed that the caspase substrate poly(ADP-ribose)polymerase is cleaved in these cells after didemnin B treatment. In both cell lines, poly(ADP-ribose)polymerase cleavage is inhibited by benzyloxycarbonyl-VAD-fluoromethylketone and also by acetyl-YVAD-chloromethylketone in Daudi cells. These results indicate that a caspase(s) other than caspase-3 is required for didemnin B-induced apoptosis. We show that caspases may be activated during apoptosis that are not required for the progression of apoptosis. J. Cell. Biochem. 72:269–278, 1999. © 1999 Wiley-Liss, Inc.

Key words: poly(ADP-ribose)polymerase; roscovitine; caspase-1; caspase-3; HL-60; Daudi

Didemnin B is a branched cyclic peptolide that is currently being tested as an anticancer agent in clinical trials [Shin et al., 1991, 1994]. It has been shown to induce complete apoptosis rapidly in human promyeloid HL-60 cells [Grubb et al., 1995], but other cell types, such as human lymphoblastoid Daudi cells, undergo apoptosis more slowly (Fig. 1). Because didemnin B has such an immediate effect on HL-60 cells growing asynchronously in culture, we used it as a model system to study the minimal requirements for apoptosis to occur. It has been shown that didemnin B causes inhibition of protein, RNA, and DNA synthesis [Crampton et al., 1984], albeit at higher concentrations than those used in our studies, and that it requires protein tyrosine kinase activity to induce apoptosis [Johnson et al., 1996].

Until recently, most studies of apoptosis have indicated that the apoptotic pathway is specific for the type of cell and inducing stimulus; however, it is becoming apparent that all these different apoptotic pathways are converging near the endpoint to use the same cellular components, particularly the caspase family of proteases. Caspases (*c*ysteine *asp*artic acid pro*teases*) were first identified to be of importance for apoptosis because of their similarity to the CED-3 protein, which is required for programmed cell death in the nematode *Caenorhabditis elegans* [Yuan and Horvitz, 1990]. CED-3 was found to have sequence homology with human interleukin-1β (IL-1β-converting enzyme (ICE) [Yuan et al., 1993]. Subsequently other human proteases with homology to CED-3 (ICE-like proteases) were identified, which have recently been renamed as caspases [Alnemri et al., 1996]. Caspases are synthesized as proenzymes that must be cleaved to become activated. ICE, now known as caspase-1, is cleaved from a 45-kDa proenzyme to a p10 and p20 subunit. The active enzyme is a tetramer consisting of two p10 and two p20 subunits [Wilson et al., 1994]. Caspases can cleave and activate other caspases initiating a proteolytic cascade. Caspases are also known to cleave many other substrates during cell death, including poly-(ADP-ribose)polymerase (PARP), DNA-dependent protein kinase, protein kinase Cδ, sterol regulatory element binding proteins, nuclear lamins A and C, the 70-kDa component of U1

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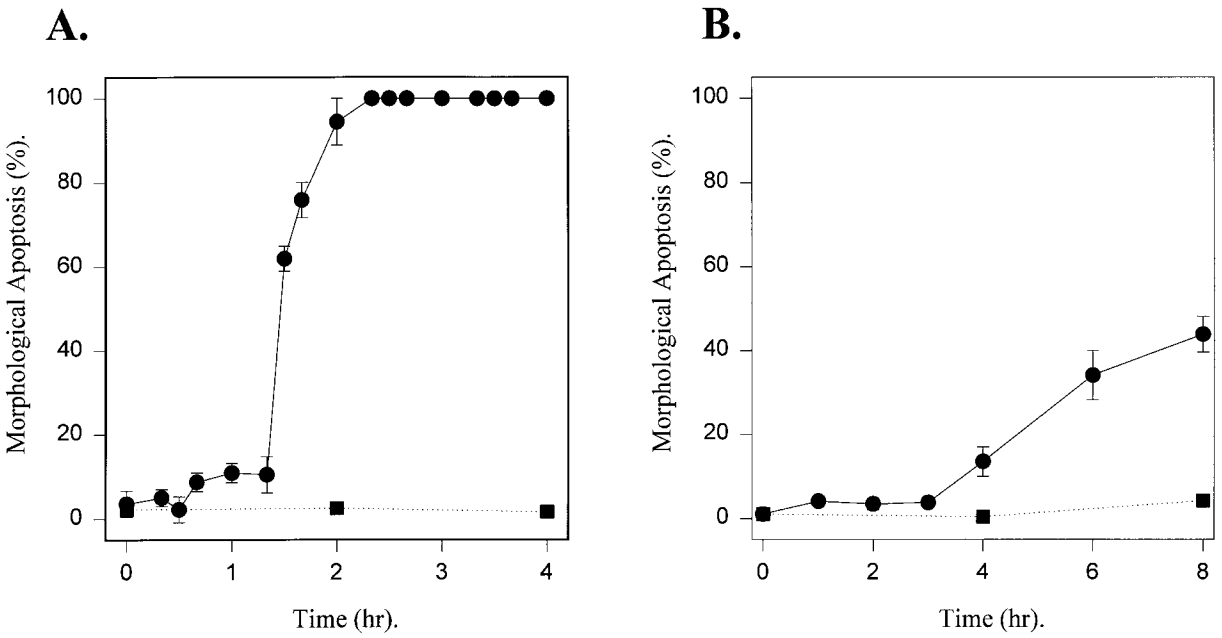

Fig. 1. Comparison of time courses of induction of apoptosis by didemnin B in HL-60 and Daudi cells. HL-60 **(a)** and Daudi **(b)** cells were treated with 1 μM didemnin B (●) or 0.1% ethanol (vehicle control, ■), and percentage apoptosis was determined as described under Materials and Methods after the indicated times. Percentage apoptosis is plotted versus time.

small nuclear ribonucleoprotein, and retinoblastoma protein [for review, see Porter et al., 1997]. Members of the caspase family have been shown to be required for apoptosis with few exceptions: evidence has been given that cell death may still occur in the presence of caspase inhibitors, but the DNA fragmentation that is considered a hallmark for apoptosis is prevented by these caspase inhibitors [Xiang et al., 1996; McCarthy et al., 1997].

It was recently shown that apoptosis may occur in the presence of z-VAD-fmk, indicating that apoptosis may proceed without activation of any of the known caspases [Monney et al., 1998]. Monney and colleagues showed that defects that they generated in the ubiquitin pathway could induce apoptosis without activation of caspases-3 or -6 or cleavage of PARP. These results suggest that apoptosis may bypass caspase activation. It is becoming clear that requirement for different caspases depends on the cell type and the inducer.

Kaufmann et al. [1993] first showed that poly(ADP-ribose)polymerase, an enzyme believed to be involved in DNA repair, is cleaved in cells undergoing apoptosis. Later, CPP32 (caspase-3) was identified as a protease with ICE (caspase-1) homology, which could cleave PARP in vitro [Nicholson et al., 1995]. Caspase-3 is a 32-kDa proenzyme which, like caspase-1, must be cleaved to be activated. It can be cleaved to a p12 and a p17 subunit. The active enzyme consists of two p12 subunits and two p17 subunits [Nicholson et al., 1995].

To investigate the requirement for caspases in didemnin B-induced apoptosis we have used caspase inhibitors and antibodies to caspases-1 and -3 to detect cleavage and consequent activation of caspases. We also studied the role of caspases in cleavage of PARP in didemnin B-induced apoptosis using both HL-60 and Daudi cells.

MATERIALS AND METHODS

Didemnin B was kindly supplied by the National Cancer Institute (NCI, Bethesda, MD), and roscovitine was provided by Laurent Meijer (C.N.R.S., Station Biologique, France). The caspase inhibitors z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD-fmk), Ac-Tyr-Val-Ala-Asp-chloromethylketone (Ac-YVAD-cmk), and Ac-Asp-Glu-Val-aspartic acid aldehyde (Ac-DEVD-CHO) were purchased from Bachem (Bubendorf, Switzerland). Mouse monoclonal anti-PARP (clone C-2–10) was from Biomol (Plymouth Meeting, PA). Rabbit polyclonal anti-caspase-1 (p20 subunit) was from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-caspase-3 (p17 subunit) was kindly provided by Donald W. Nicholson, Merck-Frosst (Quebec, Canada).

Cell Culture

Human promyeloid HL-60 cells and human lymphoblastoid Daudi cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine, and 5 mM HEPES (pH 7.4) at 37°C in the presence of 5% CO_2 in a humidified environment.

Quantification of Apoptosis

Treatment of HL-60 or Daudi cells with didemnin B or roscovitine with or without caspase inhibitors was performed at a cell density of approximately 2×10^5 cells/ml. Didemnin B was dissolved in ethanol; Ac-YVAD-cmk, z-VAD-fmk, and roscovitine were dissolved in dimethylsulfoxide (DMSO); and Ac-DEVD-CHO was dissolved in dH_2O before dilution in medium. After the indicated period of incubation with the compounds, 200-µl aliquots of cells were removed and centrifuged onto glass slides coated with 0.01% (w/v) poly-L-lysine at 2,000 rpm for 5 min in a cytospin. Cells were then fixed in ethanol/acetic acid (3:1) for 5 min, rinsed in dH_2O for 1 min, and stained with 1 µg/ml 4′,6-diamidino-2-phenylindole (2HCl) for 5 min. After rinsing in dH_2O for 1 min, slides were mounted using antifading mounting medium (10 mg/ml p-phenylenediamine in 90% glycerol, pH 9.0), and nuclear morphology was analyzed under ultraviolet (UV) light (280 nm), using a Nikon microphot-FX fluorescence microscope. The percentage of apoptosis was determined by counting at least 400 nuclei from each slide.

Immunoblotting for Caspases-1 and -3

HL-60 or Daudi cells (4×10^5) were treated with didemnin B or roscovitine with or without caspase inhibitors. After the indicated time of incubation, cells were washed once in ice-cold phosphate-buffered saline (PBS), consisting of 8.2 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 138 mM NaCl, 2.7 mM KCl (pH 7.4), and pelleted. Cells were resuspended in 40 µl 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin A, 1 mM NaF, and then vortexed. After 15 min on ice, samples were centrifuged for 15 min at 4°C; 4 × loading buffer was added to the supernatant to yield a final concentration of 45 mM Tris-HCl (pH 6.8), 15% glycerol, 1.5% sodium dodecyl sulfate (SDS), 0.001% bromophenol blue, and 1.75% β-mercaptoethanol for anti-CPP32 blots only), and lysates were incubated at 100°C for 2 min. After determining protein concentration, 20 µl lysate was resolved in a 12% or a 4–20% (Novex, San Diego, CA) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before transfer to nitrocellulose or PVDF. The membrane was blocked in 3% skim milk powder (Bio-Rad, Sydney, Australia) in PBS and incubated with 3 µg/ml anti-caspase-1 or a 1:2,000 anti-caspase-3 dilution in the blocking mixture followed by a 1:2,000 dilution of anti-rabbit horseradish peroxidase (HRP) conjugate (Amersham, Sydney, Australia) and enhanced chemiluminescence (ECL) detection (Amersham, Sydney, Australia).

Immunoblotting for PARP

HL-60 or Daudi cells (2×10^5) were treated with didemnin B or roscovitine with or without pretreatment with caspase inhibitors. At the end of the incubation periods, cells were washed once in ice-cold PBS and pelleted. Cells were resuspended in 100 µl 62.5 mM Tris-HCl (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 0.003% bromophenol blue, and 5% β-mercaptoethanol, and then sonicated for 30 s and incubated at 65°C for 15 min. A total of 50 µl lysate was then resolved on an 8% SDS-PAGE before transfer to nitrocellulose. The membrane was blocked in 5% skim milk powder, PBS, and 0.1% Tween-20 and incubated with a 1:5,000 dilution of anti-PARP in the blocking mixture, followed by 1:2,500 anti-mouse HRP conjugate (Amersham). PARP was then detected using the enhanced chemiluminescence (ECL) kit from Amersham.

RESULTS

Induction of Apoptosis by Didemnin B

We have earlier described the very rapid induction of apoptosis in HL-60 cells [Grubb et al., 1995], which is dependent on protein tyrosine kinase(s) [Johnson et al., 1996]. We have since tested a whole range of blood cell lines, most of which undergo apoptosis upon exposure to didemnin B. However, most of these lines undergo didemnin B-induced apoptosis much more slowly than do HL-60 cells (unpublished data). In this study, we have used HL-60 and Daudi (a human lymphoblastoid cell line) cells. Whereas HL-60 cells undergo apoptosis to 100% upon exposure to didemnin B within 2–3 h, only about 40% of Daudi cells exhibit apoptotic morphology after 8 h of the same treatment (Fig. 1).

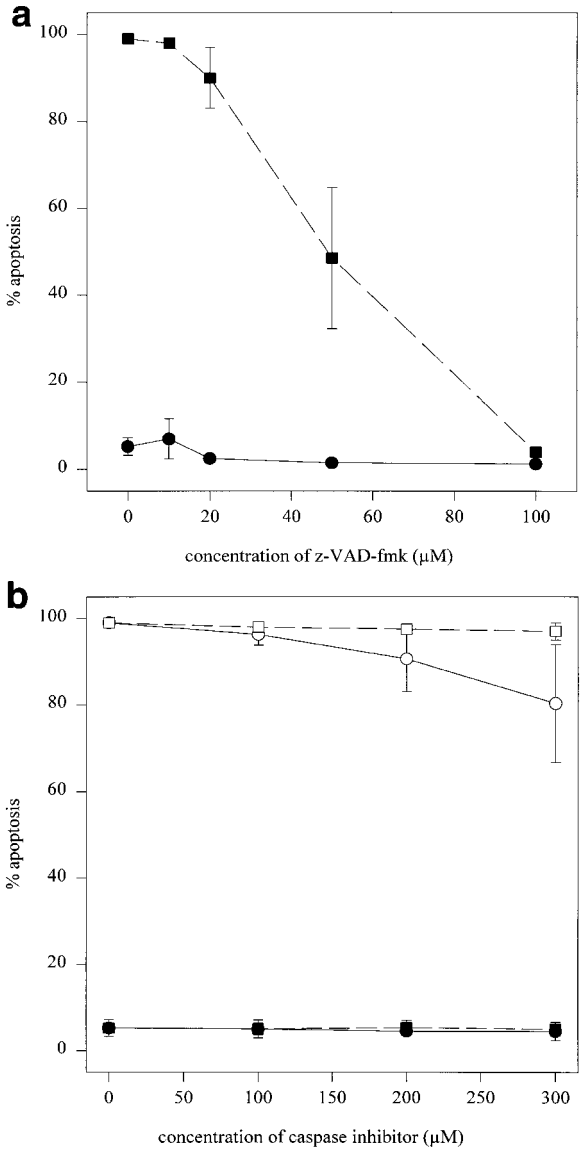

Fig. 2. Effects of z-VAD-fmk, Ac-YVAD-cmk, and Ac-DEVD-CHO on didemnin B-induced apoptosis in HL-60 cells. **a:** HL-60 cells were pretreated for 1 h with z-VAD-fmk, and percentage apoptosis was determined after a further 3-h incubation in the absence (●) or presence (■) of 1 μM didemnin B. Percentage apoptosis is plotted versus concentration of z-VAD-fmk. The data represent the mean of at least two experiments with error bars showing the standard deviation. **b:** HL-60 cells were pretreated for 1 h with Ac-YVAD-cmk (circles) or Ac-DEVD-CHO (squares), and percentage apoptosis was determined after a further 3-h incubation in the absence (●,■) or presence (○,□) of 1 μM didemnin B. Percentage apoptosis is plotted versus concentration of caspase inhibitor. The data represent the mean of at least two experiments, with error bars showing the standard deviation.

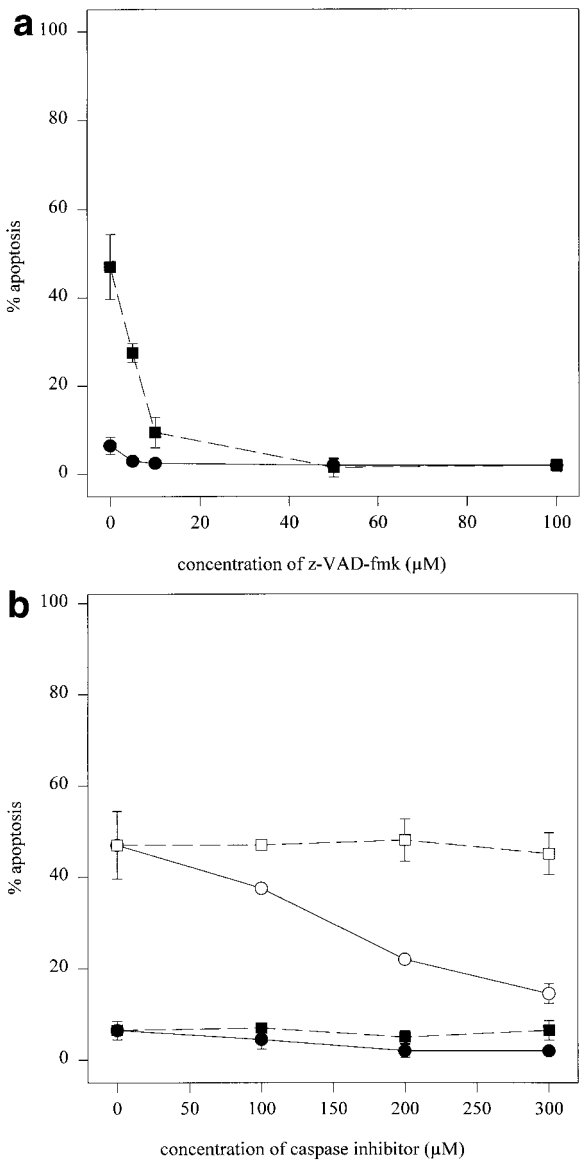

Fig. 3. Effects of z-VAD-fmk, Ac-YVAD-cmk, and Ac-DEVD-CHO on didemnin B-induced apoptosis in Daudi cells. **a:** Daudi cells were pretreated for 1 h with z-VAD-fmk, and percentage apoptosis was determined after a further 8-h incubation in the absence (●) or presence (■) of 1 μM didemnin B. Percentage apoptosis is plotted versus concentration of z-VAD-fmk. The data represent the mean of at least two experiments with error bars showing the standard deviation. **b:** Daudi cells were pretreated for 1 h with Ac-YVAD-cmk (circles) or Ac-DEVD-CHO (squares), and percentage apoptosis was determined after a further 8-h incubation in the absence (●, ■) or presence (○,□) of 1 μM didemnin B. Percentage apoptosis is plotted versus concentration of caspase inhibitor. The data represent the mean of at least two experiments, with error bars showing the standard deviation.

Effects of Caspase Inhibitors on Didemnin B-Induced Apoptosis

In order to determine whether caspases are involved in the rapid induction of apoptosis by didemnin B, HL-60 cells were induced to undergo apoptosis with didemnin B in the presence of caspase inhibitors. The tripeptide z-VAD-fmk resembles the cleavage site for caspases and is believed to be a general caspase inhibitor with irreversible inhibition [Armstrong et al.,

1996]. HL-60 cells were pretreated with 10, 20, 50, and 100 µM z-VAD-fmk for 1 h, followed by 3-h treatment with 1 µM didemnin B and morphological determination of apoptotic nuclei (Fig. 2a). Didemnin B induces complete apoptosis in HL-60 cells after 3 h, which is fully inhibited by 100 µM z-VAD-fmk, indicating that some caspase activity is essential for didemnin B-induced apoptosis in HL-60 cells. Tetrapeptide caspase inhibitors are more specific toward certain caspases than z-VAD-fmk. Ac-YVAD-cmk is the most effective peptide inhibitor for caspase-1 [Thornberry et al., 1992], and Ac-DEVD-CHO inhibits caspase-3-like activities [Nicholson et al., 1995]. HL-60 cells were pretreated with 100, 200, and 300 µM of either Ac-YVAD-cmk or Ac-DEVD-CHO, followed by 3-h treatment with 1 µM didemnin B (Fig. 2b) to determine whether a caspase-1-like or caspase-3-like activity is required. Neither inhibitor significantly inhibited didemnin B-induced apoptosis.

The same inhibitors were then tested for their effects on didemnin B-induced apoptosis in Daudi cells. Daudi cells undergo apoptosis much more slowly than HL-60 cells treated with didemnin B, only reaching approximately 50% apoptosis after 8 h. Daudi cells were pretreated with 10, 20, 50, and 100 µM z-VAD-fmk (Fig. 3a) or 100, 200, and 300 µM of either Ac-YVAD-cmk or Ac-DEVD-CHO (Fig. 3b) as indicated, followed by treatment with 1 µM didemnin B for 8 h. In these cells, z-VAD-fmk inhibits didemnin B-induced apoptosis as in HL-60 cells however at a lower concentration; only 50 µM z-VAD-fmk is required to completely inhibit apoptosis. Ac-YVAD-cmk also significantly inhibits didemnin B-induced apoptosis in Daudi cells in a concentration-dependent manner, which indicates that a caspase-1-like activity is required in Daudi cells. As in HL-60 cells, Ac-DEVD-CHO has no effect.

Because Ac-DEVD-CHO is not as cell permeable as the other inhibitors, and the concentrations used have no effect on didemnin B-induced apoptosis, it was tested for its ability to inhibit apoptosis induced by a drug other than didemnin B in these cell lines. Roscovitine is a cyclin-dependent kinase inhibitor [Meijer and Kim, 1997; Meijer et al., 1997] that can induce apoptosis in both HL-60 and Daudi cells. HL-60 cells were pretreated for 1 h with 300 µM Ac-DEVD-CHO, followed by treatment with 100 µM roscovitine or 1 µM didemnin B; the percentage of apoptosis was determined after 1, 2, 3, and 4 h (Fig. 4a). Daudi cells were pretreated for 1 h with 300 µM Ac-DEVD-CHO, followed by 8-h treatment with 25 µM roscovitine or 1 µM didemnin B (Fig. 4b). Ac-DEVD-CHO significantly inhibited roscovitine-induced apopto-

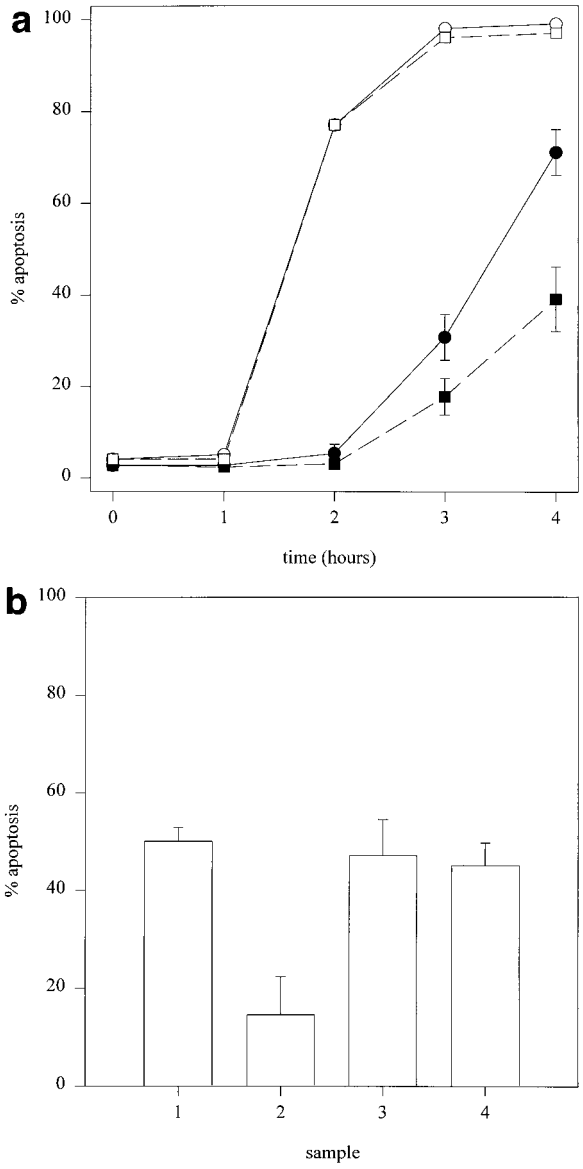

Fig. 4. Inhibition of roscovitine-induced apoptosis by Ac-DEVD-CHO in HL-60 and Daudi cells. **a:** HL-60 cells were pretreated for 1 h with 300 µM Ac-DEVD-CHO (squares) or untreated (circles) and percentage apoptosis was determined 1, 2, 3, and 4 h after addition of 100 µM roscovitine (●,■) or 1 µM didemnin B (○,□). Percentage apoptosis is plotted versus time after addition of didemnin B or roscovitine. The data represent the mean of at least two experiments, with error bars showing the standard deviation. **b:** Daudi cells were pretreated for 1 h with 300 µM Ac-DEVD-CHO (samples 2, 4) or untreated (samples 1, 3) and percentage apoptosis was determined after 8-h treatment with 25 µM roscovitine (samples 1, 2) or 1 µM didemnin B (samples 3, 4). Percentage apoptosis is plotted versus the sample. The data represent the mean of at least two experiments with error bars showing the standard deviation.

sis in both cell lines, indicating that these cells are sufficiently permeable to Ac-DEVD-CHO.

Didemnin B-Induced Activation of Caspases-1 and -3

Caspases are activated by cleavage at a site that is itself likely to be a substrate for caspases, indicating that a proteolytic cascade may be activated during apoptosis [Enari et al., 1996]. The activation of caspases can be detected by immunoblotting with an antibody to the caspase, which can also detect its cleavage product. The cleavage of procaspases-1 and -3 was investigated to determine whether these caspases are activated unspecifically during didemnin B-induced apoptosis.

HL-60 cells were treated with 1 μM didemnin B for 1, 2, 3, and 4 h, and an anti-caspase-1 (Fig. 5a) immunoblot was performed as described under Materials and Methods. Procaspase-1 cleavage, as detected by the appearance of the 20-kDa subunit of the active enzyme, starts to appear after only 2-h treatment with didemnin B, indicating that caspase-1 is activated, even though its activity is not required for didemnin B-induced apoptosis in HL-60 cells (cf. Fig. 2b). In Daudi cells treated with 1 μM didemnin B for 3, 4, 5, 6, and 7 h (Fig. 5a), caspase-1 cleavage starts to occur after 4 h. An anti-caspase-3 immunoblot (Fig. 6a) was performed for both cell lines treated under the same conditions as for the anti-caspase-1 immunoblot. Although caspase-3 activity is not required for didemnin B-induced apoptosis in either cell line, didemnin B starts to induce some caspase-3 cleavage after 1 h in HL-60 cells, and 3 h in Daudi cells. Caspase-1 cleavage appears to progress slightly more slowly than caspase-3 cleavage in both cell lines.

Effects of Caspase Inhibitors on Didemnin B-Induced Activation of Caspases-1 and -3

Didemnin B-induced activation of caspases-1 and -3 occurs in both cell lines even though the activities of caspases-1 and -3 in HL-60 cells and caspase-3 in Daudi cells is not required for didemnin B-induced apoptosis. To ensure that the inhibitors act as expected under these conditions, the effects of the caspase inhibitors on caspase activation were tested.

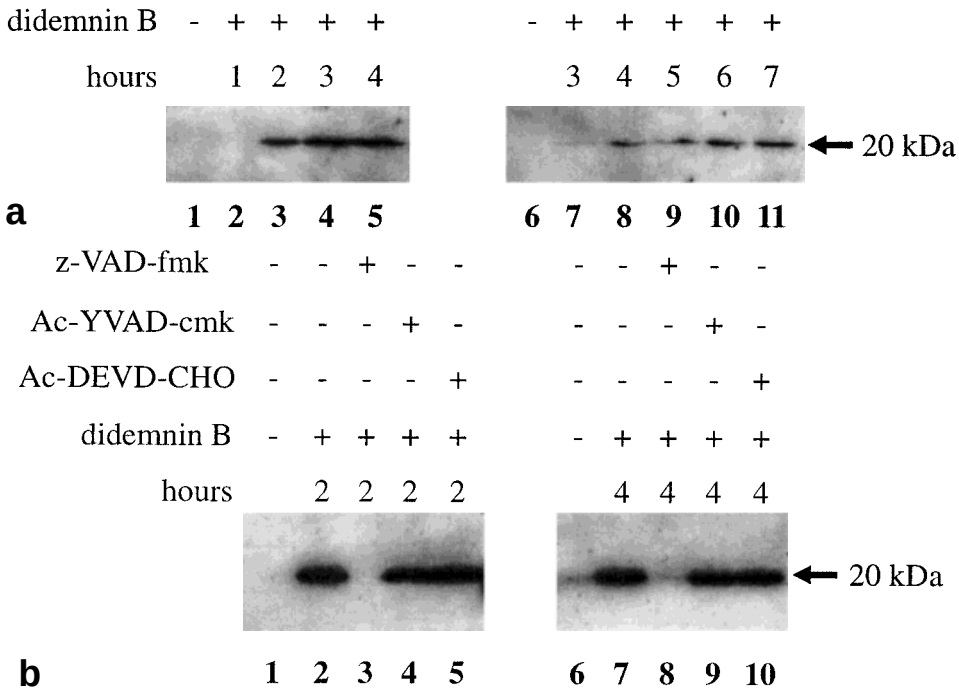

Fig. 5. Didemnin B-induced activation of caspase-1 in HL-60 and Daudi cells. HL-60 (**lanes 1–5**) and Daudi cells (**lanes 6–11**) were treated with 1 μM didemnin B for the indicated times and lysed as described under Materials and Methods, and an anti-caspase-1 immunoblot was performed. Control cells were untreated (**lanes 1, 6**). **a:** The 20-kDa cleavage product of caspase-1 appears after 2-h didemnin B treatment in HL-60 cells and after 4-h treatment in Daudi cells. **b:** Cells were pretreated for 1 h with 100 μM z-VAD-fmk (**lanes 3, 8**), 300 μM Ac-YVAD-cmk (**lanes 4, 9**), or 300 μM Ac-DEVD-CHO (**lanes 5, 10**), followed by 1 μM didemnin B-treatment for 2 h in HL-60 and 4 h in Daudi cells. Caspase-1 cleavage was inhibited by z-VAD-fmk in both HL-60 and Daudi cells.

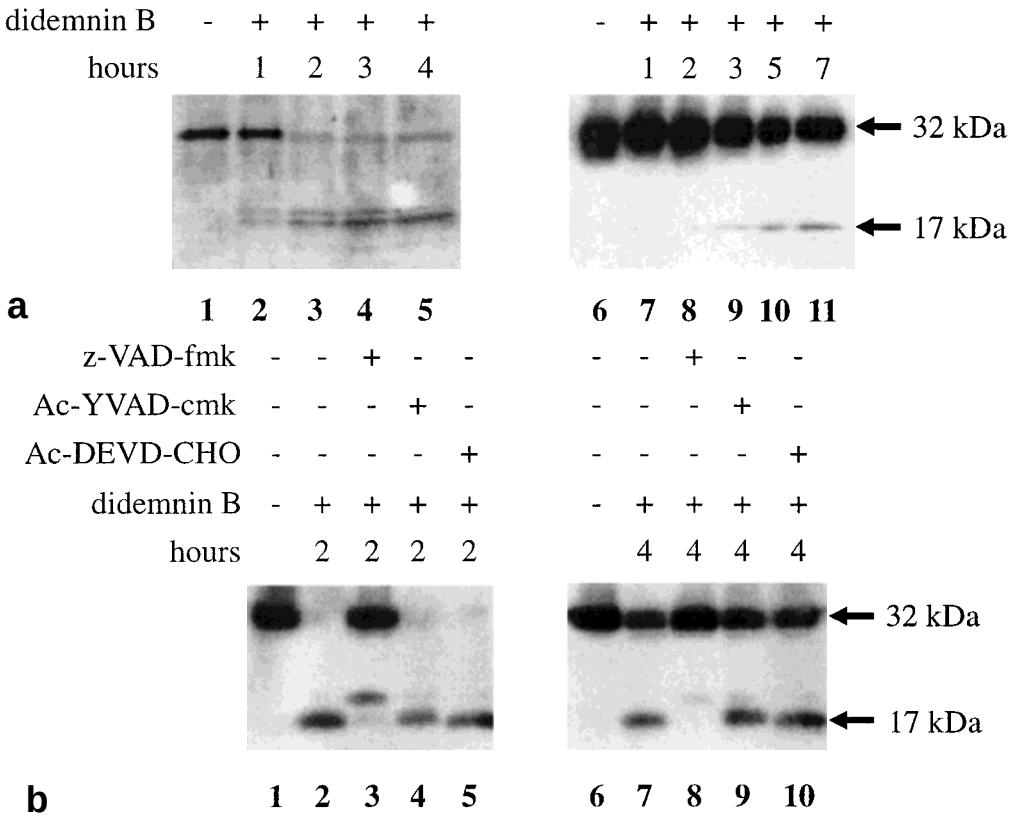

Fig. 6. Didemnin B-induced activation of caspase-3 in HL-60 and Daudi cells. HL-60 (**lanes 1–5**) and Daudi cels (**lanes 6–11**) were treated with 1 µM didemnin B for the indicated times and lysed as described in Materials and Methods, and an anti-caspase-3 immunoblot was performed. Control cells were untreated (**lanes 1, 6**). **a:** The 17 kDa cleavage product of caspase-3 starts to appear 1 hour after treatment with 1 µM didemnin B in HL-60 cells and 3 hours after treatment in Daudi cells. The 32 kDa and 17 kDa forms of caspase-3 are indicated with arrows. **b:** Cells were pretreated for 1 hour with 100 µM z-VAD-fmk (**lanes 3, 8**), 300 µM Ac-YVAD-cmk (**lanes 4, 9**), or 300 µM Ac-DEVD-CHO (**lanes 5, 10**), followed by 1 µM didemnin B-treatment for 2 hours in HL-60 and 4 hours in Daudi cells. Caspase-3 cleavage was inhibited by z-VAD-fmk in both HL-60 and Daudi cells.

HL-60 and Daudi cells were pretreated with 100 µM z-VAD-fmk, 300 µM Ac-YVAD-cmk, or 300 µM Ac-DEVD-CHO for 1 h before treatment with 1 µM didemnin B for 2 h or 4 h, respectively, and anti-caspase-1 (Fig. 5b) and anti-caspase-3 (Fig. 6b) immunoblots were performed. In HL-60 and Daudi cells, z-VAD-fmk inhibits the cleavage of caspase-1, but the other caspase inhibitors have no effect. Caspase-3 cleavage is also partially inhibited by z-VAD-fmk in HL-60 and Daudi cells. Ac-DEVD-CHO and Ac-YVAD-cmk have no effect on caspase-3 cleavage.

Cleavage of PARP in Didemnin B-Induced Apoptosis and Effects of Caspase Inhibitors

PARP cleavage is believed to be a common occurrence in apoptosis [Kaufmann et al., 1993]. To investigate whether this cleavage occurs in didemnin B-induced apoptosis, HL-60 and Daudi cells were treated with 1 µM didemnin B for 1, 2, 3, and 4 h and 1, 3, 5, and 7 h, respectively. An anti-PARP immunoblot was performed on lysates prepared as described under Materials and Methods (Fig. 7a). Cleavage of PARP starts to appear after 2-h treatment with didemnin B in HL-60 cells and after 3-h treatment in Daudi cells.

Caspase-3 was originally identified as a caspase that could cleave PARP in vitro [Nicholson et al., 1995]. However, it does not always appear to be the caspase responsible for cleaving PARP [Fernandes-Alnemri et al., 1995]. The effect of the caspase inhibitors on cleavage of PARP was determined by pretreating cells with 100 µM z-VAD-fmk, 300 µM Ac-YVAD-cmk, and 300 µM Ac-DEVD-CHO for 1 h before treating with 1 µM didemnin B for 3 h in HL-60 cells or for 5 h in Daudi cells (Fig. 7b). In HL-60 cells, z-VAD-fmk, but not Ac-YVAD-cmk or Ac-DEVD-CHO, inhibits cleavage of PARP, indicating that caspases-1 and -3 are not responsible

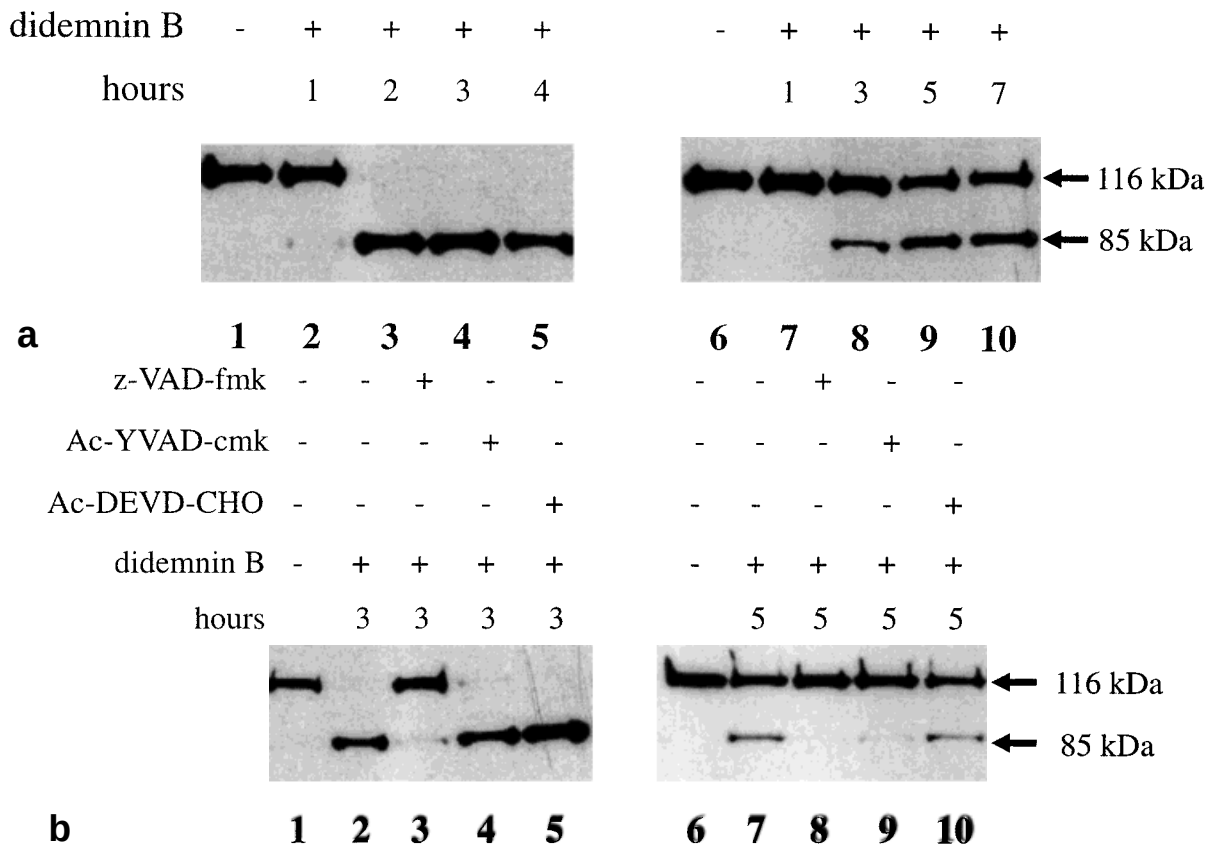

Fig. 7. Didemnin B-induced PARP cleavage in HL-60 and Daudi cells and effects of caspase inhibitors. **(a)** HL-60 (**lanes 1–5**) and Daudi (**lanes 6-10**) cells were treated with 1 μM didemnin B for the indicated time and lysed as described in Materials and Methods, and an anti-PARP immunoblot was performed. The approximately 85 kDA cleavage product of the 116 kDa PARP appears after 2 h in HL-60 and 3 h in Daudi, although to a much lesser extent. Control cells were untreated (**lanes 1, 6**). **(b)** HL-60 (**lanes 1–5**) and Daudi (**lanes 6–10**) cells were pretreated for 1 hour with 100 μM z-VAD-fmk (**lanes 3, 8**) 300 μM Ac-YVAD-cmk (**lanes 4, 9**), and 300 μM Ac-DEVD-CHO (**lanes 5, 10**), followed by didemnin B treatment for 3 h HL-60 and 5 h in Daudi cells. PARP cleavage was inhibited by z-VAD-fmk in HL-60 and z-VAD-fmk and Ac-YVAD-cmk in Daudi cells.

for PARP-cleavage. However, in Daudi cells, both z-VAD-fmk and Ac-YVAD-cmk inhibit cleavage of PARP, which correlates with their ability to inhibit apoptosis induced by didemnin B.

For comparison, the effect of roscovitine on PARP cleavage in Daudi cells was determined. Daudi cells were treated with 25 μM roscovitine for 1, 3, 5, and 7 h and an anti-PARP immunoblot was performed (Fig. 8). PARP cleavage was evident after 5-h treatment with roscovitine. Daudi cells were then pretreated with 100 μM z-VAD-fmk, 300 μM Ac-YVAD-cmk, or 300 μM Ac-DEVD-CHO, followed by 5-h treatment with 25 μM roscovitine. The anti-PARP immunoblot presented in Figure 8 shows that all three caspase inhibitors inhibit roscovitine-induced cleavage of PARP in Daudi cells. In roscovitine-induced apoptosis, Ac-DEVD-CHO inhibits apoptosis and PARP cleavage, whereas in didemnin B-induced apoptosis, Ac-DEVD-CHO does not inhibit apoptosis or PARP cleavage.

DISCUSSION

The inhibition of didemnin B-induced apoptosis by z-VAD-fmk indicates that both HL-60 and Daudi cells require some caspase activity during apoptosis induced by didemnin B. Because neither Ac-YVAD-cmk nor Ac-DEVD-CHO significantly inhibits didemnin B-induced apoptosis in HL-60 cells, the caspase(s) required for didemnin B-induced apoptosis in these cells is most likely not caspase-1 or -3. However, Figure 2b suggests that it may be a member of the caspase-1 subfamily, which includes caspases-1, -4, and -5, since caspase-4 can also be inhibited by Ac-YVAD-CHO, although not as strongly as caspase-1 [Margolin et al., 1997]. This situation is similar to that in Fas-mediated apoptosis, which has shown the

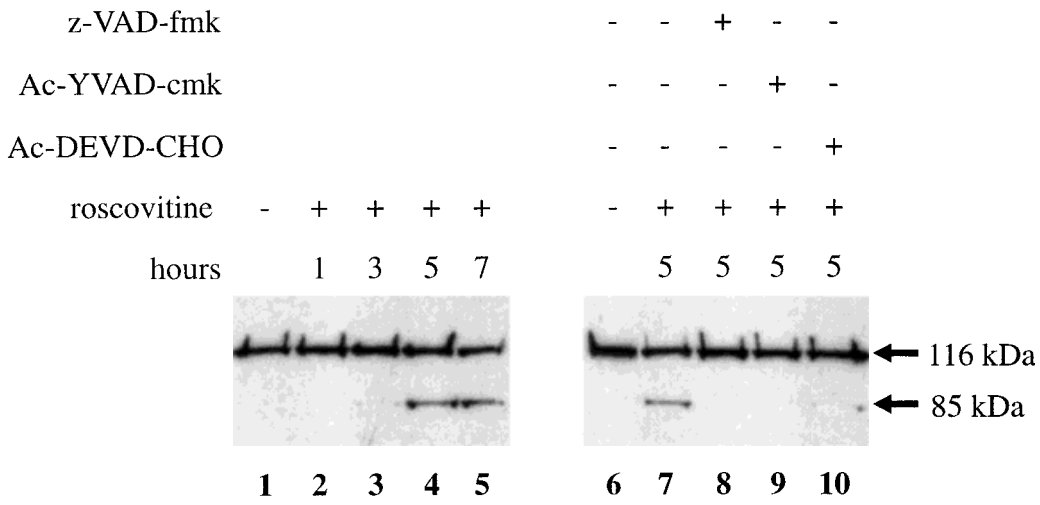

Fig. 8. Roscovitine-induced PARP cleavage in Daudi cells and effects of caspase inhibitors. Daudi cells were treated with 25 μM roscovitine for indicated time (**lanes 2–5**) or pretreated for 1 hour with 100 μM z-VAD-fmk (**lane 8**), 300 μM Ac-YVAD-cmk (**lane 9**), or 300 μM Ac-DEVD-CHO (**lane 10**) followed by treatment with 25 μM roscovitine for 5 hours. Control cells were untreated (**lanes 1, 6**). Cells were lysed as described in Materials and Methods and an anti-PARP immunoblot was performed. The 85 kDa cleavage product of PARP appears after 5 hours treatment with roscovitine, and this cleavage is inhibited by z-VAD-fmk, Ac-YVAD-cmk, and Ac-DEVD-CHO.

ability to occur in some cells in the presence of inhibitors of caspases-1 and -3 [Darmon and Bleackley, 1996]. The lack of effect of Ac-DEVD-CHO on didemnin B-induced apoptosis in Daudi cells shows that a member of the caspase-3 subfamily is not required for didemnin B-induced apoptosis in these cells either; a member of the caspase-1 subfamily is required, however, as Ac-YVAD-cmk does inhibit apoptosis. These results would suggest that since caspase-1 and -3 activity is not required for apoptosis induced by didemnin B in HL-60 cells and caspase-3 is not required in Daudi cells, these caspases would not be activated during didemnin B-induced apoptosis.

However, since caspases-1 and -3 are cleaved in both cell lines with didemnin B treatment, it appears that these caspases become activated whether their activity is required for didemnin B-induced apoptosis or not, suggesting that initiation of a caspase cascade may activate caspases that are not required for apoptosis. At least in HL-60 cells, some other caspase that is not downstream from caspases-1 and -3 must be responsible for apoptosis and may also be responsible for cleavage of caspases-1 and -3. These results show that caspases may be activated during induction of apoptosis unspecifically.

Examination of the effects of caspase inhibitors on activation of caspases-1 and -3 during apoptosis showed that the general caspase inhibitor z-VAD-fmk can inhibit didemnin B-induced activation of both of these caspases in both HL-60 and Daudi cells. However, the caspase-1 and -3 inhibitors have no effect on activation of caspases-1 and -3. These results indicate that while caspases-1 and -3 have no role in the activation of the caspase that is required for didemnin B-induced apoptosis, this caspase may be responsible for the cleavage of caspases-1 and -3 in cells treated with didemnin B. This result supports the idea that caspases are activated unspecifically by other caspases during apoptosis. Wang et al. (1996) suggested that caspase-3 could be cleaved at different sites to produce an active enzyme. Interestingly, some partial cleavage of caspase-3 still occurs in the presence of z-VAD-fmk in HL-60 cells at concentrations sufficient to inhibit apoptosis.

Examination of PARP cleavage showed that PARP is cleaved during didemnin B-induced apoptosis by some caspase other than a member of the caspase-1 or -3 subfamilies in HL-60 cells or by a member of the caspase-3 subfamily in Daudi cells. The inhibition of PARP cleavage by z-VAD-fmk in both cell lines and Ac-YVAD-cmk in Daudi cells suggests that the caspase(s) required for didemnin B-induced apoptosis either cleave PARP themselves or activate a caspase other than a member of the caspase-3 subfamily that cleaves PARP.

These results show that different cell types have different requirements for caspase activity in didemnin B-induced apoptosis. However, in both HL-60 and Daudi cells, caspases are activated that are not required for didemnin B-induced apoptosis. This activation can be inhibited by z-VAD-fmk in both HL-60 and Daudi cells; however Ac-YVAD-cmk does not inhibit activation of either caspase-1 or -3 although it inhibits apoptosis in Daudi cells. Interestingly, treatment with Ac-YVAD-cmk leads to inhibition of PARP cleavage in Daudi cells. In this instance, PARP cleavage correlates better with apoptosis than activation of caspase-3.

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