Reviews

The Cephalostatin Way of Apoptosis[⊥]

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The cephalostatins, bis-steroidal natural products from the marine tube worm *Cephalodiscus gilchristi*, were isolated by Dr. G. R. Pettit and his group. These compounds show a unique cytotoxicity profile in the in vitro screen of the National Cancer Institute, suggesting a novel mechanism of action. Indeed, cephalostatin 1 (**1**) is an extremely powerful agent that acts via an unusual apoptosis pathway. It induces selective Smac/DIABLO, but no cytochrome *c* release from mitochondria. Nevertheless, caspase-9 is required for apoptosis induction. Interestingly, caspase-9 is activated without the participation of the apoptosome, leading to the question of its mechanism of activation. We found that endoplasmic reticulum stress-associated caspase-4 contributes to nonclassical cephalostatin-mediated caspase-9 activation, additionally pointing out the unusual pathway used by this substance. Cephalostatin 1 (**1**), therefore, provides a very good tool to discover novel apoptotic pathways, which might be important in the understanding and treatment of chemo-resistant cancer.

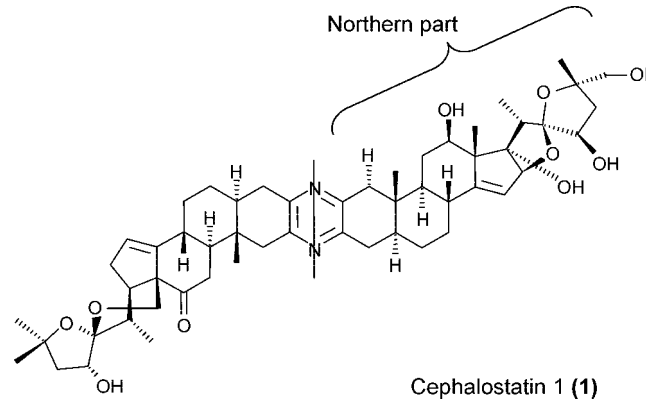
Introduction

Since ancient times natural products have played a major role in the treatment of diseases. Discoveries based upon folk medicine include, for example, the *Vinca* (*Catharanthus*) alkaloids, which have significantly contributed to the successful treatment of cancer.[1] These and further discoveries led to the initiation of a screening program for antitumor agents by the U.S. National Cancer Institute (NCI). Between 1960 and 1982 about 35 000 plant samples were tested, primarily against mouse leukemia cell lines. The most effective drug obtained from the screening was paclitaxel (Taxol), originally isolated from the bark of *Taxus brevifolia*.[1,2]

In 1985, the NCI started a new program in which extracts from plants, animals, and microorganisms (increasingly those of marine origin) were tested against a panel of 60 human cancer cell lines.[1] The intent was to identify compounds active against solid tumors, which may be missed in the initial screen against leukemia cells. Presently, almost 60% of drugs approved for cancer treatment are of natural origin.[3,4] In recent decades compounds of marine origin have gained more and more attention in this context.

Why are compounds of marine origin promising candidates for anticancer drug development? Many marine organisms are soft-bodied and are sedentary in lifestyle. Thus, they have developed the ability to produce toxic compounds or to obtain them from marine microorganisms as chemical defenses. These compounds need to be extremely toxic since they are released and diluted into seawater.[5] Although nowadays the potency of marine compounds especially for the development of new anticancer drugs is recognized, there are still major difficulties, such as sourcing the drug in sufficient availability for preclinical and clinical studies.

Without any doubt, Dr. G. Robert Pettit and his group have performed outstanding work and are regarded as pioneers in the field of marine compounds. This review will focus on this group of compounds, the cephalostatins, and their unusual mechanisms of cytotoxicity.



Cephalostatin 1 **(1)**

Figure 1. Chemical structure of cephalostatin 1 (**1**).

The Cephalostatins

The cephalostatins are a group of structurally strongly related bis-steroidal compounds isolated by Prof. Pettit's group from the South African marine tube worm *Cephalodiscus gilchristi* Ridewood (Cephalodiscidae). The chemical structure of cephalostatin 1 (**1**) is depicted in Figure 1. Up to the present, 19 derivatives have been characterized. The cephalostatins belong to the most cytotoxic marine natural products ever tested by the NCI. All cephalostatins show the same unique cytotoxicity profile in the NCI-60 cell line panel (Figure S1, Supporting Information, shows cephalostatins 1 and 2, as examples, and displays the growth inhibitory potency of these substances against 60 cancer cell lines of diverse origin).[6,7] Although the cytotoxicity profiles of the cephalostatins do not differ, their potency varies depending on chemical composition. Structure–activity studies on different cephalostatins have revealed that the "northern" part of these molecules is the most commonly shared unit among the cephalostatins and is also strongly associated with resultant antitumor activity.[8] Cephalostatins 1 and 2 are known to

[⊥] Dedicated to Dr. G. Robert Pettit of Arizona State University for his pioneering work on bioactive natural products.

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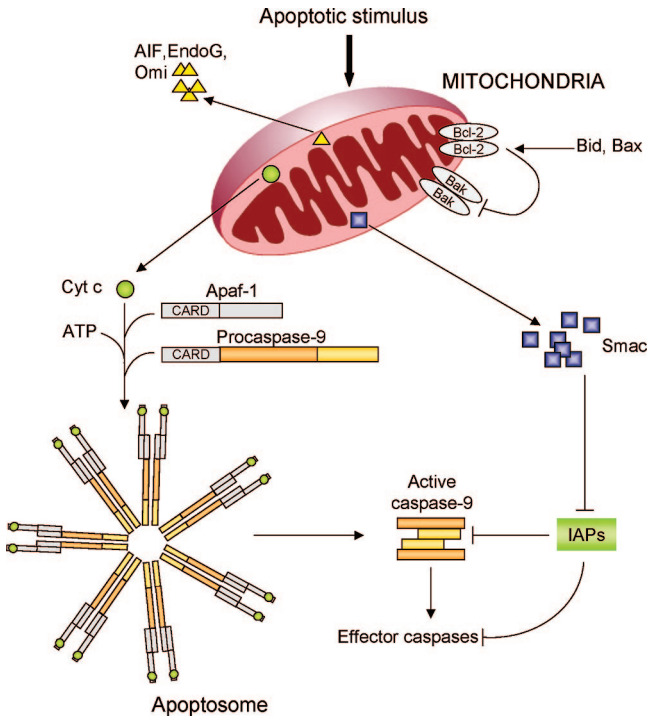

Figure 2. Intrinsic apoptotic pathway. Mitochondria are the central organelles in the intrinsic apoptosis pathway. Upon exposure to many different stimuli such as chemotherapeutic drugs, the mitochondria membrane is permeabilized and proapoptotic proteins are released into the cytosol. A complex called an apoptosome is formed consisting of cytochrome *c*, Apaf-1, and procaspase-9, where this caspase is activated and initiates the activation of effector caspases. Smac abolishes the negative regulation of caspases by IAP. AIF, Omi, and EndoG are thought to induce caspase-independent cell death. Bcl-2 family proteins (e.g., Bax, Bid) regulate the intrinsic pathway.

be the most potent cephalostatins in the NCI-60 cell line panel, with GI (50% growth inhibition) values of approximately 1 nM.[9,10] Interestingly, the cephalostatin cytotoxicity fingerprints did not show comparable correlations to any other member of the standard agent database, suggesting that the cytotoxicity of these marine compounds derives from a unprecedented, but as yet undefined, mechanism of action.[11] Initial studies carried out by our group supported this notion.[12–14] Beyond in vitro tests, cephalostatin 1 (**1**) was proven to be effective in several xenograft models, such as melanoma, sarcoma, leukemia, and human mammary carcinoma.[15]

Apoptosis Signal Transduction

Mitochondria. Apoptosis, or the so-called programmed cell death, is a tightly regulated and highly conserved event requiring the interaction of multiple factors. Defects in apoptosis-inducing pathways may lead to cancer, which is characterized by an imbalance between cell proliferation and cell death.[16] Moreover, since cancer chemotherapeutic drugs induce death in malignant cells by triggering apoptosis, defects in apoptosis signaling pathways may lead to resistance in chemotherapy.[17,18]

Apoptosis can be triggered by various stimuli from outside (receptor-mediated extrinsic pathway) or inside (mitochondria-mediated intrinsic pathway) the cell. Upon binding of death receptor ligands such as CD95L or TNF, a so-called death-inducing signaling complex (DISC) is formed, leading to activation of a initiator caspase, i.e., caspase-8. The central event of the intrinsic pathway (see Figure 2) is the mitochondrial membrane permeabilization (MMP) of the outer and the inner mitochondrial membrane. MMP causes the dissipation of the mitochondrial membrane potential ($\Delta\Psi$m), which is required for mitochondrial functions such as ion transport and energy conservation.[19,20] Mitochondria-mediated apoptosis is strictly controlled by members of the Bcl-2 family, which promote or inhibit the formation of pores triggering the release of death-inducing molecules from the mitochondrial intermembrane space.[21–23]

Besides other factors, cytochrome *c* and Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low PI) are the main pro-apoptotic molecules released from mitochondria as a consequence of MMP. Cytochrome *c* mediates the classical apoptosis pathway. It prompts the ATP-dependent assembly of cytochrome *c*, Apaf-1, and procaspase-9 into a complex called the "apoptosome", leading to efficient processing and activation of the initiator caspase-9.[24] This, in turn, activates downstream caspases, such as caspase-3, resulting in DNA fragmentation and apoptosis. Smac provides an additional mechanism to promote caspase activation by binding to and antagonizing IAPs (inhibitors of apoptosis proteins). These proteins can counteract both the activation and the activity of caspases.[25]

Besides the mitochondria, other organelles or cellular compartments (e.g., the endoplasmic reticulum (ER), the cytoskeleton, lysosomes, and the nucleus) may contribute to apoptosis signaling by sensing damage or integrating pro-apoptotic signals.[26]

Endoplasmic Reticulum. As mentioned above, other cellular compartments can be involved in apoptotic signaling. Stress induction in the ER has also been linked to apoptosis induction (for overview see Figure 3).[27,28] ER stress leads to the activation of genes possessing an unfolded protein response (UPR) element, which controls the levels of molecular chaperones, such as M_r 78 000 glucose-regulated stress protein (BiP/GRP78) involved in protein folding in the ER.[29] Furthermore, the eukaryotic initiation factor-2 (eIF2) is phosphorylated by the PKR-like ER-localized eIF2alpha kinase (PERK) in response to ER stress, leading to an attenuation of translational initiation and protein synthesis.[28] When these stress modulators are unable to rescue cells, various apoptotic pathways are activated. Recruitment of TNF receptor-associated factor 2 (TRAF2) to activated stress sensor proteins, called IREs, induces the apoptosis signal-regulating kinase 1 (ASK1)/c-Jun N-terminal kinase (JNK) cascade.[30] In addition, ER-specific caspases, such as caspase-12 in the murine system, seem to aggregate at the ER membrane surface through TRAF2 proteins, resulting in their cleavage and activation.[31] In humans, caspase-4 has been proposed to play a role as an ER stress-specific caspase in a similar manner to caspase-12.[32] Furthermore, the UPR increases the transcription of the transcription factor C/EBP homologous protein (CHOP), which is closely associated with cell death.[33,34]

Since most of the chemotherapeutic drugs used in cancer treatment signal through mitochondrial cytochrome *c* release, it is very difficult to distinguish whether the ER plays a role as stress sensor for chemotherapeutic agents that reroute the signal directly through mitochondria and the apoptosome or whether the ER is able to activate its own death pathway. In this respect, it is important to characterize specific elements of the ER stress response, because they could represent novel targets for the development of new cancer chemotherapeutic strategies.

Cephalostatin-Induced Apoptosis Pathways in Leukemia

New drugs that induce apoptosis by mechanisms differing from those of classical chemotherapeutic drugs might provide a chance of overcoming chemoresistance. The unique differential cytotoxicity profile of the cephalostatins obtained from the 60-cell-line in vitro screen of the NCI strongly suggested that these compounds may utilize a new molecular mechanism to trigger cell death. Thus, cephalostatin 1 (**1**)-induced cell death was characterized using the well-established Jurkat leukemia T cells as a model.

Morphology. The overall morphology of cephalostatin 1 (**1**)-induced cell death in leukemia Jurkat T cells corresponds very well

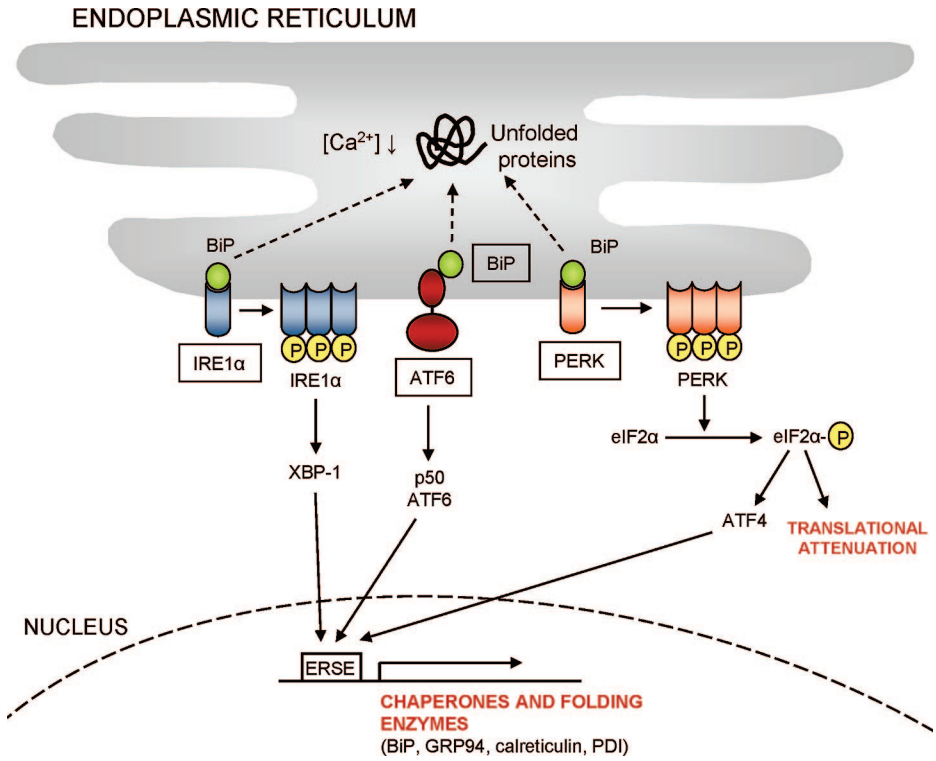

Figure 3. Apoptosis induction by the ER. The UPR is activated by the accumulation of unfolded proteins in the ER. The transmembrane proteins IRE1, PERK, and ATF6 act as sensors and induce the transcription factors XBP-1, p50ATF6, and ATF4, which in turn stimulate the transcription of their target genes. Target genes of the UPR share a consensus sequence in their promoters, the ER stress response element (ERSE), and code for chaperones and other proteins involved in protein folding such as BiP/GRP78, GRP94, calreticulin, and PDI (protein disulfide isomerase). Phosphorylation of eIF2α by PERK leads to an attenuation of general protein synthesis.

to classical signs of apoptosis (Figure 4), with cells beginning to shrink and cellular granularity increasing. Hoechst staining of the nucleus and fluorescence microscopy revealed classical DNA fragmentation and chromatin condensation in response to treatment with **1**. Cephalostatin 1 (**1**) triggered phosphatidylserine translocation to the outside of the plasma membrane, which is an early sign of apoptosis.[12] Phosphatidylserine is an aminophospholipid normally located in the cytosolic layer of the plasma membrane and translocates to the outer side of the membrane by activation of scramblases under apoptotic conditions. Once outside, it serves as an "eat me" signal for phagocytes and other neighboring cells, thus triggering removal of the apoptotic cells.

Analysis of death morphology by electron microscopy, however, indicated some differences from classical apoptotic organelle morphology. Thus, in addition to the nuclei and mitochondria, the ER seemed to be affected by treatment with **1**. The ER membranes were enlarged and vesicles that were presumably ER-derived accumulated in the cytoplasm.[12] This will be important in the future when looking at the signaling pathways used by cephalostatin 1 (**1**).

Selective Smac Release. Interestingly, although cephalostatin 1 (**1**) led to dissipation of the mitochondrial membrane potential, cytochrome *c* and AIF were not released from the mitochondrial intermembrane space. In contrast, Smac/DIABLO was heavily unleashed as an early event of treatment with **1** (Figure 4).[12] This particular selectivity for the release of Smac/DIABLO from mitochondria was not restricted to cephalostatin 1 (**1**). Cephalostatin 2 showed the same behavior. This interesting finding was also not specific to leukemic cells since other cell lines, such as SK-Mel-5 and MCF-7 cells, responded in a similar way on treatment with cephalostatin 2 (unpublished results). Studies clarifying the particular role of Smac for cephalostatin-mediated cell death are ongoing.

Caspase Activation. In addition to this unusual finding, cephalostatin 1 (**1**) treatment of Jurkat leukemia T cells led to the activation of caspases-9, 8, and 3, an essential step in cephalostatin-induced apoptosis since the inhibition of caspases completely

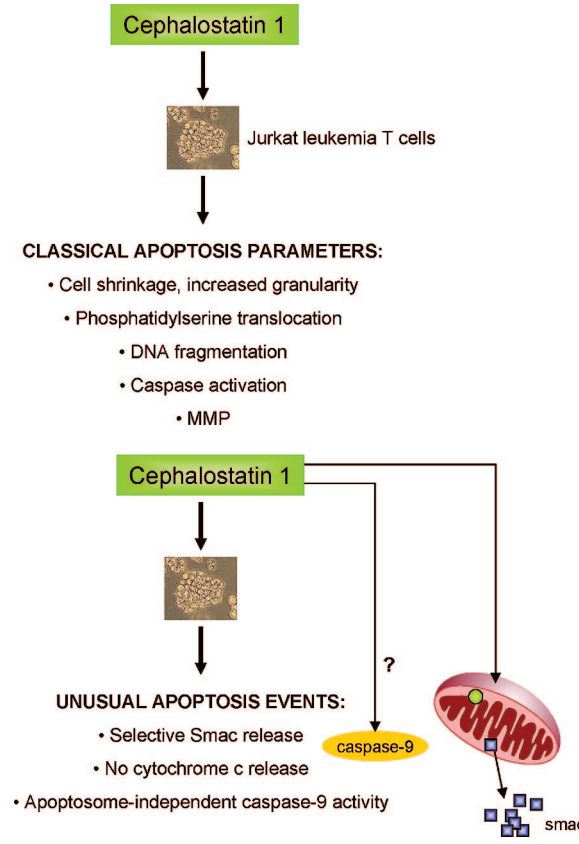

Figure 4. Cephalostatin 1 (**1**) induces classical apoptosis parameters as well as unusual signaling events.

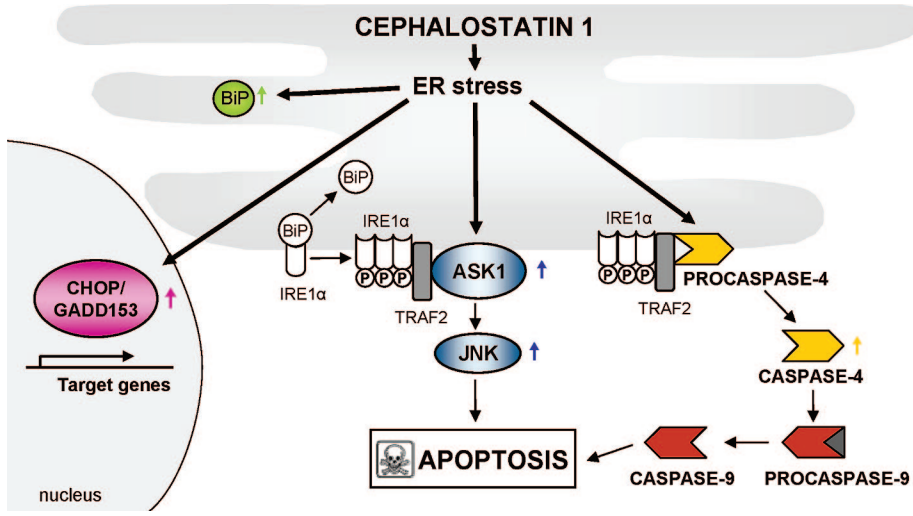

Figure 5. Proposed mechanism of cephalostatin 1 (**1**)-induced ER stress and apoptosis.

prevented cell death. Caspase-8 is not an essential caspase for the mediation of apoptosis by cephalostatin 1 (**1**) since Jurkat caspase-8 knockout cells and the corresponding control cells responded equally toward **1**. Furthermore, an impact of CD95 signaling effected via caspase-8[35] could be excluded in cephalostatin 1-mediated apoptosis: Jurkat cells deficient in CD95 (Jurkat R[36]) showed no difference in their reactivity toward cephalostatin 1 (**1**) compared to control cells.[12]

On the basis of these results we investigated further the importance of caspase-9, the initiator caspase involved in the mitochondrial apoptosis pathway. To determine its relevance, we used a Jurkat cell line deficient in caspase-9. Interestingly, apoptosis induced by cephalostatin 1 (**1**) was almost completely inhibited in caspase-9-deficient cells, whereas cells stably expressing full-length caspase-9 died normally when exposed to **1**.[14]

Apoptosome-Independent Activation of Caspase-9. Caspase-9 is normally activated by its recruitment into the apoptosome. Apoptosome formation requires mitochondria-derived cytochrome *c*, the cytosolic factor Apaf-1, ATP, and procaspase-9. Therefore, we tested whether caspase-9 activation by cephalostatin 1 (**1**) treatment involves an association of caspase-9 with Apaf-1. Immunoprecipitation experiments showed that **1** does not induce a binding between caspase-9 and Apaf-1. This fact, together with the lack of cytochrome *c* release upon cephalostatin treatment, suggests that caspase-9 activation in response to cephalostatin occurs independently of the formation of an apoptosome.

To further support this unique finding, Bak-deficient cells and Apaf-1 siRNA were used. Since Jurkat cells do not express Bax,[37] the Bak−/− Jurkat cells are resistant to apoptotic stimuli, such as etoposide, that use the intrinsic mitochondrial pathway hallmarked by release of cytochrome *c*. Importantly, Bak−/− cells are equally sensitive to cephalostatin 1 (**1**) as Bak-reconstituted control cells, further pointing to a cytochrome *c*-independent signaling pathway. To confirm the induction of apoptosis by **1** without apoptosome formation, Apaf-1 was silenced via siRNA in Jurkat cells. The lack of Apaf-1 had no effect on cephalostatin 1 (**1**), whereas the apoptotic response to etoposide was significantly diminished.[14] But how is caspase-9 apoptosome independently activated by cephalostatin 1 (**1**)?

Role of ER Stress for Activation of Caspase-9 by Cephalostatin 1 (1). As mentioned above, electron microscopy showed that the ER was affected by cephalostatin treatment. The ER is mainly responsible for proper calcium homeostasis and post-translational modification of proteins to obtain their mature conformation. When a cytotoxic condition such as an alteration in calcium homeostasis exceeds the capacity of the ER, a highly conserved unfolded protein response (UPR) is activated. If this alteration persists, cells can undergo apoptosis. At least three pathways are known to be involved in ER stress-associated apoptosis. All of them deserve attention as potential ER-specific targets for cancer chemotherapeutic strategies. The first is the transcriptional activation of the gene for C/EBP homologous protein (CHOP). The second is activation of the c-Jun N-terminal kinase pathway that is mediated by activation of apoptosis signal-regulating kinase 1 (ASK1). The third is activation of ER-associated caspase-12 or caspase-4 in humans, respectively. Cephalostatin 1 (**1**) activates all three pathways, indicating the important role of ER stress response for its chemotherapeutic profile.[13,14]

Cephalostatin 1 (**1**) increases the expression of BiP, induces phosphorylation of the eukaryotic initiation factor-2 (eIF2), and augments the expression of CHOP. As overexpression of CHOP has been shown to lead to cell cycle arrest and/or apoptosis, pharmacological or genetic increase of CHOP expression may represent a new anticancer strategy. The second pathway in ER stress is activation of the c-Jun N-terminal kinase. JNK activation can promote cell survival as well as death. However, sustained activation of JNK is known to lead to apoptosis and requires activation of ASK1. Both kinases are activated by cephalostatin 1 (**1**), and experiments using cells with a dominant negative form of ASK1 pointed to its participation in apoptosis, shown by diminished DNA fragmentation.[13,14]

In addition, cephalostatin 1 (**1**) activates the ER stress-associated caspase-4. Since this activation also occurred in caspase-9-deficient cells, we hypothesized that caspase-4 might be an initiator caspase, acting upstream of caspase-9. Indeed, pretreatment of normal Jurkat cells with the peptidic caspase-4 inhibitor zLEVDfmk completely abrogated caspase-9 activity induced by **1**. These results were verified in Jurkat cells with strongly reduced caspase-4 expression caused by siRNAs. Such cells showed a marked inhibition of the activation of caspase-9 in response to cephalostatin 1 (**1**), confirming the upstream role of caspase-4 in caspase-9 activation.[14]

Taken together cephalostatin 1 (**1**) is proposed to activate caspase-9 independent from apoptosome formation via ER stress-induced caspase-4 (Figure 5).

Summary

The marine compound cephalostatin 1 (**1**) is a very potent substance that is able to induce apoptosis in an unusual pathway (Figure 6). One prominent feature of this type of activity by cephalostatin 1 (**1**) is the triggering of selective Smac/DIABLO release from mitochondria. The release was found to occur independently of caspase activation. The functional role of Smac

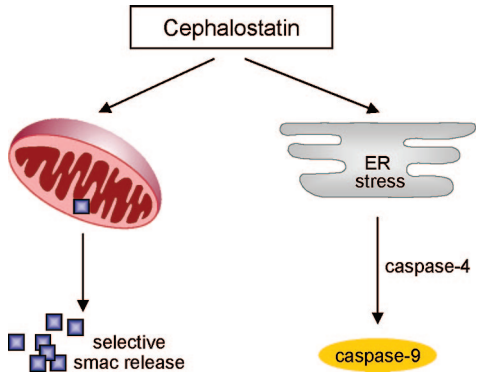

Figure 6. Hallmarks of cephalostatin-induced apoptosis.

for apoptosis induction and the molecular players contributing to Smac/DIABLO liberation are currently under investigation by our group.

A further important observation is that cephalostatin 1 (**1**) activates caspase-9 without the formation of an apoptosome. Interestingly, in this context, **1** induces an endoplasmic reticulum stress response via increased expression of BiP and CHOP and augmented phosphorylation of the eukaryotic initiation factor-2 (eIF2). Furthermore, ASK1 and JNK are activated. Most importantly, ER stress-related caspase-4 is activated on treatment with **1**, which is involved subsequently in the apoptosome-independent activation of caspase-9.

Overall, these results characterize the apoptosis-inducing mechanism of cephalostatin 1 (**1**) and clearly indicate a unique, cytochrome *c*-independent apoptosis signaling triggered by this marine bis-steroidal compound. Since most currently available cancer chemotherapeutic drugs act in a manner dependent on cytochrome *c*, it will be very interesting to see whether **1** is effective either in treating chemo-resistant tumors or in sensitizing tumors against chemotherapy. In either case, we have identified a tool that will allow us further insights into apoptosis signaling.

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Supporting Information Available: Fingerprints of cytotoxic profiles of cephalostatins 1 and 2. This information is available free of charge via the Internet at http://pubs.acs.org.

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