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Cephalostatin 1 inactivates Bcl-2 by hyperphosphorylation independent of M-phase arrest and DNA damage

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Running title: Cephalostatin 1 and Bcl-2

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Abbreviations:

Apaf-1, apoptotic protease-activating factor 1; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; ASK, apoptosis signal regulating kinase; Bcl-2, anti-apoptotic protein first identified in B-cell lymphoma; BH3, Bcl-2 homology domain 3; ECL, enhanced chemoluminescence; EDTA, ethylene diamine tetraacetic acid; ER, endoplasmic reticulum; JNK, c-Jun N-terminal kinase; MIA, microtubule-interfering agent; PMSF, phenylmethanesulfonyl fluoride; SAPK, stress-activated protein kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Smac/DIABLO, second mitochondria-derived activator of caspases/ direct IAP-binding protein with a low isoelectric point; TBS-T, Tris-buffered saline with tween;

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Abstract

Cephalostatin 1 is a marine product that induces a novel cytochrome c-independent apoptotic pathway in Jurkat leukemia T cells (Dirsch et al. (2003) *Cancer Res* **63**, 8869-8876). Here we show that overexpression of the anti-apoptotic protein Bcl-2 protects cells only partially against cephalostatin 1-induced apoptosis. The mechanism of Bcl-2 inactivation by cephalostatin 1 is based on hyperphosphorylation of Bcl-2 on Thr^{69} and Ser^{87} since Jurkat cells overexpressing a Bcl-2 protein with mutations on both phosphorylation sites were completely protected against cephalostatin 1. In search of the kinase responsible for Bcl-2 phosphorylation, JNK was found to be activated by cephalostatin 1. Reduction of Bcl-2 phosphorylation by the specific JNK inhibitor SP600125 suggested a crucial role for JNK in this process. JNK activation was not a consequence of DNA damage, a known stimulus of JNK, since cephalostatin 1 did not induce DNA lesions as shown by the comet assay. Arrest in M-phase is also demonstrated to be associated with JNK activation. However, cephalostatin 1 does not evoke an arrest in M-phase as shown by flow cytometry. Taken together, cephalostatin 1 is shown to induce JNK activation with subsequent Bcl-2 phosphorylation and inactivation. Reported triggers, such as the induction of a M-phase arrest or DNA damage are not involved in this process suggesting a novel mechanism for cephalostatin 1-mediated Bcl-2 hyperphosphorylation.

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Introduction

The cephalostatins, isolated from the Indian ocean hemichordate *Cephalodiscus gilchristi* Ridewood for the first time in 1988 (Pettit *et al.*, 1988), belong to the most cytotoxic marine natural products ever tested by the National Cancer Institute/USA. Cephalostatin 1 proved to be the most active of the 19 cephalostatins (~1 nM mean GI_{50} in the 2-day NCI-60 screen) (LaCour *et al.*, 1999). Besides the *in vitro* tests, it was shown to inhibit murine leukemia and brain tumor xenografts *in vivo* (Pettit, 1994).

We showed recently that cephalostatin 1 induces a unique apoptotic signaling pathway that activates caspase-9 independently of an apoptosome since neither the release of cytochrome c from mitochondria nor an interaction of Apaf-1 with caspase-9 was detected. Remarkably, the protein Smac/DIABLO was selectively released from mitochondria in response to cephalostatin 1 (Dirsch *et al.*, 2003).

We show here that overexpression of Bcl-2 in Jurkat cells confers only partial protection against cephalostatin 1-induced apoptosis whereas overexpression of Bcl-x_L was found to abolish cephalostatin 1-mediated cell death (Dirsch *et al.*, 2003).

Bcl-2 and Bcl-x_L are member of an evolutionarily conserved family of proteins that control apoptosis. Both proteins belong to the anti-apoptotic subgroup of the family while the BH3-only polypeptides and the Bax/Bak-like proteins are involved in the initiation of apoptosis. Bcl-2 is localized on the mitochondrial outer membrane but was also found at membranes of the ER and nucleus (Cory and Adams, 2002).

Because of its important role in regulation of apoptotic processes, the Bcl-2 family has been associated with cancer development and with resistance to anticancer treatment. Occurrence of Bcl-2 overexpression, first found in human follicular B-cell lymphomas and generated by the chromosomal translocation t(14;18) (Reed *et al.*,

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1988), has been discovered in most chronic lymphocytic lymphomas (Hanada *et al.*, 1993), indolent lymphomas and several other tumors (Reed, 1999). High expression of Bcl-2 alone is only weakly oncogenic (Strasser *et al.*, 1993) but provides an extended life-span of the cell for secondary mutations to develop (Kaufmann and Vaux, 2003). As a result, the two events in concert are able to provoke formation of neoplasms. Studies using Bcl-2 overexpressing leukemic cell lines showed that Bcl-2 conveys resistance to a large number of anti-cancer drugs including DNA damaging agents (Pratesi *et al.*, 2001). The clinical manifestation of raised Bcl-2 levels in AML, ALL and also prostate cancer is correlated with a poor prognosis (Campos *et al.*, 1993). Hence, finding substances that abrogate the antiapoptotic function of Bcl-2 and thereby enhance sensitivity of the cell to drug treatment is of great therapeutic importance.

This prompted us to investigate whether cephalostatin 1 is able to inactivate Bcl-2 and to characterize the underlying mechanism.

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Materials and Methods

Materials – Cephalostatin 1 was isolated from *Cephalodiscus gilchristi* Ridewood as described previously (Pettit *et al.*, 1988). Etoposide and the JNK inhibitor SP600125 were purchased from Calbiochem (Bad Soden, Germany). Paclitaxel and propidium iodide were obtained from Sigma (Deisenhofen, Germany). All used anticancer drugs were dissolved in dimethyl sulfoxide.

Cells – Jurkat human T cells (clone J16) and Jurkat cells stably transfected with vector control, *Bcl-2* or a mutant form of Bcl-2 (Yamamoto *et al.*, 1999) (kindly provided by Prof. Korsmeyer, Boston, USA and Drs. P.H. Krammer, H. Walczak, Heidelberg, Germany) were maintained in RPMI 1640 containing 2 mM L-glutamine (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (PAA Laboratories, Cölbe, Germany). Medium of transfected cells was supplemented with 1 mg/ml G418 (Life Technologies, Inc., Eggenstein, Germany) every fifth passage.

Quantification of Apoptosis and Cell Cycle Analysis – Quantification of apoptosis was carried out according to Nicoletti *et al.* (Nicoletti *et al.*, 1991). Briefly, cells were incubated for 24 h in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton-X-100 and 50 µg/ml propidium iodide) and analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, Heidelberg, Germany). Nuclei left to the G1-peak containing hypodiploid DNA were considered to be apoptotic.

Western Blot Analysis – Western blotting was performed as described previously (Antlsperger *et al.*, 2003). Briefly, cells were lysed by adding lysis buffer (2 mM EDTA, 137 mM NaCl, 10% glycerol, 2 mM tetrasodium pyrophosphate, 20 mM Tris, 1% Triton-X-100, 20 mM sodium glycerophosphate hydrate, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM PMSF, supplemented with the protease inhibitor complete™ (Roche, Mannheim, Germany)). Equal amounts of protein were

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separated by SDS-PAGE (12%) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Eschborn, Germany). Equal protein loading was controlled by Coomassie Blue staining of gels. Membranes were blocked by 5% fat free milk powder in TBS-T and incubated with specific antibodies against Bcl-2 (Upstate, Lake Placid, USA), phospho-Bcl-2 (Cell Signaling, Frankfurt, Germany) and phospho-JNK (Cell Signaling, Frankfurt, Germany). Detection of the proteins of interest was accomplished with secondary antibodies conjugated to horseradish peroxidase and ECL Plus substrate solution (Amersham Biosciences, Freiburg, Germany).

Comet assay – A single cell gel electrophoresis assay was performed using the Trevigen Comet assay kit (Trevigen, Gaithersburg, MD, USA) under alkaline conditions according to the manufacturer's conditions. After treatment, cells were suspended in low point melting agar and placed onto a microscope slide. After solidification of agarose, slides were submerged in lysis solution (2.5 M sodium chloride, 100 mM EDTA, pH 10, 10 mM Tris Base, 1% sodium lauryl sarcosinate, 1% Triton-X-100) for 40 min and then transferred to alkaline solution for 40 min. Electrophoresis was performed at 300 mA for 30 min. Finally, slides were neutralized and DNA was stained with SYBR® Green. Pictures were taken at an Axiovert 25 microscope (Zeiss, Munich, Germany).

Statistical analysis. All experiments were performed three times. Results are expressed as mean ± SE. Statistical analysis was performed by ANOVA followed by a Bonferroni multiple comparison test or by an unpaired two-tailed Student *t*-test. P values < 0.05 were considered significant.

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Results

Overexpression of Bcl-2 protects Jurkat cells only partially against cephalostatin 1-induced apoptosis.

Both, Bcl-2 and Bcl-x_L inhibit apoptosis at the mitochondrial level. In a previous study we showed that Bcl-x_L overexpression confers full protection against cephalostatin 1-induced apoptosis in Jurkat cells (Dirsch *et al.*, 2003). Interestingly, Bcl-2-overexpressing cells were only partially protected (Fig. 1B) against cephalostatin 1 (Fig. 1A, chemical structure).

Inactivation of Bcl-2 by cephalostatin 1 is provoked by Bcl-2 hyperphosphorylation.

The incomplete protection in Bcl-2-overexpressing cells suggests that cephalostatin 1 is able to inactivate Bcl-2. Since hyperphosphorylation at Thr^{69} and Ser^{87} has been reported to be one mechanism of inactivation (Ruvolo *et al.*, 2001), we clarified whether cephalostatin 1 utilizes this mechanism to disable Bcl-2. As depicted in Fig. 2A, Bcl-2 is indeed hyperphosphorylated 8 h after cephalostatin 1 treatment visible by an additional upper band at 28 kDa in the Western blot. Etoposide (E; 10 µM, 16 h) used as control showed no effect. Fig. 2B demonstrates that the overall level of Bcl-2 remained unchanged upon cephalostatin 1 treatment.

In order to prove that the observed Bcl-2 hyperphosphorylation is indeed the mechanism of Bcl-2 inactivation we used Jurkat cells overexpressing a mutant form of Bcl-2. In this mutant Bcl-2 protein, all three phosphorylation sites (Thr^{69}, Ser^{70}, Ser^{87}) are substituted by alanine to prevent phosphorylation and thus inactivation of Bcl-2 (Yamamoto *et al.*, 1999). In comparison to cells carrying the vector alone (Jurkat/*neo*) and cells overexpressing the wildtype Bcl-2 protein (Jurkat/*bcl-2*) the mutant cell line (Jurkat/*mbl-2*) was completely protected against cephalostatin 1 (Fig.

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2C). In order to prove that all three cell lines respond as they are reported to (Yamamoto *et al.*, 1999), they were exposed to paclitaxel (1 μM, 24 h). Fig. 2D shows that the Jurkat/*mbcl-2* cells were more protected against paclitaxel-induced apoptosis than cells overexpressing the wild-type Bcl-2 protein while Jurkat/*neo* cells succumbed freely to apoptosis. In summary, hyperphosphorylation of Bcl-2 by cephalostatin 1 diminishes the protective function of Bcl-2.

Jun-terminal kinase is involved in cephalostatin 1-triggered Bcl-2 phosphorylation.

Next, we examined which kinase may be responsible for the cephalostatin 1-induced Bcl-2 hyperphosphorylation. As depicted in Fig. 3A, both JNK1 and JNK2 are phosphorylated already 2 h after cephalostatin 1 treatment and phosphorylation increases up to 8 h after stimulation. In order to link JNK activation to Bcl-2 phosphorylation, we pretreated Jurkat cells with the specific JNK inhibitor SP600125 (Fig. 3B, chemical structure) prior to stimulation with cephalostatin 1. Fig. 3C reveals that the inhibitor reduces Bcl-2 hyperphosphorylation after treatment with cephalostatin 1 or paclitaxel. Etoposide showed no effect.

Notably, paclitaxel seems to lead to a stronger hyperphosphorylation than cephalostatin 1. To clarify whether this difference in Bcl-2 hyperphosphorylation has an impact on Bcl-2 inactivation, we compared the levels of apoptosis induced by these two drugs in Jurkat/*neo*, Jurkat/*bcl-2* and Jurkat/*mbcl-2* cells as shown in Fig. 3D. As expected, Bcl-2 overexpressing cells are more sensitive to paclitaxel than to cephalostatin 1 suggesting that the stronger hyperphosphorylation induced by paclitaxel leads indeed to a stronger inactivation of Bcl-2. However, Jurkat/*mbcl-2* cells showed also a higher sensitivity towards paclitaxel compared to cephalostatin 1

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suggesting that paclitaxel may have - next to the mechanism of Bcl-2 hyperphosphorylation - a further, unknown mechanism to inactivate Bcl-2.

Bcl-2 phosphorylation is not mediated by G2/M-phase-dependent events.

The majority of compounds reported to induce Bcl-2 hyperphosphorylation activate JNK and induce a cell cycle arrest in M-phase, as e.g. microtubule-damaging agents (Ruvolo *et al.*, 2001). A causal link between M-phase arrest, JNK activation and subsequent Bcl-2 hyperphosphorylation, however, has not been shown in all cases. In order to investigate whether cephalostatin 1-induced JNK activation and successive Bcl-2 phosphorylation depend on an M-phase arrest, we analyzed the cell cycle distribution of untreated Jurkat cells and cells incubated with cephalostatin 1 (1 µM) or paclitaxel (1 µM) for 8 h, a time point at which JNK activation was evident. As demonstrated by Fig. 4A and B, paclitaxel induces a potent M-phase block whereas cephalostatin 1 did not interfere with cell cycle progression. Interestingly, cells in G1 and S-phase seem to be more susceptible to cephalostatin 1 since the overall percentage of cells in G1- and S-phase decreased after cephalostatin 1 stimulation (Fig. 4B). The appearance of a sub-G1 peak, (Fig. 4A, *middle panel*) reveals that cells formerly present in G1 and S underwent apoptosis. The percentage of cells in the G2/M-phase was not altered after cephalostatin 1 treatment compared to control. These results indicate that both JNK activation and Bcl-2 phosphorylation induced by cephalostatin 1 occur independent of a M-phase blockade.

Cephalostatin 1 does not induce Bcl-2 phosphorylation via *DNA damage.*

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JNK may be activated by cellular stress. Many genotoxic agents mediate JNK activation (Saleem *et al.*, 1995) and some have been described to phosphorylate Bcl-2 (Pratesi *et al.*, 2000b). To elucidate whether cephalostatin 1 leads to DNA lesions, we performed a comet assay employing cells treated with cephalostatin 1 (1 µM) or etoposide (10 µM) for 4 h. Both drugs induced comparable levels of apoptosis at these concentrations (data not shown). Fig. 5 provides clear evidence that etoposide induces DNA damage visible as the typical comet tail whereas cephalostatin 1 does not. This experiment suggests that DNA damage is not the type of cell stress leading to cephalostatin 1-induced JNK activation.

Discussion

Bcl-2 overexpression has been shown to convey resistance to various chemotherapeutic agents (Domen and Weissman, 2003). Here we show that cephalostatin 1 is able to inactivate Bcl-2 in Jurkat T cells *via* a mechanism that includes hyperphosphorylation.

Several mechanisms are reported to inactive Bcl-2: activated caspases have been shown to cleave Bcl-2 generating a 23 kDa pro-apoptotic product (Fadeel *et al.*, 1999;Del Bello *et al.*, 2001). Furthermore, down-regulation of Bcl-2 mRNA or Bcl-2 protein has been observed after treatment with several anti-cancer drugs (Sawada *et al.*, 2000; Bandyopadhyay *et al.*, 2003). In cephalostatin 1-induced apoptosis, total Bcl-2 levels are not altered suggesting that cleavage of the protein or alterations of mRNA levels do not apply here. Beyond that, Bcl-2 phosphorylation has been observed as mechanism for altering the activity of the protein. Under physiological conditions, Bcl-2 is phosphorylated on Ser^{70} during M-phase (Ito *et al.*, 1997). The kinases suggested to be responsible for this phosphorylation are PKC (Ito *et al.*,

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1997;May *et al.*, 1994) and ERK (Deng *et al.*, 2000). Phosphorylation of Ser^{70} seems to be important for the anti-apoptotic function of the protein (Ito *et al.*, 1997). In contrast, hyperphosphorylation on Thr^{69} and Ser^{87} was proposed to inactivate Bcl-2 (Yamamoto *et al.*, 1999) and abrogate its protective role by impairing its interaction with the pro-apoptotic protein Bax (Scatena *et al.*, 1998).

Hyperphosphorylation of Bcl-2 was shown to be induced by drugs, such as anti-mitotic agents (Ruvolo *et al.*, 2001) and some DNA damaging chemotherapeutics (Pratesi *et al.*, 2000). However, cephalostatin 1-induced Bcl-2 hyperphosphorylation occurs independent of a mitotic arrest or DNA-damage and is sufficient for the observed Bcl-2 inactivation. Compared to paclitaxel, the degree of hyperphosphorylation induced by cephalostatin 1 seemed to be lower corresponding to the lower sensitivity of Bcl-2 overexpressing cells towards cephalostatin 1. Interestingly, Jurkat T cells carrying mutated phosphorylation sites (Jurkat/*mbcl-2*) displayed a higher apoptosis rate after paclitaxel treatment compared to cephalostatin 1. This observation may be explained by an additional inactivating mechanism of paclitaxel, such as cleavage of Bcl-2 (Blagosklonny *et al.*, 1999).

Several previous studies have focused on the signaling pathway leading to Bcl-2 hyperphosphorylation. Among the key enzymes responsible for Bcl-2 inactivation various kinases have been described (Raf-1 (Blagosklonny *et al.*, 1997), protein kinase A (Srivastava *et al.*, 1999), ASK (Tang *et al.*, 1994), JNK (Yamamoto *et al.*, 1999)) depending on cell type and stimulus. Activated JNK has been implied in the hyperphosphorylation of Bcl-2 in response to numerous anti-mitotic agents such as paclitaxel, *Vinca* alkaloids or cryptophycins (Mollinedo and Gajate, 2003). In some settings, JNK activation but no Bcl-2 phosphorylation was evident (Figueroa-Masot *et al.*, 2001) or no causal link between JNK activation and Bcl-2 phosphorylation

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could be proven (Wang *et al.*, 1999). In fact, protein kinase A was favored as the kinase exclusively responsible for Bcl-2 phosphorylation (Srivastava *et al.*, 1999).

In the present study cephalostatin 1-triggered Bcl-2 hyperphosphorylation was strongly impaired by the specific JNK inhibitor SP600125 pointing again to JNK as the crucial kinase upstream of Bcl-2.

How does cephalostatin 1 induce JNK activation and subsequent Bcl-2 phosphorylation? JNK/SAPK (stress-activated protein kinase) activation is involved in the regulation of cell cycle progression at the transition from G1- to S-phase (MacCorkle-Chosnek *et al.*, 2001). JNK also fulfills functions in the M-phase (Yamamoto *et al.*, 1999). Beyond that, it is induced by diverse extracellular stimuli such as UV irradiation, pro-inflammatory cytokines, heat shock and numerous cytotoxic agents (Minden and Karin, 1997). Among these, microtubuli-interfering agents (MIA) inducing an arrest in G2/M-phase activate JNK as major pro-apoptotic player (Mollinedo and Gajate, 2003). JNK activation by MIAs was often linked to Bcl-2 hyperphosphorylation suggesting that an arrest in M-phase is a crucial factor in the signaling pathway leading to Bcl-2 inactivation and subsequent apoptosis (Tseng *et al.*, 2002;Fan *et al.*, 2000;Yamamoto *et al.*, 1999). Cephalostatin 1, however, does not arrest cells in G2/M-phase contradicting the view that an arrest in M-phase may be a prerequisite for JNK activation with a subsequent Bcl-2 hyperphosphorylation and inactivation.

JNK activation was also found in response to DNA damaging agents (Saleem *et al.*, 1995b; Hayakawa *et al.*, 2003). Some of them, such as platinum compounds and doxorubicin were shown to induce Bcl-2 phosphorylation (Pratesi *et al.*, 2000). Up to now, the pathway leading to Bcl-2 phosphorylation in DNA damage-induced apoptosis has not been investigated but involvement of JNK is conceivable.

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Cephalostatin 1, however, does not induce DNA damage excluding this mechanism for JNK activation.

In conclusion, the activation of JNK by cephalostatin 1 which leads to hyperphosphorylation and inactivation of Bcl-2 is mediated by a mechanism yet to be identified. A promising possibility may be ER stress as activator of JNK (Urano *et al.*, 2000). As to whether cephalostatin 1 induces ER stress and whether this ER stress accounts for JNK activation and Bcl-2 phosphorylation awaits to be shown. In any case, cephalostatin 1 may have a potential for the treatment of drug-resistant cancers due to its ability to inactivate the anti-apoptotic protein Bcl-2. Studies testing this hypothesis are in progress.

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Figure legends

Figure 1. Overexpression of Bcl-2 protects Jurkat cells only partially against cephalostatin 1-induced apoptosis. *A,* Chemical structure of cephalostatin 1. *B*, Control cells (Jurkat/*neo*) and cells overexpressing Bcl-2 (Jurkat/*bcl-2*) were stimulated with cephalostatin 1 (1 µM) for the indicated periods of time, stained with PI and analyzed by flow cytometry. Apoptosis is expressed as percentage of cells with subdiploid DNA content. Data are the mean ± SE of three independent experiments performed in triplicate. *, $p < 0.05$, *** $p < 0.001$ (ANOVA/Dunnett), compared to untreated cells. *C*, Cell lysates of Jurkat/*neo* and Jurkat/*bcl-2* were analyzed for Bcl-2 expression by Western blot analysis.

Figure 2. Cephalostatin 1 induces hyperphosphorylation of Bcl-2. *A*-*B***,** Jurkat/*bcl-2* cells were incubated with 1 µM cephalostatin 1 for 2 – 24 h or as positive control with etoposide (E; 25 µg/ml, 16 h). Cell lysates were analyzed by Western blotting for hyperphosphorylated Bcl-2 (ppBcl-2) (*A*) and total unphosphorylated Bcl-2 (*B*). *C-D,* Control cells (Jurkat/*neo*), cells overexpressing wildtype Bcl-2 (Jurkat/*bcl-2*) or Bcl-2 with alanine-substituted phosphorylation sites (Thr^{69}, Ser^{70}, Ser^{87}) (Jurkat/*mbcl-2*) were stimulated with cephalostatin 1 (1 µM) for the indicated periods of time (*C*) or with paclitaxel (1 µM, 24 h) (*D*). Apoptotic cells were quantified by flow cytometry as described under *Materials and Methods.* For the sake of clearness part of results shown in *C* and *D* (24 h) are again presented in Fig. 3 C. Data are the mean ± SE of three independent experiments performed in triplicate. ***, $p < 0.001$ (ANOVA/Dunnett).

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Figure 3. Jun-terminal kinase is involved in cephalostatin 1-triggered Bcl-2 phosphorylation. *A,* Jurkat/*bcl-2* cells were stimulated with cephalostatin 1 (1 µM) for the indicated periods of time. Etoposide (E; 25 µg/ml, 8 h) was used as positive control. Cell lysates were analyzed with anti-phospho-JNK antibody for the activated forms of JNK1 (p46) and JNK2 (p54). *B,* Chemical structure of SP600125. *C*, Jurkat cells were incubated with (+) or without (-) SP600125 (10 µM) for 0.5 h and further stimulated with cephalostatin 1 (1 µM), paclitaxel (1 µM) or etoposide (2 µM) for 16 h. Lysates were immunoblotted with anti-phospho-Bcl-2 antibody. *D,* Jurkat/*neo*, Jurkat/*bcl-2* and Jurkat/*mbcl-2* were left untreated, treated with cephalostatin 1 (1 µM) or paclitaxel (1 µM) for 24 h (as described in Fig. 2 *C* and *D*). The diagram shows the percentage of apoptotic cells at 24 h of Jurkat/*bcl-2* and Jurkat/*mbcl-2* compared to Jurkat/*neo* cells (= 100%).

Figure 4. Cephalostatin 1 does not interfere with cell cycle progression. *A,* Jurkat cells were incubated with cephalostatin 1 (1 µM) and paclitaxel (1 µM) for 8 h, stained with PI and analyzed by flow cytometry as described under *Experimental Procedures*. The histograms show the distribution of cells according to their DNA content. Cell cycle phases are marked by arrows. *B,* Quantification of cells in G_0/G1, S and G2/M-phase. The data shown are the mean ± SE of three independent experiments performed in triplicate.

Figure 5. Cephalostatin 1 does not lead to DNA lesions. Jurkat cells were either left untreated (CO) , stimulated with cephalostatin 1 (CPH-1; 1 µM) or etoposide (10 µM) for 4 h. DNA damage analysis was performed by comet assay as described

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under *Materials and Methods*. Representative pictures of three independent experiments are shown.

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Fig. 1

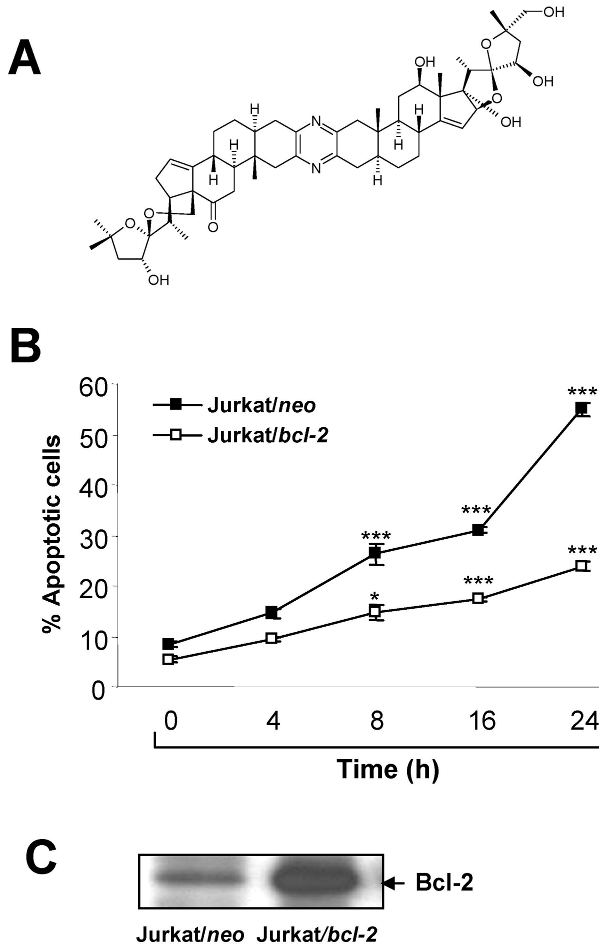


Fig. 2

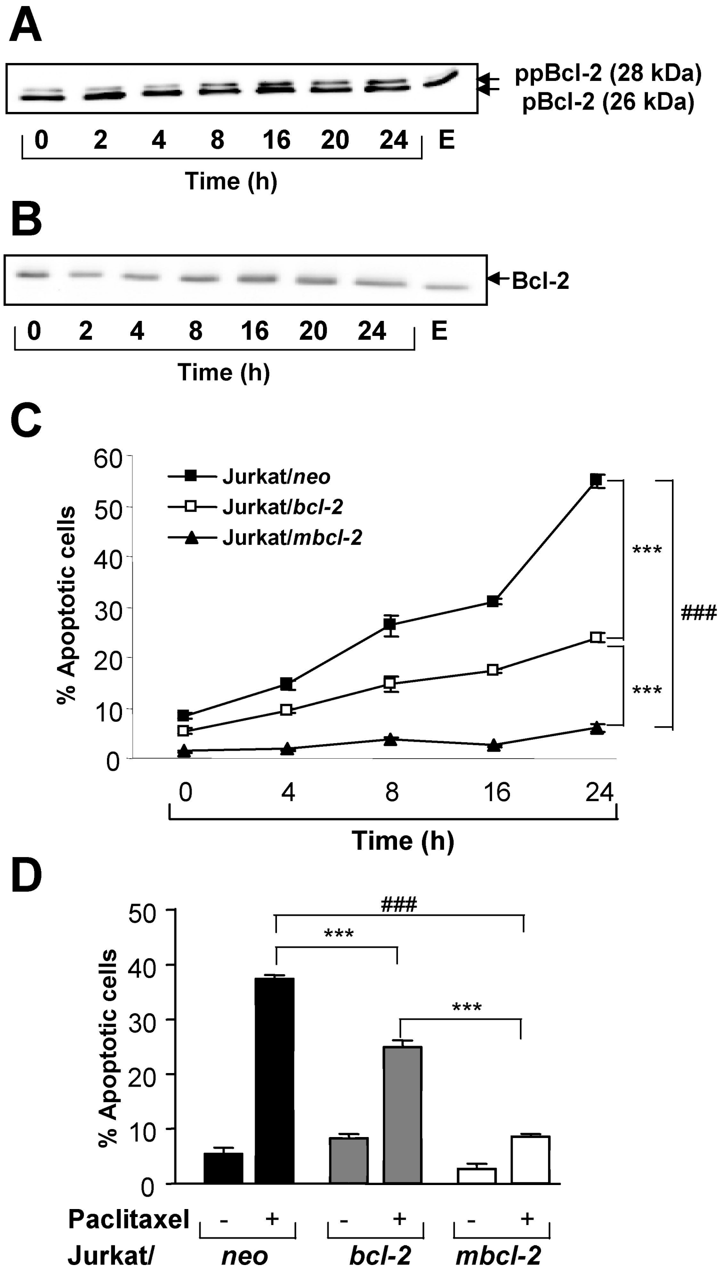


Fig. 3

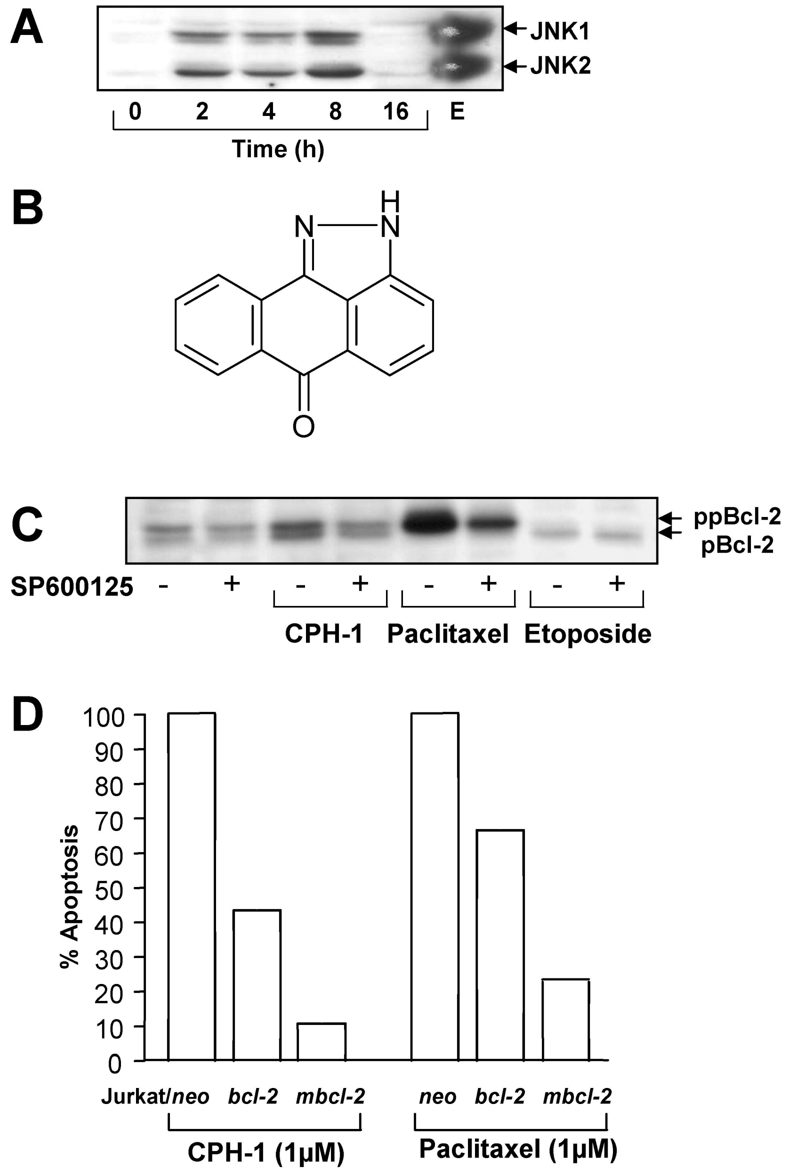
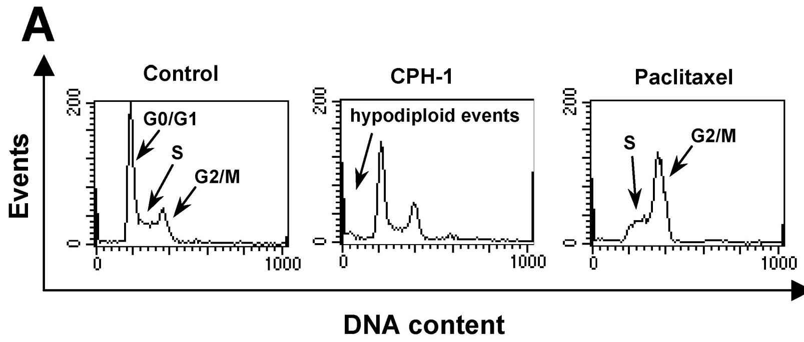


Fig. 4



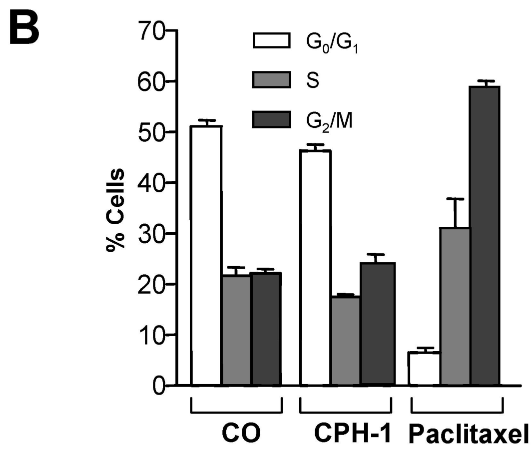


Fig. 5