Helenalin Triggers a CD95 Death Receptor-independent Apoptosis That Is Not Affected by Overexpression of Bcl-xL or Bcl-2

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ABSTRACT

Apoptosis is required for proper tissue homeostasis. Defects in apoptosis signaling pathways, thus, contribute to carcinogenesis and chemoresistance. A major goal in chemotherapy is, therefore, to find cytotoxic agents that restore the ability of tumor cells to undergo apoptosis. We show here that the sesquiterpene lactone helenalin (10–50 μM) induces apoptosis in leukemia Jurkat T cells even if they lack the CD95 death receptor or overexpress the antiapoptotic proteins Bcl-xL or Bcl-2. Activated peripheral blood mononuclear cells, however, are not affected (10–50 μM helenalin). Helenalin led to a time-dependent (0–24 h) cleavage of the specific caspase-3-like substrate Asp-Glu-Val-Asp-tetrafluoromethylcoumarin as well as to the proteolytic processing of procaspase-3 and -8. Caspase activation was a necessary requirement for apoptosis because the broad-spectrum caspase inhibitor benzoyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (zVAD-fmk, 50 μM) completely abrogated helenalin-induced DNA fragmentation as well as phosphatidylserin translocation. Although the initiator caspase-8 was activated, the helenalin-induced signaling pathway did not require the CD95 death receptor as shown using cells without or with an antibody (ZB4)-blocked CD95 receptor. Helenalin also did not induce CD95 or CD95-ligand expression. On the other hand, helenalin was found to induce the release of cytochrome c from mitochondria that was not inhibited by the caspase inhibitor zVAD-fmk, which indicated that cytochrome c release precedes caspase activation. Cytochrome c release was accompanied by dissipation of the mitochondrial transmembrane potential (ΔΨm), which was partly inhibited by zVAD-fmk, which suggests that caspases are involved in loss of ΔΨm. Most importantly, overexpression of the mitochondria protecting proteins Bcl-xL or Bcl-2 failed to confer resistance to helenalin-induced apoptosis, although the data presented here suggest that helenalin induces a mitochondria-dependent pathway. Thus, helenalin is a promising experimental cytotoxic agent that possibly points to new strategies to overcome apoptosis resistance attributable to overexpression of antiapoptotic Bcl-2 proteins.

INTRODUCTION

Chemotherapeutic drugs have been shown to use apoptotic pathways to mediate their cytotoxic effect (1). Two major routes have been identified through which cytotoxic drugs induce apoptosis. One involves the activation of the CD95 (Apo-1/Fas) receptor system, which results in a caspase-8-dependent caspase cascade and subsequent cell death. The other pathway is dependent on mitochondrial cytochrome c release, leading to activation of caspase-9 and a caspase cascade downstream of mitochondria (2). Both pathways are highly regulated. Consequently, defects in the regulation of apoptosis can render tumor cells resistant to death stimuli. Various mechanisms underlying tumor cell resistance to apoptosis are currently discussed (3).

Resistance to death receptor-induced apoptosis can be caused by mechanisms such as down-regulation of the receptor and deficient up-regulation of CD95-L, as well as mutations within the gene encoding the receptor. Moreover, defects in the apoptotic signaling pathway downstream of the CD95 receptor, e.g., by elevated expression of proteins like FLIP, a homologue of caspase-8 that lacks proteolytic activity, can lead to decreased sensitivity toward apoptotic stimuli (3, 4).

Members of the antiapoptotic Bcl-2 family proteins confer protection against most apoptotic stimuli that act via mitochondria. Bcl-2 and Bcl-xL were shown to prevent mitochondrial cytochrome c release and subsequent caspase activation and cell death (3, 5, 6). In a large percentage of human neoplasias, antiapoptotic Bcl-2 proteins were found to be overexpressed or proapoptotic Bcl-2 homologues like Bax that appear to be reduced or functionally inactive (3, 7). These alterations in expression or functionality of Bcl-2 family members can render tumor cells more resistant to a wide variety of cell death stimuli, including essentially all classical chemotherapeutic drugs (3).

An important goal in chemotherapy is, therefore, to find new cytotoxic agents that are able to increase or restore the ability of tumor cells to undergo apoptosis.

In this respect, sesquiterpene lactones are promising compounds of natural origin. They represent a structural class of phytochemicals typically found in the composite family (Asteraceae). Since decades ago, these plant constituents are known to have cytotoxic capacity. The cytotoxicity of sesquiterpene lactones is suggested to be attributable to their ability to react with sulfhydryl groups, e.g., of cysteine residues in a Michael-type addition (8, 9). Thus, the primary cellular target of sesquiterpene lactones differs from those of classical chemotherapeutic drugs like doxorubicin, cisplatin (interaction with DNA), etoposide (inhibition of topoisomerase II), methotrexate (antagonization of folic acid), or vincristine (inhibition of mitosis).

Although the cytotoxicity of sesquiterpene lactones is well documented (8–10), it is not known whether sesquiterpene lactones are able to induce apoptosis in tumor cells.

The aims of the present study were, therefore, first, to examine whether helenalin, one of the best investigated sesquiterpene lactones with regard to cytotoxicity (8–11), is able to induce apoptosis in leukemia T cells, and, second, to characterize the apoptotic pathways that are involved.

MATERIALS AND METHODS

Compounds. Helenalin [4-hydroxy-5,8-dimethyl-3-methylene-3a,4a,5,8,9,9a-hexahydro-3H,4H-azuleno(6,5-b)furan-2,6-dione] was isolated from Psilotrope cooperi (A. Gray) Green as described previously (12). Purity was 99.0% as judged by high-performance liquid chromatography. Helenalin was dissolved in DMSO and further diluted in PBS. Final DMSO concentration did not exceed 0.1%, a concentration ascertained not to interfere with the experimental results.

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3 The abbreviations used are: CD95-L, CD95-ligand; zVAD-fmk, benzoxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PL, propidium iodide; FACS, fluorescence-activated cell sorter/sorting; afc, 7-amino-4-tetrafluoromethylcoumarin; DEVD-afc, Asp-Glu-Val-Asp-afc; JC-1, 5,5′,6–6′-tetrachloro-1,1′,3′,3′-tetratetraethylbenzimidazol carbocyanine iodide; MMP, mitochondrial membrane permeabilization; ΔΨm, mitochondrial transmembrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PTP, peripheral transfer pore; ANT, adenine nucleotide translocator; MAPK, mitogen-activated protein kinase.

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Helenalin Induces Apoptosis in Jurkat T Cells. Helenalin was applied to Jurkat T cells at a concentration that was shown to effectively impair cell viability (MTT assay; IC_{50} 6 µM; data not shown). Treated cells had the typical appearance of apoptosis, i.e., they showed shrinkage and apoptotic bodies as well as fragmented apoptotic nuclei visualized by fluorescence microscopy after DNA staining with Hoechst 33342 (data not shown). Helenalin treatment also led to the exposure of phosphatidylserine on the outside of the plasma membrane as detected by Annexin V-FITC staining (Fig. 1A). Furthermore, analysis of PI-stained nuclei by flow cytometry showed that helenalin-treated cells displayed a population of nuclei with hypodiploid DNA (Fig. 1B). Dose-response studies revealed that helenalin (10–50 µM, 24 h) dose-dependently increased the percentage of apoptotic cells up to 80% (Fig. 2A). Helenalin (10 µM)-triggered apoptosis also occurred time dependently (4–24 h). First significant apoptosis could be detected 16 h after cell stimulation (Fig. 2B).

The apoptosis-inducing capacity of helenalin in leukemia T cells raised the question as to whether helenalin also affects healthy human PBMCs. Interestingly, dose-response studies revealed that PBMCs activated with PHA (1 µg/ml, 48 h) were resistant toward helenalin up to 50 µM. In contrast, staurosporine (0.5 µM), applied as positive control, readily induced DNA fragmentation in these cells (Fig. 2C).

Helenalin-induced Apoptosis Is Dependent on Caspase Activation. To investigate whether helenalin-triggered apoptosis requires the activation of caspases, cells were pretreated with the broad-spectrum caspase inhibitor zVAD-fmk. The inhibitor completely abrogated helenalin-induced apoptosis (Fig. 3A) which indicated that caspases are essential components in this apoptotic pathway. In addition, helenalin was found to increase caspase-3-like activity time dependently reaching the level of significance 16 h after cell exposure to helenalin (Fig. 3B). To further characterize the involved caspases, the activation of two key components of the caspase cascade, the downstream effector caspase-3 and the initiator caspase-8 were examined below. The remaining pellet of permeabilized cells was lysed in 0.1% Triton/PBS (15 min, 4°C), centrifuged (12,000 × g, 4°C, 10 min), and the supernatant containing mitochondrial cytochrome c analyzed by SDS-PAGE.

Western Blot Analysis. Cells were collected by centrifugation, washed with ice-cold PBS, and lysed in 1% Triton X-100, 0.15 M NaCl, and 10 mM Tris-HCl (pH 7.4) with the protease inhibitor complete (Roche, Mannheim, Germany) for 30 min. Lysates were homogenized through a 22-gauge needle and centrifuged at 10,000 × g for 10 min at 4°C. Equal amounts of protein were separated by SDS-PAGE (10% for caspase-8, 12% for Bcl-2 proteins, 15% for caspase-3 and cytochrome c), transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Eschborn, Germany). Equal protein loading was controlled by Coomassie Blue staining of gels. Membranes were blocked with 2% BSA in PBS containing 0.05% Tween 20 (1 h) and incubated with specific antibodies against caspase-8 (mouse monoclonal antibody C-15, 1:5 dilution of hybridoma supernatant; kindly provided by Dr. P. H. Krammer, Heidelberg, Germany), caspase-3 (mouse monoclonal antibody clone 19; Transduction Laboratories, Heidelberg, Germany), cytochrome c (mouse monoclonal antibody 7H8.2C12; Pharmingen, Heidelberg, Germany), Bcl-xL or Bcl-2 (rabbit polyclonal antibodies, clones S18 and N-19, respectively; Santa Cruz Biotechnology, Heidelberg, Germany) overnight at 4°C. Specific proteins were visualized by secondary antibodies conjugated to horseradish peroxidase and the Renaissance Plus reagent (NEN Life Science, Zaventem, Belgium). Pictures were taken on a Kodak Digital Science Image station 440CF (NEN, Life Science, Zaventem, Belgium).

Statistical Analysis. All of the experiments were performed at least three times. Results are expressed as mean ± SE. Statistical comparisons were made by ANOVA followed by a Dunnett multiple comparisons test or by an unpaired two-tailed Student’s t test. P < 0.05 were considered significant.

RESULTS

Analysis of Caspase-3-like Activity. Cells were collected by centrifugation, washed with ice-cold PBS, and lysed in 5 mM MgCl2, 1 mM EGTA, 0.1% Triton X-100, and 25 mM HEPES (pH 7.5) containing the protease inhibitor complete (Roche, Mannheim, Germany). Cytosol was prepared by centrifugation at 14,000 × g (15 min, 4°C). The fluorometric DEVD-afc cleavage assay was measured as described above using a mouse antihuman CD95-L antibody (NOK-1; Pharmingen, Heidelberg, Germany), with the exception that cells were fixed in 1 ml of paraformaldehyde (4%; 15 min, 4°C), washed with PBS, permeabilized with ice-cold ethanol (100%, 60 min, 4°C), and washed again prior to incubation with the antihuman CD95-L antibody.

Analysis of Cytochrome c Release. Release of cytochrome c from mitochondria was analyzed according to Leist et al. (21). Briefly, cell pellets were resuspended in permeabilization buffer [210 mM D-mannitol, 70 mM sucrose, 10 mM HEPES, 5 mM succinate, 0.2 mM EGTA, 0.15% BSA, and 80 µM digitonin (pH 7.2)] at 4°C and gently shaken at 4°C for 10 min. Permeabilized cells were centrifuged (300 × g); the supernatant was removed, and the cells were centrifuged again (10 min, 13,000 × g). The obtained cytosol was separated on a 15% SDS-PAGE and probed for cytochrome c as described below. The remaining pellet of permeabilized cells was lysed in 0.1% Triton/PBS (15 min, 4°C), centrifuged (12,000 × g, 4°C, 10 min), and the supernatant containing mitochondrial cytochrome c analyzed by SDS-PAGE.
Fig. 1. Helenalin induces apoptosis in Jurkat leukemia T cells. A, FACS analysis of Annexin V-FITC and PI-stained cells, either left untreated (Control), treated with helenalin (6 μM, 24 h) or as a positive control with actinomycin D (2 μg/ml, 24 h). Cells appearing in the lower right quadrant show positive Annexin V-FITC staining, which indicates phosphatidyserine translocation to the cell surface, and no DNA staining with PI, which demonstrates intact cell membranes, both features of early apoptosis. B, FACS analysis of PI-stained nuclei of cells treated as indicated under A. The histograms show the distribution of nuclei with different DNA content. Counts left of the "G0-peak" (gated) demonstrate the appearance of nuclei with subdiploid DNA content.

Fig. 2. Helenalin induces apoptosis dose- and time-dependently in Jurkat leukemia T cells but not in healthy human PBMCs. A, dose-dependent induction of apoptosis by helenalin. Cells were incubated with increasing concentrations of helenalin for 24 h. B, time-dependency of helenalin-induced apoptosis. Cells were incubated with 10 μM helenalin for the indicated time periods. C, influence of increasing concentrations of helenalin (24 h) on PHA-activated healthy human PBMCs. Staurosporine (50n, 500 nM, 24 h) was used as positive control. % Apoptotic cells, percentage of cells with subdiploid DNA content as described under “Materials and Methods,” **Bars, the mean ± SE of at least three independent experiments performed in duplicate; *, P < 0.05; **, P < 0.001 (ANOVA/Dunnett).

Helenalin Induces MMP. Many apoptotic signals transduce their death-inducing message via mitochondria. A key event in mitochondria-controlled apoptotic pathways is the outer and/or inner MMP, involving the release of proteins, such as cytochrome c, from the intermembrane space and the dissipation of the electrochemical gradient (∆Ψm), created by the proteins of the respiratory chain located on the inner mitochondrial membrane (23). Fig. 5A shows that, indeed, helenalin caused a time-dependent release of cytochrome c into the cytosol, indicating the occurrence of outer MMP. Considerable amounts of cytochrome c in the cytosol were detectable as early as 8 h after cell treatment. Preincubation with the caspase inhibitor zVAD-fmk (1 h, 50 μM) was unable to prevent cytochrome c release, which indicated that cytochrome c release preceded caspase activation. Helenalin also led to a dissipation of ∆Ψm as detected by flow cytometry using the fluorochrome JC-1 (19, 20). Substantial loss of ∆Ψm was evident 16 h after the addition of helenalin as seen by a decrease in FL-2 intensity and an increase in FL-1 intensity (Fig. 5B). Pretreatment with zVAD-fmk (1 h, 50 μM) reduced ∆Ψm (Fig. 5C), suggesting that caspases are involved in helenalin-induced inner MMP. Thus, helenalin-mediated apoptosis involves inner and outer MMP.

Influence of Antiapoptotic Bcl-xL and Bcl-2 Proteins on Helenalin-mediated Apoptosis. Antiapoptotic members of the Bcl-2 family like Bcl-xL and Bcl-2 were shown to prevent cytochrome c release and subsequent apoptotic cell death by a variety of apoptotic stimuli (3, 5, 6). Because the broad-spectrum caspase inhibitor zVAD-fmk was unable to block cytochrome c release in response to helenalin, we assumed that caspase activation and subsequent cell death occurs as a consequence of outer MMP downstream of mitochondria. To verify this notion, Jurkat cells overexpressing Bcl-xL or Bcl-2 protein (Fig. 6A) were exposed to helenalin. Surprisingly, Bcl-xL as well as Bcl-2 failed to abrogate helenalin-mediated apoptotic cell

Fig. 3C shows the time-dependent processing of the inactive M₆, 32,000 caspase-3 precursor to the active p17 subunit by Western blot analysis. Cleavage products were detectable as early as 8 h after cell stimulation but increased up to 16 h. Caspase-8, expressed in two functionally active isoforms, caspase-8α and caspase-8β, is processed on activation to p43 and p41 intermediates and to the p18 active subunit (22). Helenalin also led to activation of caspase-8. Cleavage products of procaspase-8 were detectable 16 h after helenalin treatment, which indicates a delayed activation compared with caspase-3 (Fig. 3D).

Helenalin-induced Apoptosis Occurs Independent of the CD95 System. Several cytotoxic drugs were shown to mediate apoptosis by increasing the expression of the CD95 receptor or inducing the CD95-L (2, 4). We, therefore, monitored changes in the expression of the CD95 receptor as well as of the CD95-L. The expression of the CD95 receptor, remained unchanged up to 24 h after cell exposure to helenalin (Fig. 4A, left). Examining CD95-L expression, no up-regulation could be detected over a period of 24 h. In contrast, helenalin treatment decreased CD95-L expression (Fig. 4A, right).

Although helenalin treatment did not lead to increased CD95 receptor or CD95-L expression, helenalin might activate the CD95 receptor system by other mechanisms. Therefore, helenalin was applied to the subclone JurkatΔ6 which lacks the CD95 receptor (13). Helenalin induced apoptosis in Jurkat and JurkatΔ6 cells to a similar extent (Fig. 4B), indicating that apoptosis occurs independently of the CD95 receptor. To further corroborate this outcome, we used an antagonistic anti-CD95 antibody (ZB4). Jurkat cells preincubated for 1 h with the ZB4 antibody did not undergo apoptosis on exposure to soluble CD95-L, whereas on helenalin treatment, antibody-blocked Jurkat cells died similarly to the Jurkat cells that were not preincubated with ZB4 (Fig. 4C).
death, although apoptosis was slightly reduced when helenalin was applied at lower concentrations (10–20 μM). Fig. 6B (top graph) shows the dose-response studies of helenalin performed with Jurkat cells stably transfected with vector alone (Jurkat/neo), Bcl-xL (Jurkat/bcl-xL), or Bcl-2 (Jurkat/bcl-2). To verify that the used transfected Jurkat clones were working properly, we applied staurosporine, known to induce a mitochondrial caspase cascade that is inhibited by Bcl-2 and Bcl-xL (24, 25). Fig. 6B (bottom graph) demonstrates that staurosporine (50–400 nM) dose-dependently triggers apoptosis in Jurkat/neo cells whereas Jurkat/Bcl-xL and Jurkat/Bcl-2 cells are protected against staurosporine. Thus, helenalin seems to be able to trigger apoptosis either by a mechanism that bypasses mitochondrial events or by inactivating effectively mitochondrial protection by Bcl-xL and Bcl-2.
We demonstrated that the sesquiterpene lactone helenalin triggers apoptosis in leukemia Jurkat T cells as judged by classical morphological as well as biochemical criteria. Activation of caspases was shown to be a critical event in helenalin-induced apoptosis. Proteolytic cleavage of the initiator caspase-8, which was demonstrated in response to helenalin, typically occurs after triggering cell surface death receptors like the CD95 receptor (5, 26). Moreover, in various cell types, chemotherapeutic drugs were demonstrated to activate the CD95 receptor pathway by increasing the CD95 receptor expression or induce the CD95-L (2, 4). Therefore, we first investigated whether this mechanism applies also to helenalin. Flow cytometric experiments, however, revealed that helenalin does neither increase CD95 receptor expression nor induce CD95-L. In addition, CD95-resistant Jurkat T cells that lack the CD95 receptor were susceptible to helenalin-induced apoptosis, as are parental cells, indicating that CD95 is not required for helenalin-mediated apoptosis. This result was further corroborated using the antagonistic anti-CD95 antibody ZB4. Preincubation with ZB4 completely blocked CD95-L-induced apoptosis, whereas helenalin-mediated cell death was unaffected. Thus, helenalin is able to trigger apoptosis independent of the CD95 receptor.

Besides the caspase cascades initiated by death receptors, another cascade that is essentially controlled by mitochondria exists (2, 5, 7, 23). A central event in mitochondria-controlled cell death seems to be the occurrence of outer and/or inner MMP (23). Outer MMP leads to the release of mitochondrial intermembrane proteins like cytochrome \( c \) into the cytosol. Released cytochrome \( c \) then triggers the assembly of the so-called apoptosome by interacting with the apoptotic pro-
Helenalin, indeed, initiated redistribution of cytochrome c into the cytosol as early as 8 h after cell treatment. Thus, cytochrome c release seemed to be an early event in the helenalin-mediated apoptosis that preceded caspase-3-like activity. Interestingly, the broad-spectrum caspase inhibitor zVAD-fmk was unable to prevent cytochrome c release, which was evidence that caspase-activation occurred solely downstream of mitochondria.

Inner MMP leading to loss of $\Delta \Psi_m$ mostly accompanies outer MMP (23). In fact, helenalin clearly induced dissipation of $\Delta \Psi_m$. Interestingly, loss of $\Delta \Psi_m$ appeared to be delayed compared with cytochrome c release but correlated with the onset of caspase-3-like activity, which suggests a possible link between caspas and mitochondrial perturbation. Indeed, zVAD-fmk was able to considerably reduce dissipation of $\Delta \Psi_m$. This outcome fits into recently published data proposing a feedback amplification loop between caspase activation and mitochondrial dysfunction (25, 27–31). This link is mediated either by cytosolic factors that serve as caspase substrates and lead, once cleaved, to cytochrome c release (25, 28, 29) or directly by caspases that affect mitochondrial membrane proteins like the anti- apoptotic Bcl-2. Bcl-2 cleavage products then cause cytochrome c release and caspase activation (27, 30, 31).

Considering our data which strongly implicate a helenalin-initiated caspase cascade downstream of mitochondria, the most interesting finding was that the mitochondria-protecting proteins Bcl-xL and Bcl-2 were unable to prevent helenalin-induced apoptotic cell death. This outcome suggests that helenalin triggers a (second) pathway that bypasses mitochondria and/or that helenalin is able to inactivate the mitochondrial protection conferred by Bcl-2 proteins. Bcl-2 family members are localized at the membranes of mitochondria, in which it is suggested that they control membrane permeability either by collaborating with proteins of the PTP or by forming autonomous pores (7, 32). Inactivation of Bcl-2 proteins, e.g., by mutation, phosphorylation, or cleavage can abrogate their mitochondria protective effects (3, 6, 7, 31). Interestingly, an additional mechanism by which the effect of antiapoptotic Bcl-2 members can be overcome seems to be the thiol cross-linking of the ANT, a protein of the PTP localized on the inner mitochondrial membrane (7). Sesquiterpene lactones are known to react covalently with thiol groups (8, 9). It is, therefore, tempting to speculate on a direct effect of helenalin on the PTP by binding to the ANT. Additional studies will address whether helenalin may also signal independently of mitochondrial events like cytochrome c release or rather whether it targets mitochondrial proteins to overcome Bcl-2 and Bcl-xL-mediated protection.

In view of the apoptosis-inducing potential of helenalin even in Bcl-xL- and Bcl-2-overexpressing cells, it is a remarkable finding that activated PBMCs are resistant toward helenalin. Activated T cells, a major population in PHA-stimulated PBMCs, were shown to possess increased apoptosis resistance by transiently increasing the expression of Bcl-xL (33). In fact, PHA-activated PBMCs used in the present study were found to express considerable levels of Bcl-xL. Bcl-xL overexpression in Jurkat cell, however, failed to confer resistance toward helenalin-induced apoptosis, making it unlikely that Bcl-xL accounts for the remarkable resistance of PBMCs toward helenalin. Other studies suggest that, besides the increase in Bcl-xL, a lack of recruitment of caspase-8 to the death-inducing signaling complex (DISC) might be responsible for the resistance of activated peripheral T cells toward CD95 receptor-mediated apoptosis (34).

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$^a$ Unpublished data.
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