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Mistletoe Lectin Activates Caspase-8/FLICE Independently of Death Receptor Signaling and Enhances Anticancer Drug-induced Apoptosis[1]

Heike Bantel,[2] Ingo H. Engels,[2] Wolfgang Voelter, Klaus Schulze-Osthoff, and Sebastian Wesselborg[3]

Department of Internal Medicine I [H. B., I. H. E., K. S-O., S. W.] and Department of Physical Biochemistry of the Institute of Physiological Chemistry [W. V.], Eberhard-Karls-University, D-72076 Tübingen, Germany

ABSTRACT

Mistletoe lectin I (ML-I) is a major active component in plant extracts of *Viscum album* that is increasingly used in adjuvant cancer therapy. ML-I exerts potent immunomodulating and cytotoxic effects, although its mechanism of action is largely unknown. We show that treatment of leukemic T- and B-cell lines with ML-I induced apoptosis, which required the prior activation of proteases of the caspase family. The involvement of caspases is demonstrated because (*a*) a peptide caspase inhibitor almost completely prevented ML-I-induced cell death and (*b*) proteolytic activation of caspase-8, caspase-9, and caspase-3 was observed. Because caspase-8 has been implicated as a regulator of apoptosis mediated by death receptors, we further investigated a potential receptor involvement in ML-I-induced effects. Cell death triggered by ML-I was neither attenuated in cell clones resistant to CD95 nor in cells that were rendered refractory to other death receptors by overexpressing a dominant-negative FADD mutant. In contrast, ML-I triggered a receptor-independent mitochondria-controlled apoptotic pathway because it rapidly induced the release of cytochrome *c* into the cytosol. Because ML-I was also observed to enhance the cytotoxic effect of chemotherapeutic drugs, these data may provide a molecular basis for clinical trials using MLs in anticancer therapy.

INTRODUCTION

Extracts of mistletoe (*Viscum album*) have been widely used in adjuvant chemotherapy of human cancer for a long time. Their therapeutically active molecules are lectin components comprising ML[4]-I, ML-II, ML-III, and a recently isolated chitin-binding protein called ViscalbCBA (1–3). The classical MLs I, II, and III consist of two subunits that are linked by a disulfide bridge (4). They differ in their relative sugar-binding specificities. Although ML-I shows specificity to D-galactose, ML-II and ML-III preferentially bind to N-acetylgalactosamine (5). A main effector molecule in mistletoe extracts is the β-galactoside-specific lectin ML-I (6). Its B-chain is a 34 kDa-protein that binds to the cell membrane and subsequently delivers the 29 kDa-A-subunit into the cytosol. The complete amino acid sequence of the A-chain (7), as well as the B-chain, (8) has been recently determined and found to be closely related to those of the plant lectins ricin and abrin, which are known to act as type-II ribosome-inactivating proteins. After internalization, the A-chain catalytically inactivates the 60S ribosomal subunit by depurination of a single adenosine residue within the 28S rRNA. It is assumed that, similar to ricin, the A-chain exerts cytotoxic properties through the inhibition of protein synthesis (9).

Experiments in cell cultures and animal models have revealed that ML-I elicits several types of cellular responses that may support the adjuvant effect of mistletoe extracts in cancer therapy. ML-I exerts a broad immunostimulatory activity (10). Thus, incubation of peripheral blood mononuclear cells or monocytic cell lines with ML-I results in increased expression of various cytokines, such as interleukin-1, interleukin-6, TNF-α, and granulocyte macrophage colony-stimulating factor (1, 11, 12). Furthermore, an increase in the number of natural killer cells and phagocytic activity has been observed. It has also been shown that the administration of ML-I is followed by the release of β-endorphin into the plasma (13). β-endorphin is an oligopeptide that decreases the pain response in the central nervous system (14). Thereby, MLs may improve the life quality of cancer patients.

An important activity of ML-I includes its cytostatic and cytotoxic effect on different tumor cells of lymphoid origin, in particular. Some recent evidence suggests that this cytotoxicity may be mediated by induction of apoptosis, a highly conserved mechanism of cell death. It has been observed that incubation of different cell lines with MLs results in cell death associated with typical apoptotic alterations such as cell shrinkage, chromatin condensation, and internucleosomal DNA cleavage (15–17). However, it remains unclear from these studies by which mechanism and signal transduction pathway MLs induce programmed cell death.

Recent studies have shown that apoptosis is essentially controlled by a family of conserved proteases, called caspases, that are currently considered as the central executioners of many apoptotic pathways. In mammalian cells, at least 12 different caspase members exist; these are cysteine proteases that cleave their substrates after aspartate residues (18, 19). Caspases are synthesized as inactive proenzymes and proteolytically processed to form an active complex composed of two heterodimeric subunits of about 10 and 20 kDa. An increasing number of proteins have been found to be cleaved by caspases and, for some of them, an apoptotic function could be attributed. Among different substrates are enzymes involved in genome function such as the DNA repair enzyme PARP, regulators of the cell cycle such as retinoblastoma protein and MDM-2, and structural proteins of the nucleus and cytoskeleton such as lamins, Gas2, gelsolin, and fodrin (18–20). Furthermore, DNA cleavage is triggered on caspase-mediated degradation of the inhibitory subunit of a novel endonuclease, designated CAD for caspase-activated DNase (21).

One of the best defined apoptotic pathways is mediated by the death receptor CD95 (APO-1/Fas; Refs. 22 and 23). Triggering of CD95 by its natural ligand or agonistic antibodies induces the formation of a DISC that consists of the adapter protein FADD and FLICE/caspase-8 (24, 25). Complex formation is initiated through homophilic interaction of the death domains present in the intracellular part of both CD95 and FADD. FADD, in addition, contains a second interacting region called the DED, which couples to caspase-8 as the most proximal element in the caspase cascade. Further downstream, caspase-8 presumably triggers the proteolytic activation of other caspases and cleavage of cellular substrates.

It has also been shown that mitochondria play an important role in

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[2] These authors contributed equally to this work.

[3] To whom requests for reprints should be addressed, at Department of Internal Medicine I, Eberhard-Karls-University, Otfried-Müller-Str. 10, D-72076 Tübingen, Germany. Phone: 49-7071-29-80660; Fax: 49-7071-29-5865; E-mail: snwessel@med.uni-tuebingen.de.

[4] The abbreviations used are: ML, mistletoe lectin; Apaf, apoptotic protease-activating factor; CARD, caspase recruitment domain; DED, death effector domain; $\Delta\Psi_m$, mitochondrial transmembrane potential; DISC, death-inducing signaling complex; PARP, poly(ADP-ribose)polymerase; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; zVAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; mAb, monoclonal antibody.

the regulation of apoptosis. An early event in this process is the translocation of cytochrome *c* from the mitochondria into the cytosol, which is inhibited by antiapoptotic Bcl-2 proteins (26, 27). In the cytosol, cytochrome *c* interacts with Apaf-1, the mammalian homologue of the *Caenorhabditis elegans* cell death regulator Ced-4 (28). Binding of cytochrome *c* then presumably leads to a conformational change in Apaf-1 and exposes an interaction motif, the so-called CARD. This region serves as protein interface by binding to caspases that have a similar domain at their NH_2 terminus. A redistribution of cytochrome *c* into the cytosol is observed in a variety of apoptotic conditions, such as CD95 ligation or treatment of cells with chemotherapeutic drugs and UV-irradiation (26–30). During CD95-mediated apoptosis, cytochrome *c* release is mediated by caspase-8-triggered cleavage of the proapoptotic Bcl-2 member Bid (31, 32). It has also been shown that in some cases several unrelated apoptotic stimuli, such as anticancer drugs and UV-irradiation, obviously require a functional CD95 pathway to induce apoptosis (33–35).

In the present study, we investigated the mechanism of ML-I-induced cytotoxicity in leukemic T- and B-cell lines. We show that treatment of cells with ML-I activated caspase-8/FLICE, caspase-9, and caspase-3 and subsequent apoptotic cell death, which was almost completely prevented by a caspase inhibitor. Due to the activation of caspase-8, we initially speculated that ML-I-induced apoptosis might involve the CD95 pathway, either through the up-regulation of its ligand or a direct lectin-mediated receptor cross-linking. However, cell clones resistant to CD95 and other death receptors activated caspases and underwent apoptosis to a similar extent as wild-type cells, indicating that death receptors were not involved. Instead, ML-I-induced-apoptosis was associated with the mitochondrial release of cytochrome *c* and required internalization of the lectin. In addition, we show that ML-I potentiated anticancer drug-induced cytotoxicity. Our data, therefore, demonstrate that ML-I induces caspase activation and apoptosis by a death receptor-independent, but mitochondria-controlled pathway, and thereby may provide a rational basis for further clinical trials using MLs in adjuvant cancer therapy.

MATERIALS AND METHODS

Cells and Reagents. The human leukemic T-cell line Jurkat and B-cell line BJAB were maintained in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin (all from Life Technologies, Inc., Eggenstein, Germany). The CD95-resistant Jurkat subline Jurkat-R was generated by continuous culture in the presence of anti-CD95 mAb (IgG3, 1 μg/ml; Cell Diagnostica, Münster, Germany) for 6 months. BJAB FADD-DN cells, which were stably transfected with a dominant-negative FADD mutant lacking the NH_2-terminal DED region (36), were originally obtained from Dr. V. M. Dixit (University of Michigan, Ann Arbor, MI). ML-I was prepared and purified by lactose affinity chromatography, as described previously (7). The chemotherapeutic drugs etoposide and mitomycin C were obtained from the clinical pharmacy (Medical Clinics, Tübingen, Germany). Mitomycin C was dissolved in methanol and etoposide in ethanol and kept as stock solutions at −70°C. Brefeldin A was purchased from Sigma Chemical Co. (Deisenhofen, Germany). The broad-range caspase inhibitor zVAD-fmk was purchased from Enzyme Systems (Dublin, CA).

Cytofluorometric Analysis of Cell Death, Apoptosis, and Reduction of $\Delta\Psi_m$. For determination of apoptosis, 4×10^4 cells/well were seeded in microtiter plates and treated with the indicated concentrations of the apoptotic agents. Cell viability was measured by uptake of 2 μg/ml propidium iodide in PBS into nonfixed cells and subsequent analysis in a flow cytometer. The leakage of fragmented DNA from apoptotic nuclei was determined as described by the method of Nicoletti *et al.* (37). Briefly, apoptotic nuclei were prepared by lysing cells in a hypotonic buffer (1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide) and subsequently analyzed by flow cytometry. Nuclei to the left of the 2N peak containing hypodiploid DNA were considered as apoptotic. To investigate the effect of ML-I on anticancer drug-induced cytotoxicity, cells were incubated with different concentrations of ML-I and etoposide. The combined effects of both drugs were calculated by isobologram analysis according to the method of Berenbaum (38). To determine the reduction of the $\Delta\Psi_m$, cells were incubated at 37°C for 15 min in PBS containing 80 nM of the cationic fluorochrome 3,3′-dihexyloxacarbocyanine iodide (Sigma Chemical Co.; Ref. 39). Subsequently, cells were kept on ice and measured in a flow cytometer at 530 nm. All flow cytometric analyses were performed on a FACScalibur (Becton Dickinson, Heidelberg, Germany) using CellQuest analysis software.

Cell Extracts and Immunoblotting. Cleavage of caspases and the caspase-specific substrates PARP and Bid was detected by immunoblotting. Cells (2×10^6) were seeded in 24-well plates and treated with the apoptotic stimuli. After the indicated time periods, cells were washed in cold PBS and lysed in 1% Triton X-100, 50 mM Tris (pH 7.6), and 150 mM NaCl containing 3 μg/ml aprotinin, 3 μg/ml leupeptin, 3 μg/ml pepstatin A, and 2 mM phenylmethylsulfonyl fluoride. Subsequently, proteins were separated under reducing conditions on a SDS-polyacrylamide gel (8–15% gradient gel for caspase-8 and PARP and 15% gel for caspase-3, caspase-9, and Bid, respectively) and electroblotted to a polyvinylidene difluoride membrane (Amersham, Braunschweig, Germany). Membranes were blocked for 1 h with 5% nonfat dry milk powder in Tris-buffered saline and then immunoblotted for 1 h with rabbit anti-PARP polyclonal antibody (Boehringer-Mannheim), mouse mAbs directed against caspase-8 (Cell Diagnostica), caspase-3 (Transduction Laboratory, Lexington, KY), caspase-9 [kindly provided by Dr. Y. A. Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); Ref. 40] or rat anti-Bid polyclonal antibody [kindly provided by Dr. J. Yuan (Harvard Medical School, Boston, MA); Ref. 31]. Membranes were washed four times with Tris-buffered saline/0.02% Triton X-100 and incubated with the respective peroxidase-conjugated affinity-purified secondary antibody for 1 h. After extensive washing, the reaction was developed by enhanced chemiluminescent staining using enhanced chemiluninescence reagents (Amersham).

Measurement of Cytochrome *c* Release. For analysis of cytochrome *c* release, 7.5×10^6 cells were collected by centrifugation, washed with ice-cold PBS, and resuspended in five volumes of buffer A containing 250 mM sucrose, 20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The cells were homogenized with 10–15 strokes in a douncer, and the homogenates were centrifuged at $1000 \times g$ to remove cell nuclei. The supernatants were transferred to a fresh tube and centrifuged at $10000 \times g$ for 10 min at 4°C to deplete mitochondria. The resulting supernatants designated as cytosolic S10 fraction from each sample were loaded on a 15% SDS polyacrylamide gel. Cytochrome *c* release was analyzed by immunoblotting with the mouse mAb 7H8.2C12 (PharMingen, Hamburg, Germany).

RESULTS

ML-I Induces Caspase-dependent Apoptosis. We investigated the cytotoxic activity of ML-I in Jurkat leukemic T cells. As measured by the formation of hypodiploid DNA, ML-I dose-dependently triggered apoptosis (Fig. 1, *A* and *B*). Induction of an apoptotic form of cell death was also observed by classical morphological alterations, such as cell shrinkage and membrane blebbing (data not shown). Because caspases have been implicated in the execution of apoptosis, we analyzed their involvement in ML-I-induced cytotoxicity. Pretreatment of cells with zVAD-fmk, a broad peptide caspase inhibitor, almost completely abrogated ML-I-induced cell death (Fig. 1*B*). The results, therefore, indicate that caspases are the critical executioners of ML-I-induced cytotoxicity.

Caspases comprise a family of different cysteine proteases that are synthesized as inactive zymogens and converted to an active complex composed of two heterodimeric subunits (18, 19). To investigate which caspase members are activated during ML-I-induced cell death, we monitored the processing of procaspases in immunoblot analyses using antibodies specific to individual proteases. Jurkat cells were treated with ML-I for 6 h, after which protein extracts were prepared and fractionated by SDS-PAGE. Treatment of cells with ML-I resulted in the conversion of the inactive 32-kDa caspase-3 precursor to

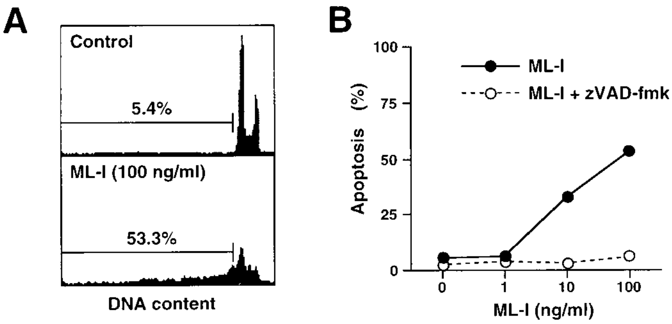

Fig. 1. ML-I activates caspase-dependent apoptosis in Jurkat leukemic T cells. *A*, flow cytometric detection of hypodiploid DNA. Typical DNA histograms of control nuclei (*top*) and cells treated for 6 h with 100 ng/ml ML-I (*bottom*) are shown. Nuclei were stained with propidium iodide and analyzed for DNA content. A total of 5×10^3 cells was used to create each histogram. Peaks representing fragmented hypodiploid DNA and the G_0/G_1, and S/G_2 phases of the cell cycle are shown. *B*, inhibition of ML-I-induced apoptosis by the caspase inhibitor zVAD-fmk. Cells were pretreated with either control medium (●) or 100 μM zVAD-fmk (○) for 1 h and then stimulated with the indicated concentrations of ML-I. Induction of cell death was quantified after 24 h by propidium iodide staining of hypodiploid apoptotic nuclei and subsequent flow cytometry.

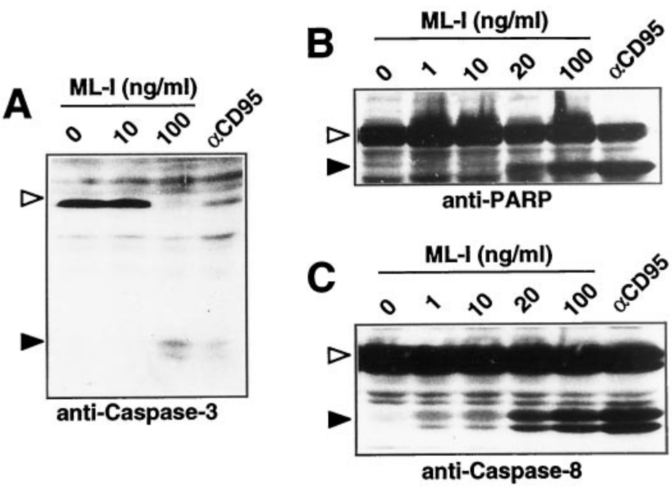

Fig. 2. ML-I induces the proteolytic processing of several caspase members. Jurkat cells were incubated with the indicated concentrations of ML-I. After 6 h, total cell lysates were prepared, separated by SDS-PAGE, and subjected to immunoblotting. The blots were developed using specific antibodies, followed by enhanced chemiluminescence staining. *A*, proteolytic processing of caspase-3. The blot indicates the cleavage of the 32-kDa caspase-3 precursor (*open arrowhead*) to the p17-activated subunit (*closed arrowhead*). *B*, cleavage of PARP. The blot shows the 116-kDa full-length PARP (*open arrowhead*) and the characteristic 89-kDa caspase cleavage product (*closed arrowhead*). *C*, processing of caspase-8. The blot shows a double band of 54 kDa corresponding to the proforms caspase-8a and caspase-8b (*open arrowhead*) and the intermediate cleavage products of 43 kDa and 41 kDa (*closed arrowhead*).

the proteolytically cleaved p17 subunit, indicating that caspase-3 was activated during ML-I induced apoptosis (Fig. 2*A*). In a detailed dose-response assay, we further measured the cleavage of PARP, an enzyme involved in DNA repair, which is specifically cleaved by caspases during apoptosis. Fig. 2*B* demonstrates that PARP, a 116-kDa protein, was cleaved into the characteristic 89-kDa fragment in the course of ML-I treatment at concentrations as low as 20 ng/ml.

We next investigated the processing of caspase-8, the most proximal caspase during CD95-mediated apoptosis. Caspase-8 is synthesized as an inactive precursor of 54 kDa and, following formation of a 43-kDa intermediate cleavage product, processed to a p18 and p10 heterodimer (25, 41). In untreated Jurkat cells, an antibody against the p18 subunit of caspase-8 detected a doublet protein band of 54 kDa, which represents the isoforms procaspase-8a and procaspase-8b (42). ML-I treatment of Jurkat cells resulted in the conversion of the protein doublet to the 43- and 41-kDa intermediate fragments, which became visible already at a concentration of 1 ng/ml of ML-I (Fig. 2*C*). These results, therefore, demonstrate that several caspase members, including caspase-3 and caspase-8, are activated during ML-I-induced apoptosis.

ML-I-induced Caspase-8 Activation and Apoptosis Are Independent of CD95 and Death Receptor Signaling. Caspase-8 has been originally identified as a proximal regulator in apoptosis mediated by the surface receptor CD95 (25). In this pathway, caspase-8 is recruited to the DISC on homophilic binding to the DED-containing adapter protein FADD. Because apoptosis mediated by other apoptotic agents, such as chemotherapeutic drugs, has been previously proposed to involve a CD95 signaling, we investigated the requirement of a functional CD95 pathway for ML-I-induced apoptosis. In such a scenario, it is conceivable that ML-I either induces the expression of CD95 ligand or, by direct binding to the glycosylated surface receptor, triggers CD95 cross-linking, which is necessary for signal transduction. To investigate whether the CD95 receptor/ligand interaction is involved in ML-I-induced cytotoxicity, we used the subclone Jurkat-R, which is resistant to CD95 signaling. When CD95-sensitive Jurkat and resistant Jurkat-R cells were treated with different concentrations of ML-I, both cell lines underwent apoptosis with a very similar dose-dependency (Fig. 3*A*). In contrast, an agonistic anti-CD95 antibody induced apoptosis in Jurkat cells, but not in Jurkat-R cells, confirming that these cells were, indeed, CD95-resistant (Fig. 3*B*).

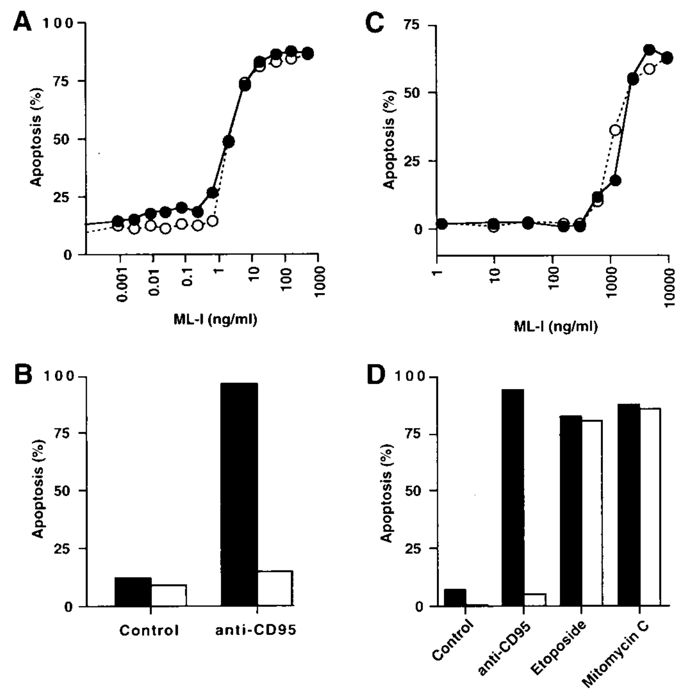

Fig. 3. ML-I-induced apoptosis is independent of CD95 and death receptor signaling. *A*, effects of ML-I on apoptosis in CD95-sensitive and -resistant Jurkat cells. CD95-sensitive Jurkat (●) or -resistant Jurkat-R (○) cells (4×10^4) were stimulated with the indicated concentrations of ML-I. Induction of cell death was assessed after 24 h by propidium iodide staining of hypodiploid apoptotic nuclei. *B*, effect of anti-CD95 on apoptosis in Jurkat and Jurkat-R cells. Both cell types were stimulated with agonistic anti-CD95 antibodies (1 μg/ml) and measured for apoptosis after 24 h. The results confirm that Jurkat-R cells (□) are resistant, whereas Jurkat cells (■) are sensitive to CD95-mediated apoptosis. *C*, effect of ML-I on BJAB cells stably expressing a dominant-negative FADD mutant or its empty vector control. BJAB-vector (●) and BJAB FADD-DN (○) cells were stimulated with the indicated concentrations of ML-I. After 36 h, apoptosis was assessed by measuring formation of hypodiploid DNA. *D*, induction of apoptosis in vector control and FADD-DN-expressing BJAB cells by anti-CD95 and chemotherapeutic drugs. Both cell types were treated for 36 h with anti-CD95 (1 μg/ml), etoposide (25 μg/ml), or mitomycin C (25 μg/ml) and assessed for apoptosis, as described above. ■, BJAB vector; □, BJAB FADD-DN.

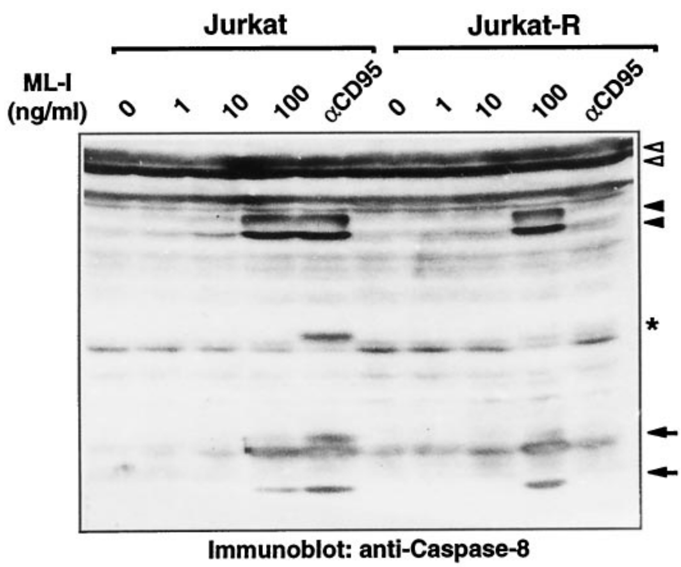

Fig. 4. Caspase-8 is activated by ML-I in the absence of CD95 signaling. Jurkat and Jurkat-R cells (2×10^6) were stimulated with the indicated concentrations of either ML-I for 6 h or 1 μg/ml anti-CD95 for 3 h. Cellular proteins were separated by SDS-PAGE, and processing of procaspase-8 was detected by immunoblotting with caspase-8-specific antibodies. *Open arrowheads* indicate the two different isoforms of procaspase-8 (caspase-8a and caspase-8b), which are cleaved into the intermediate forms p43 and p41 (*closed arrowheads*) and finally processed to the active subunit of 18 and 16 kDa (*closed arrow*). The IgG light chain of stimulatory anti-CD95 antibody is indicated with an *asterisk*.

To further exclude a potential role of other death receptors, we analyzed the effect of ML-I in BJAB B-cells, which were stably transfected with a dominant-negative mutant of the adapter protein FADD, lacking the essential DED region. It has been previously reported that FADD also transduces apoptotic signals triggered by TNF receptor 1 and the TRAIL receptors (43–46). In accordance, BJAB FADD-DN cells are resistant to anti-CD95 and TRAIL and, furthermore, have an intrinsic resistance against TNF-induced apoptosis. A dose-response experiment revealed that, compared with Jurkat cells, a higher dose of ML-I was required to induce apoptosis in both BJAB FADD-DN and BJAB cells transfected with the vector control (Fig. 3*C*). However, both cell lines underwent apoptosis in a similar concentration-dependent manner. Also, the chemotherapeutic drugs etoposide and mitomycin C induced apoptosis in both cell lines (Fig. 3*D*). In contrast, when the CD95 pathway was stimulated, cell death was induced in BJAB vector cells, but not in BJAB FADD-DN cells.

Because activation of caspase-8 is recruited to the death receptor signaling complex, we next investigated whether ML-I could also induce caspase-8 activation in CD95-resistant Jurkat cells. Interestingly, in both CD95-sensitive and -resistant cells, proteolytic processing of caspase-8 was observed in response to the same concentrations of ML-I (Fig. 4). Anti-CD95, however, activated caspase-8 only in sensitive, but not CD95-resistant, Jurkat cells. Collectively, these results demonstrate that neither ML-I-triggered caspase-8 activation nor subsequent apoptosis require CD95 or another death receptor pathway that triggers apoptosis through a FADD-containing signaling complex.

ML-I Is Internalized and Triggers a Mitochondria-controlled Apoptotic Pathway. MLs bind to the cell surface with their B-subunit and then deliver the A-chain into the cytosol. For ricin, a related lectin, it is known that the A-chain enters the cell via endocytosis and is subsequently translocated from an intracellular compartment to the cytosol (47). To investigate whether such a pathway is also required for ML-I-induced apoptosis, we used brefeldin A, a fungal inhibitor that disrupts vesicular transport. As shown in Fig. 5*A*, the addition of brefeldin A protected Jurkat cells from ML-I-induced cytotoxicity. This inhibitory effect was visible, in particular, at high lectin concentrations because brefeldin A itself was cytotoxic. In contrast to ML-I, cell death mediated by CD95 was not inhibited on incubation of cells with brefeldin A (Fig. 5*B*). The results demonstrate that both apoptotic pathways differ strongly in terms of intracellular routing and that ML-I-induced apoptosis requires a retrograde endosomal transport to induce apoptosis.

Recent evidence has demonstrated that mitochondria play a key role in the events leading to caspase activation. Induction of cell death is associated with the mitochondrial release of cytochrome *c* (26, 27). In the cytosol, cytochrome *c* forms a complex with the Ced-4 homologue Apaf-1, which results in the cleavage of procaspase-9 and subsequent activation of other caspases. Although the mechanism of cytochrome *c* release from mitochondria is unknown, it has been hypothesized that the leakage of cytochrome *c* results from the opening of a membrane permeability pore and the loss of the $\Delta\Psi_m$ (48). To investigate the involvement and temporal relationship of mitochondrial events in ML-I-induced apoptosis, we stimulated Jurkat cells with ML-I and analyzed the translocation of cytochrome *c* into the cytosol and reduction of the $\Delta\Psi_m$ in a time-course experiment. Fig. 6*A* shows that ML-I induced the release of cytochrome *c* after 5 h of stimulation. The release of cytochrome *c* largely preceded the reduction of $\Delta\Psi_m$ and the onset of apoptosis (Fig. 6, *B* and *C*). Interestingly, the release of cytochrome *c*, as well as the breakdown of the $\Delta\Psi_m$, was blocked by the caspase inhibitor zVAD-fmk (Fig. 6, *A* and *D*).

Because the mitochondrial cytochrome *c*/Apaf-1-pathway is characterized by the activation of procaspase-9, we next investigated the sequence of proteolytic events involved in ML-I-mediated toxicity. Therefore, Jurkat cells were stimulated with ML-I for different times, and the activation of procaspase-9, procaspase-3, and procaspase-8 was monitored by immunoblot analysis. In addition, we also deter-

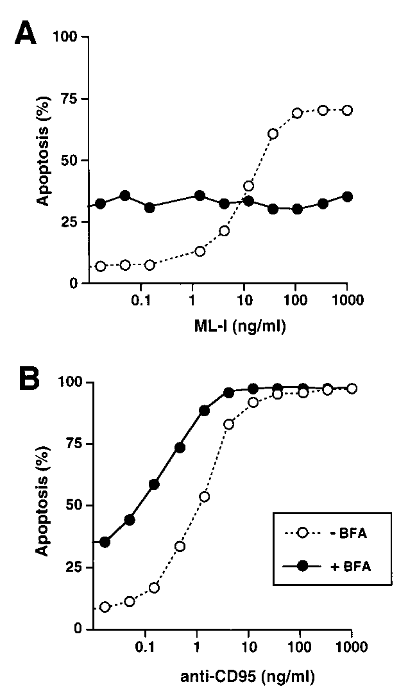

Fig. 5. Brefeldin A inhibits ML-I-induced, but not anti-CD95-induced, cell death. Jurkat cells were either left untreated (○) or pretreated for 30 min with 1 μg/ml brefeldin A (*BFA*; ●) and then stimulated with the indicated concentrations of ML-I (*A*) or anti-CD95 (*B*). Cell death was determined by flow cytometric staining of propidium iodide uptake into cells.

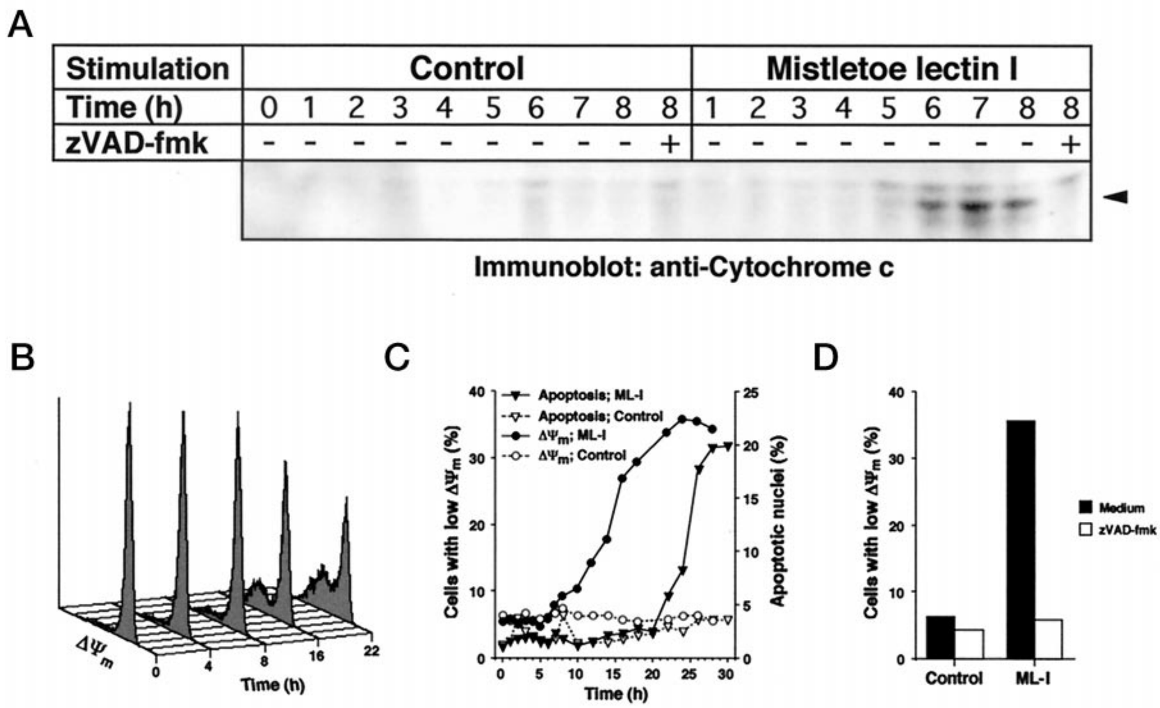

Fig. 6. ML-I-induced release of cytochrome *c* precedes the loss of $\Delta\Psi_m$ and apoptosis. *A*, time-course of mitochondrial cytochrome *c* release: Jurkat cells were pretreated with medium or 100 μM zVAD-fmk for 1 h and then either left untreated or incubated with 10 ng/ml ML-I. After the indicated time, cells were homogenized, and the S10 fraction depleted of mitochondria was loaded on a 15% SDS-PAGE. The release of cytochrome *c* into the cytosol was determined by immunoblotting. *B*, histogram analysis of the ML-I-induced reduction of $\Delta\Psi_m$: cells were treated with 10 ng/ml ML-I and, after the indicated times, $\Delta\Psi_m$ was determined by staining cells with the potential-sensitive fluorochrome 3,3′-dihexyloxacarbocyanine iodide. *C*, time-course of ML-I-induced loss of $\Delta\Psi_m$ (*circles*) and onset of apoptosis (*triangles*): The percentage of cells with a low $\Delta\Psi_m$ and hypodiploid DNA was evaluated in a flow cytometer. The *dashed lines* show the values of untreated cells, and the *closed lines* show the values of cells treated for the indicated time with ML-I. *D*, effect of zVAD-fmk on ML-I-induced loss of $\Delta\Psi_m$: the graph indicates $\Delta\Psi_m$ values that were assessed 24 h after stimulation of cells in the presence (□) or absence (■) of zVAD-fmk.

mined the proteolytic cleavage of PARP and of Bid, a proapoptotic member of the Bcl-2-family that has been demonstrated recently to be cleaved by caspase-8 (31, 32). Fig. 7 shows that proteolytic cleavage of all procaspases and caspase substrates occurred with a roughly similar time-dependency. Procaspase-8 and procaspase-9 were cleaved after 5–6 h of treatment with ML-I, which seemed to occur slightly before the activation of procaspase-3. The proteolytical processing of the caspase-3-substrate PARP coincided with the activation of caspase-3. Bid, a substrate of caspase-8, was also degraded completely in a time-dependent manner (Fig. 7). Again, inhibition of caspase activity by zVAD-fmk prevented the proteolytical degradation of all proteins tested. These results, therefore, strongly imply that ML-I-induced apoptosis is mediated via the mitochondrial cytochrome *c*/Apaf-1-pathway, which acts upstream of $\Delta\Psi_m$ reduction.

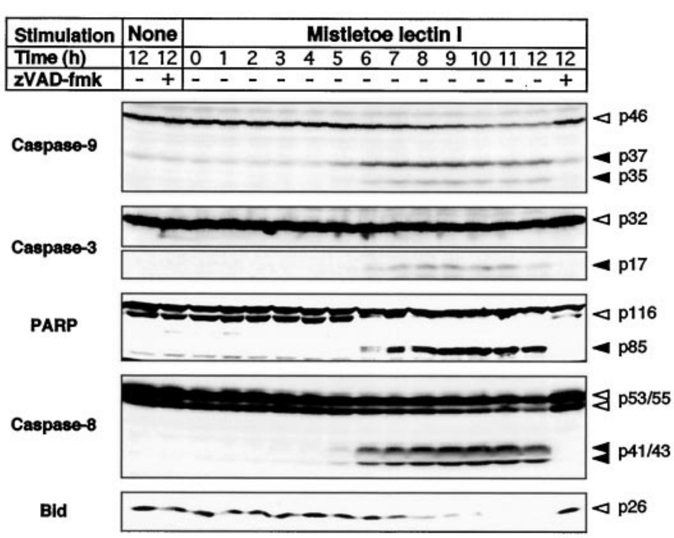

Fig. 7. Time-course of ML-I-induced proteolytical cleavage of caspase-9, caspase-8, caspase-3, PARP, and Bid. Jurkat cells were pretreated in the absence or presence of 100 μM zVAD-fmk for 1 h and subsequently stimulated for the indicated time with medium (*None*) or 10 ng/ml ML-I. Cellular proteins were separated by SDS-PAGE, and proteolytic processing of caspase-9, caspase-3, caspase-8, PARP, and Bid was detected by immunoblotting. *Open arrowheads*, the uncleaved form of the indicated proteins; *closed arrowheads*, the cleaved form of the indicated proteins.

ML-I Enhances Anticancer Drug-induced Apoptosis. As MLs are used in adjuvant anticancer therapy, we investigated whether chemotherapeutic drug-induced cell death is augmented by ML-I. To this end, Jurkat cells were treated with serial concentrations of different apoptotic agents, including the topoisomerase inhibitor etoposide and anti-CD95 in the presence of ML-I. It was found that low concentrations of ML-I (2.5–5 ng/ml), which had only low cytotoxic effects alone, markedly enhanced the cytotoxicity of etoposide, whereas CD95-mediated apoptosis was not affected (Fig. 8). This sensitizing effect of ML-I on etopside-induced apoptosis was also confirmed by isobologram analysis, which revealed supra-additive effects of a combinatorial treatment with both drugs (Fig. 8*A*).

DISCUSSION

On the basis of their broad immunostimulatory activity and antitumor effects, MLs are increasingly used in adjuvant cancer therapy. A direct cytotoxic effect of MLs has been demonstrated in both cell cultures and animal models. Thus, a decrease in cell viability and proliferative capacity induced by MLs has been found in different tumor cell lines of murine or human origin (15, 49–51). In some

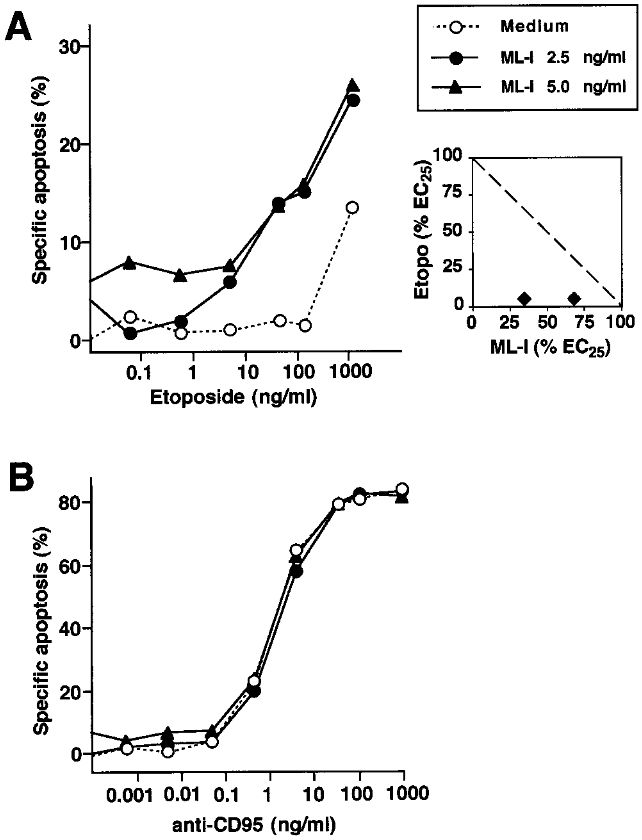

Fig. 8. ML-I enhances anticancer drug-induced cytotoxicity. Jurkat cells were either left untreated (○) or incubated with 2.5 ng/ml (●) or 5 ng/ml (▲) ML-I. At the same time, cells were coincubated with the indicated concentrations of either etoposide (*A*) or anti-CD95 (*B*). Cell death was determined after 24 h by the uptake of propidium iodide into cells. Note that at the concentrations used ML-I had only slight cytotoxic effects on its own. The inset in *A* depicts an isobologram analysis of the combined effects of ML-I and etoposide. The graph was constructed from the EC_{25} values of both agents, which represent the drug concentrations resulting in apoptosis of 25% cells after monotreatment. In the experiments, the EC_{25} values for ML-I and etoposide were 7.5 ng/ml and 1.2 μg/ml, respectively. According to the isobole analysis, the *dashed line* connecting the EC_{25} values represents additivity. Data points of a combined treatment, which fall to the left of the *dashed line,* indicate synergy or supra-additivity.

cases, it has been observed that treatment of cells with ML causes apoptotic cell death (15–17). Moreover, it could be shown that s.c. injection of remarkably low doses of ML-I (1 ng/kg) into mice was able to exert antitumor effects in a lymphosarcoma and fibrosarcoma, as well as a xenotransplanted leiomyosarcoma model (1, 52). Recently, it has been also reported that mistletoe extracts lead to the inhibition of lung metastasis and increased survival of mice transplanted with B16 melanoma cells (53). Finally, a pilot study in advanced tumor patients established a beneficial effect and partial tumor remission after treatment with mistletoe extracts (54). Thus far, however, the molecular mechanism of ML-induced cytotoxicity remains largely unknown.

In the present study, we investigated the mechanism of ML-I-induced cytotoxicity in leukemic T- and B-cell lines. In particular, Jurkat T cells were found to be highly sensitive to very low concentrations of ML-I. In both B- and T-cell lines, ML-I-triggered cell death resulted from induction of apoptosis, as assessed by classical criteria including cell shrinkage, membrane blebbing, and formation of hypodiploid DNA. We demonstrate that induction of apoptosis by ML-I was entirely dependent on the intracellular activation of caspases because (*a*) cell death was completely prevented by zVAD-fmk, a broad caspase inhibitor, and (*b*) proteolytic cleavage of the multiple procaspases to their active enzymes, as well as cleavage of the caspase-specific substrates, could be observed.

An interesting finding was the observation that ML-I was able to induce the activation of caspase-8/FLICE, the role of which, thus far, seemed to be restricted to apoptosis mediated by death receptors including CD95, TNF receptor 1, and TRAIL receptors. In the death receptor pathway, caspase-8 is a crucial component of the DISC, where it is recruited through its DEDs that interact with receptor-associated FADD (25, 43, 45). Because caspase-8 besides caspase-10 is the only DED-containing protease, it is assumed to act as a proximal initiator caspase, which subsequently processes downstream effector caspases. Initially, the activation of caspase-8, therefore, led us to suggest that death receptors such as CD95 might be involved in ML-I-induced apoptosis. It could be speculated that ML-I induces the *de novo* expression of CD95 ligand, resulting in subsequent CD95-dependent apoptosis by an auto- or paracrine mechanism. Such a scenario has been previously proposed for apoptosis mediated by anticancer drugs in some experimental systems (33, 34, 55). Another possibility was that ML-I directly triggered the cross-linking of CD95 or other glycosylated death receptors, similar to a mechanism as concanavalin A-induced activation of the T-cell receptor. To investigate the involvement of CD95 in ML-I-induced apoptosis, we made use of Jurkat T-cell clones that had been selected for resistance to CD95. In these cells, ML-I induced apoptosis with a very similar dose-response and kinetics as in the parental cells, indicating that CD95 was not required for ML-I-induced cytotoxicity. This assumption was also supported by experiments in BJAB transfectants that, due to overexpression of a dominant-negative FADD mutant, were resistant to apoptosis mediated by other death receptors. Both BJAB FADD-DN cells, as well as their nontransfected counterparts, underwent ML-I-induced apoptosis with a similar dose-dependency.

Recently, it has been reported that T-cell apoptosis can be triggered by galectin-1, a galactose-binding protein expressed in thymic stromal cells (56). To investigate whether a similar pathway may be engaged by ML-I, we analyzed cell death in the Jurkat clone JCAM, which, due to a deficiency of the phosphatase CD45, is resistant to galectin-1-mediated apoptosis (56). We could not find significant differences in the sensitivity of Jurkat cells either expressing or lacking CD45 (data not shown). Finally, we demonstrate that, in contrast to CD95, apoptosis mediated by ML-I was strongly prevented by brefeldin A, an inhibitor of vesicular transport. Collectively, these observations demonstrate that ML-I-induced apoptosis requires lectin internalization, but is not dependent on a surface receptor-mediated pathway.

Besides the death receptor/FADD pathway, it has become clear that a second, either independent or interconnected pathway exists, which is essentially controlled by the release of mitochondrial components. An early event in this process is the redistribution of cytochrome *c* into the cytosol, which is inhibited by antiapoptotic members of the Bcl-2 family (26, 27). In the cytosol, cytochrome *c* interacts with Apaf-1, an event that exposes the so-called CARD in Apaf-1. Caspases with a similar CARD-motif at their NH_2 terminus, such as caspase-9, can interact with Apaf-1, leading to their recruitment and activation. To investigate whether a mitochondria-controlled pathway is triggered by ML-I treatment, we measured the release of cytochrome *c* into cytosolic fractions. ML-I caused a time-dependent redistribution of cytochrome *c*, which was associated with the proteolytic activation of caspase-9. Interestingly, Bid, which has been recently proposed to mediate cytochrome *c* release after CD95-triggered caspase-8 activation (31, 32), was cleaved after ML-I treatment, also. Thus, these findings clearly implicate a mitochondrial death receptor-independent signaling pathway in ML-I-induced apoptosis. Our data also imply that caspase-8 can be activated not only at the level of a death receptor signaling complex, but also by cytochrome *c* translocation.

The activation of individual caspases by ML-I occurred with a roughly similar time-course, which makes it difficult to predict unequivocally the sequence of mitochondrial events. Therefore, for delineating of the exact caspase cascade, approaches with dominant-negative caspase mutants have to be used in future experiments. However, both the release of cytochrome *c* and activation of caspases clearly occurred before the loss of mitochondrial permeability transition, which has been recently observed also in apoptosis after Bax overexpression or anticancer drug-treatment (26, 57–58).

We assume that the apoptotic effect of ML-I involves its ribosome-inactivating activity and inhibition of protein synthesis. This effect is exerted by the A-chain of ML-I, whereas the B-chain is required for the binding to the cell membrane and the internalization of the A-chain. Although it has been shown that the B-chain itself has some biological activities, such as induction of cytokine synthesis (1), we could not detect a strong induction of apoptosis by either polypeptide alone. A low apoptotic effect after treatment of cells with the B-chain was probably caused by a residual contamination with the hololectin that was present in our B-chain preparation (data not shown). That inhibition of protein synthesis may be sufficient to induce apoptosis is also supported by the observation that cycloheximide induces caspase activation and apoptosis in Jurkat cells (data not shown).

An interesting finding was the observation that zVAD-fmk not only blocked caspase activation and mitochondrial permeability transition, but also cytochrome *c* release in ML-I treated cells, which is in contrast to apoptosis induced by etoposide or Bax overexpression (59, 60). It might be hypothesized that during ML-I treatment short-lived caspase inhibitors are depleted, which normally function to suppress residual caspase activity in living cells. Likely candidates in this respect include members of the inhibitor of apoptosis protein family which directly bind to and inhibit caspase-3, caspase-7, and caspase-9 (61). Consequently, depletion of inhibitor of apoptosis protein expression by ML-I should result in activation of caspase-9 which may then activate caspase-3 and caspase-8 and subsequently lead to Bid cleavage, cytochrome *c* release and an amplification of the caspase cascade. Another possibility might be that ML-I inhibits the synthesis of survival molecules such as Bcl-2 homologues or protein kinases which by phosphorylating regulatory proteins exert antiapoptotic effects.

A remarkable finding of our study was the observation that ML-I at low concentrations was able to potentiate the cytotoxic effect of anticancer drugs. It will be, therefore, interesting to investigate whether ML-I can overcome the resistance of drug-refractory tumor cells. In addition, we observed that, in contrast to several chemotherapeutic drugs, ML-I did not induce the activation of transcription factor nuclear factor κB in Jurkat cells (data not shown). Nuclear factor κB has been implicated recently in counteracting apoptosis by the inducible expression of still unknown antiapoptotic gene products (62). In summary, we demonstrate that MLs are potent inducers of apoptosis. These results may, therefore, provide promising insights and a molecular rationale to determine the therapeutic efficacy and clinical benefit of MLs in the treatment of different human cancers.

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Heike Bantel, Ingo H. Engels, Wolfgang Voelter, et al.

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