0026-895X/05/6705-1684–1689$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2005 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 67:1684–1689, 2005

Vol. 67, No. 5
4234/1198159
Printed in U.S.A.

Cephalostatin 1 Inactivates Bcl-2 by Hyperphosphorylation Independent of M-Phase Arrest and DNA Damage

Irina M. Müller, Verena M. Dirsch, Anita Rudy, Nancy López-Antón, George R. Pettit, and Angelika M. Vollmar

Department of Pharmacy, Center of Drug Research, University of Munich, Munich, Germany (I.M.M., V.M.D., A.R., N.L.-A., A.M.V.); and Cancer Research Institute, Arizona State University, Tempe, Arizona (G.R.P.)

Received June 25, 2004; accepted February 8, 2005

ABSTRACT

Cephalostatin 1 is a marine product that induces a novel cytochrome c-independent apoptotic pathway in Jurkat leukemia T cells (*Cancer Res* **63:**8869–8876, 2003). Here, we show that overexpression of the antiapoptotic 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} because 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, c-Jun NH_2-terminal kinase (JNK) was found to be activated by cephalostatin 1. Reduction of Bcl-2 phosphorylation by the specific JNK inhibitor (anthra(1,3-*cd*)pyrazol-6(2*H*)-one) SP600125 suggested a crucial role for JNK in this process. JNK activation was not a consequence of DNA damage, a known stimulus of JNK, because 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. Together, cephalostatin 1 is shown to induce JNK activation with subsequent Bcl-2 phosphorylation and inactivation. Reported triggers, such as the induction of an M-phase arrest or DNA damage are not involved in this process, suggesting a novel mechanism for cephalostatin 1-mediated Bcl-2 hyperphosphorylation.

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 (Bethesda, MD). 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 because neither the release of cytochrome *c* from mitochondria nor an interaction of apoptotic protease-activating factor 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 members of an evolutionarily conserved family of proteins that control apoptosis. Both proteins belong to the antiapoptotic subgroup of the family, whereas the Bcl-2 homology domain 3-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., 1988), has been discovered in most chronic lymphocytic lymphomas (Hanada

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 369, Vo 376/10-1)

[1] Current address: Institute of Pharmacognosy, University of Vienna, Vienna, Austria.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.004234.

ABBREVIATIONS: ER, endoplasmic reticulum; JNK, c-Jun NH_2-terminal kinase; Smac/DIABLO, second mitochondria-derived activator of caspases/direct IAP-binding protein with a low isoelectric point; ANOVA, analysis of variance; SP600125, anthra(1,3-*cd*)pyrazol-6(2*H*)-one.

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 anticancer drugs, including DNA damaging agents (Pratesi et al., 2001). The clinical manifestation of raised Bcl-2 levels in acute myelogenous leukemia, acute lymphocytic leukemia, 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.

Materials and Methods

Materials. Cephalostatin 1 was isolated from *C. gilchristi* 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 Chemie (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 Dr. S. Korsmeyer, Harvard Medical School, Boston, MA, and Drs. P. H. Krammer and H. Walczak, German Cancer Research Center, Heidelberg, Germany) were maintained in RPMI 1640 medium 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 (Geneticin; Invitrogen, Eggenstein, Germany) every fifth passage.

Quantification of Apoptosis and Cell Cycle Analysis. Quantification of apoptosis was carried out according to Nicoletti et al. (1991). In brief, 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 (BD Biosciences, Heidelberg, Germany). Nuclei left to the G_1 peak containing hypodiploid DNA were considered apoptotic.

Western Blot Analysis. Western blotting was performed as described previously (Antlsperger et al., 2003). In brief, 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, and 1 mM phenylmethylsulfonyl fluoride, supplemented with the protease inhibitor complete; Roche Diagnostics, Mannheim, Germany). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis (12%) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Eschborn, Germany). Equal protein loading was controlled by Coomassie Blue staining of gels. Membranes were blocked by 5% fat free milk powder in Tris-buffered saline with Tween and incubated with specific antibodies against Bcl-2 (Upstate Biotechnology, Lake Placid, NY), phospho-Bcl-2 (Cell Signaling Technology Inc., Frankfurt, Germany), and phospho-JNK (Cell Signaling Technology Inc.). Detection of the proteins of interest was accomplished with secondary antibodies conjugated to horseradish peroxidase and ECL Plus substrate solution (Amersham Biosciences Inc., Freiburg, Germany).

Comet Assay. A single cell gel electrophoresis assay was performed using the Trevigen Comet assay kit (Trevigen, Gaithersburg, MD) 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, and 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 (Carl Zeiss, Munich, Germany).

Statistical Analysis. All experiments were performed three times. Results are expressed as mean ± S.E. Statistical analysis was performed by ANOVA followed by a Bonferroni multiple comparison test or by an unpaired two-tailed Student's *t* test. p values <0.05 were considered significant.

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).

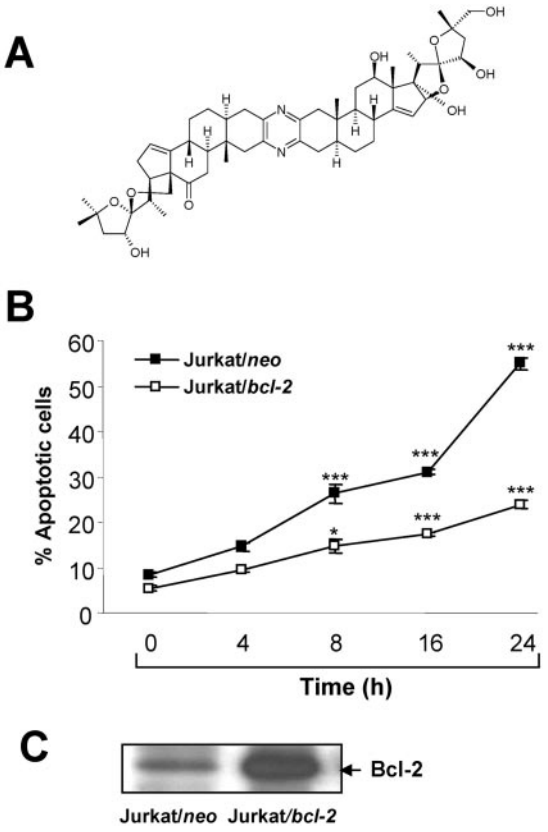

Fig. 1. Overexpression of Bcl-2 protects Jurkat cells only partially against cephalostatin 1-induced apoptosis. A, chemical structure of cephalostatin 1. B, control cells (Jurkat/neo) and cells overexpressing Bcl-2 (Jurkat/*bcl-2*) were stimulated with 1 μM cephalostatin 1 for the indicated periods of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

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. Because 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 uses 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; 25 μg/ml, 16 h) used as control showed no effect. Figure 2B demonstrates that the overall level of Bcl-2 remained unchanged upon cephalostatin 1 treatment.

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}, and Ser^{87}) are substituted by alanine to prevent phosphorylation and thus inactivation of Bcl-2 (Yamamoto et al., 1999). In comparison with cells carrying the vector alone (Jurkat/neo) and cells overexpressing the wild-type Bcl-2 protein (Jurkat/*bcl-2*), the mutant cell line (Jurkat/*mbl-2*) was completely protected against cephalostatin 1 (Fig. 2C). To prove that all three cell lines respond as reported previously (Yamamoto et al., 1999), they were exposed to 1 μM paclitaxel for 24 h. Figure 2D shows that the Jurkat/*mbcl-2* cells were more protected against paclitaxel-induced apoptosis than cells overexpressing the wild-type Bcl-2 protein, whereas Jurkat/neo cells succumbed freely to apoptosis. In

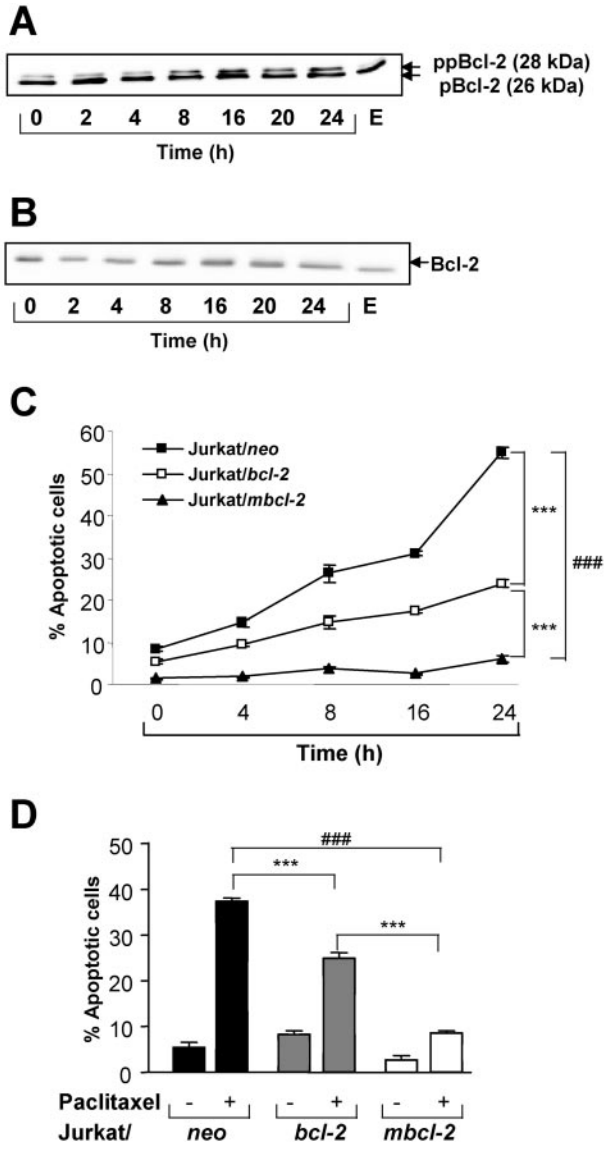

Fig. 2. Cephalostatin 1 induces hyperphosphorylation of Bcl-2. A and B, Jurkat/*bcl-2* cells were incubated with 1 μM cephalostatin 1 for 2 to 24 h or as positive control with etoposide (E; 25 μg/ml, 16 h). Cell lysates were analyzed by Western blotting for hyperphosphorylated Bcl-2 (ppBcl-2) (A) and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

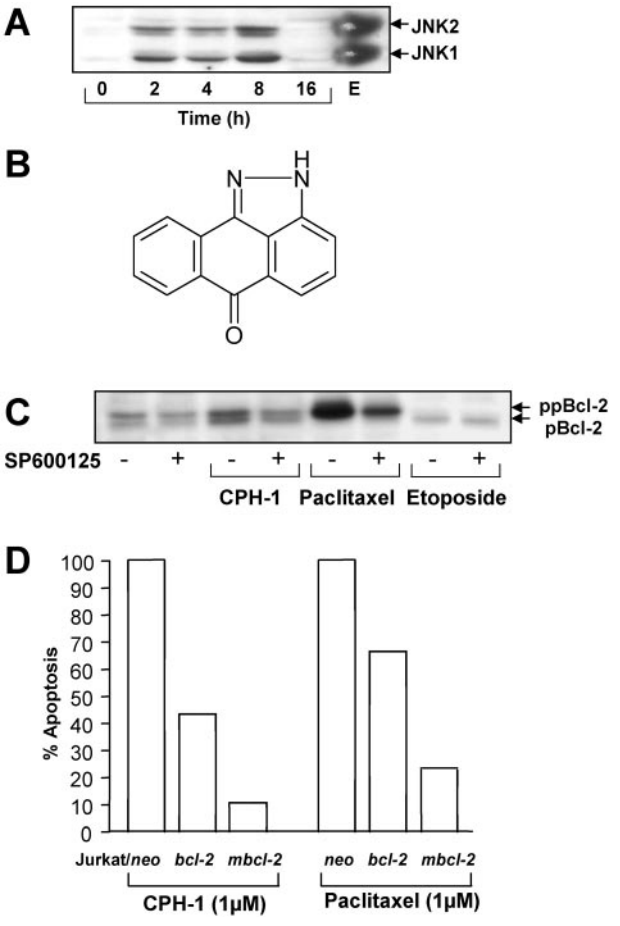

Fig. 3. Jun-terminal kinase is involved in cephalostatin 1-triggered Bcl-2 phosphorylation. A, Jurkat/*bcl-2* cells were stimulated with 1 μM cephalostatin for the indicated periods of time. Etoposide (E; 25 μg/ml, 8 h) was used as positive control. Cell lysates were analyzed with anti-phospho-JNK antibody for the activated forms of JNK1 (p46) and JNK2 (p54). B, chemical structure of SP600125. C, Jurkat cells were incubated with (+) or without (−) 10 μM SP600125 for 0.5 h and further stimulated with 1 μM cephalostatin 1, 1 μM paclitaxel, or 2 μM etoposide 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 1 μM cephalostatin 1 or 1 μM paclitaxel 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 with Jurkat/neo cells (=100%).

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. To link JNK activation to Bcl-2 phosphorylation, we pretreated Jurkat cells with the specific JNK inhibitor SP600125 (Fig. 3B, chemical structure) before stimulation with cephalostatin 1. Figure 3C reveals that the inhibitor reduces Bcl-2 hyperphosphorylation after treatment with cephalostatin 1 or paclitaxel. Etoposide showed no effect.

It is noteworthy that 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 toward paclitaxel compared with cephalostatin 1, 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 G_2/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, for example, 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. 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 1 μM cephalostatin 1 or 1 μM paclitaxel for 8 h, a time point at which JNK activation was evident. As demonstrated by Fig. 4, A and B, paclitaxel induces a potent M-phase block, whereas cephalostatin 1 did not interfere with cell cycle progression. Interestingly, cells in G_1 and S phase seem to be more susceptible to cephalostatin 1 because the overall percentage of cells in G_1 and S phase decreased after cephalostatin 1 stimulation (Fig. 4B). The appearance of a sub-G_1 peak (Fig. 4A, middle) reveals that cells formerly present in G_1 and S underwent apoptosis. The percentage of cells in the G_2/M phase was not altered after cephalostatin 1 treatment compared with control. These results indicate that both JNK activation and Bcl-2 phosphorylation induced by cephalostatin 1 occur independently of an M-phase blockade.

Cephalostatin 1 Does Not Induce Bcl-2 Phosphorylation via DNA Damage. 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., 2000). To elucidate whether cephalostatin 1 leads to DNA lesions, we performed a comet assay using cells treated with 1 μM cephalostatin 1 or 10 μM etoposide for 4 h. Both drugs induced comparable levels of apoptosis at these concentrations (data not shown). Figure 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

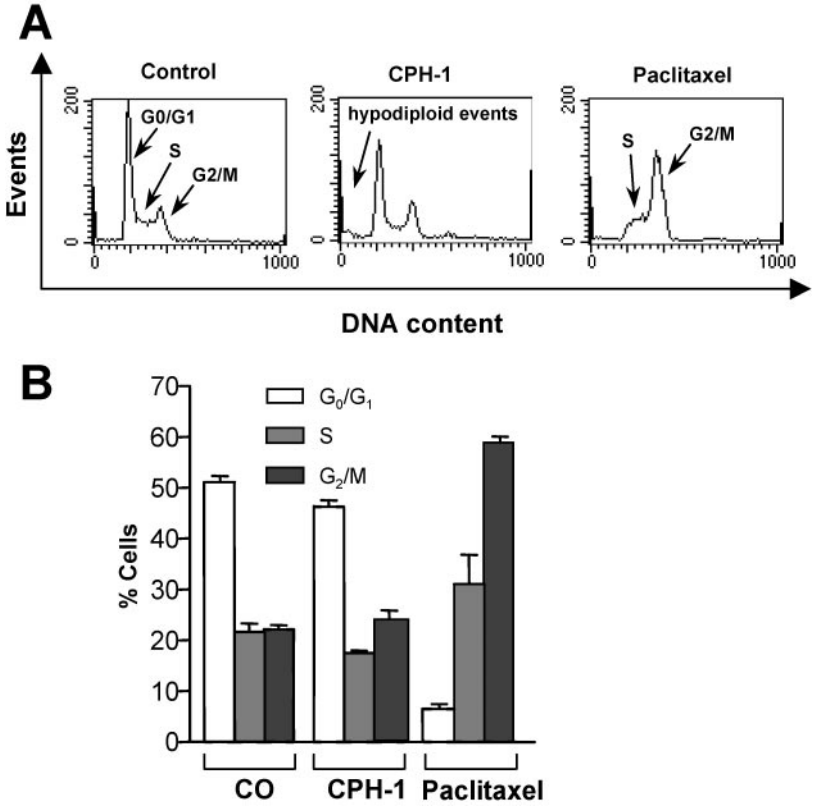

Fig. 4. Cephalostatin 1 does not interfere with cell cycle progression. A, Jurkat cells were incubated with 1 μM cephalostatin 1 and 1 μM paclitaxel for 8 h, stained with propidium iodide, and analyzed by flow cytometry as described under *Materials and Methods*. The histograms show the distribution of cells according to their DNA content. Cell cycle phases are marked by arrows. B, quantification of cells in G_0/G_1, S, and G_2/M phase. The data shown are the mean ± S.E. of three independent experiments performed in triplicate.

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 inactivate Bcl-2: activated caspases have been shown to cleave Bcl-2, generating a 23-kDa proapoptotic 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 anticancer 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 (May et al., 1994; Ito et al., 1997) and extracellular signal-regulated kinase (Deng et al., 2000). Phosphorylation of Ser^{70} seems to be important for the antiapoptotic 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 proapoptotic protein Bax (Scatena et al., 1998).

Hyperphosphorylation of Bcl-2 was shown to be induced by drugs, such as antimitotic agents (Ruvolo et al., 2001) and some DNA damaging chemotherapeutics (Pratesi et al., 2000). However, cephalostatin 1-induced Bcl-2 hyperphosphorylation occurs independently of a mitotic arrest or DNA damage and is sufficient for the observed Bcl-2 inactivation. Compared with paclitaxel, the degree of hyperphosphorylation induced by cephalostatin 1 seemed to be lower, corresponding to the lower sensitivity of Bcl-2-overexpressing cells toward cephalostatin 1. Interestingly, Jurkat T cells carrying mutated phosphorylation sites (Jurkat/*mbcl-2*) displayed a higher apoptosis rate after paclitaxel treatment compared with 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); apoptosis signal-regulating kinase-1, Tang et al. (1994); and 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 antimitotic 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 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).

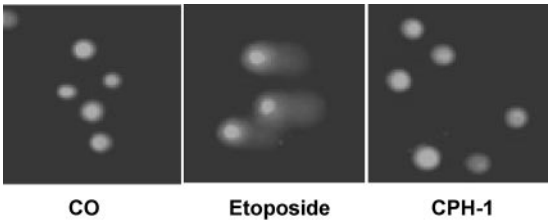

Fig. 5. Cephalostatin 1 does not lead to DNA lesions. Jurkat cells were either left untreated (CO) or stimulated with 1 μM cephalostatin 1 (CPH-1) or 10 μM etoposide for 4 h. DNA damage analysis was performed by comet assay as described under *Materials and Methods*. Representative pictures of three independent experiments are shown.

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/stress-activated protein kinase activation is involved in the regulation of cell cycle progression at the transition from G_1 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, proinflammatory cytokines, heat shock, and numerous cytotoxic agents (Minden and Karin, 1997). Among these, microtubule-interfering agents inducing an arrest in G_2/M phase activate JNK as major proapoptotic player (Mollinedo and Gajate, 2003). JNK activation by microtubule-interfering agents 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 (Yamamoto et al., 1999; Fan et al., 2000; Tseng et al., 2002). Cephalostatin 1, however, does not arrest cells in G_2/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., 1995; 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. 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. Regardless, cephalostatin 1 may have a potential for the treatment of drug-resistant cancers because of its ability to inactivate the antiapoptotic protein Bcl-2. Studies testing this hypothesis are in progress.

Acknowledgments

We thank Drs. Stanley Korsmeyer and Peter H. Krammer and Henning Walczak for supplying the used Jurkat T cell clones. We

also thank Dr. Sören Eichhorst for helpful suggestions. We acknowledge the excellent technical assistance of Liliana Schyschka.

References

Antlsperger DS, Dirsch VM, Ferreira D, Su JL, Kuo ML, and Vollmar AM (2003) Ajoene-induced cell death in human promyeloleukemic cells does not require JNK but Is amplified by the inhibition of ERK. *Oncogene* **22:**582–589.

Bandyopadhyay S, Sengupta TK, Fernandes DJ, and Spicer EK (2003) Paclitaxel- and okadaic acid-Induced destabilization of Bcl-2 mRNA is associated with decreased binding of proteins to a Bcl-2 instability element. *Biochem Pharmacol* **66:**1151–1162.

Blagosklonny MV, Chuman Y, Bergan RC, and Fojo T (1999) Mitogen-activated protein kinase pathway is dispensable for microtubule-active drug-induced Raf-1/Bcl-2 phosphorylation and apoptosis in leukemia cells. *Leukemia* **13:**1028–1036.

Blagosklonny MV, Giannakakou P, el Deiry WS, Kingston DG, Higgs PI, Neckers L, and Fojo T (1997) Raf-1/Bcl-2 phosphorylation: a step from microtubule damage to cell death. *Cancer Res* **57:**130–135.

Campos L, Rouault JP, Sabido O, Oriol P, Roubi N, Vasselon C, Archimbaud E, Magaud JP, and Guyotat D (1993) High expression of Bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* **81:**3091–3096.

Cory S and Adams JM (2002) The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2:**647–656.

Del Bello B, Valentini MA, Zunino F, Comporti M, and Maellaro E (2001) Cleavage of Bcl-2 in oxidant- and cisplatin-induced apoptosis of human melanoma cells. *Oncogene* **20:**4591–4595.

Deng X, Ruvolo P, Carr B, and May WS Jr (2000) Survival function of ERK1/2 as IL-3-activated, staurosporine-resistant Bcl2 kinases. *Proc Natl Acad Sci USA* **97:**1578–1583.

Dirsch VM, Muller IM, Eichhorst ST, Pettit GR, Kamano Y, Inoue M, Xu JP, Ichihara Y, Wanner G, and Vollmar AM (2003) Cephalostatin 1 selectively triggers the release of Smac/DIABLO and subsequent apoptosis that is characterized by an increased density of the mitochondrial matrix. *Cancer Res* **63:**8869–8876.

Domen J and Weissman IL (2003) Hematopoietic stem cells and other hematopoietic cells show broad resistance to chemotherapeutic agents in vivo when overexpressing Bcl-2. *Exp Hematol* **31:**631–639.

Fadeel B, Hassan Z, Hellstrom-Lindberg E, Henter JI, Orrenius S, and Zhivotovsky B (1999) Cleavage of Bcl-2 is an early event in chemotherapy-induced apoptosis of human myeloid leukemia cells. *Leukemia* **13:**719–728.

Fan M, Goodwin M, Vu T, Brantley-Finley C, Gaarde WA, and Chambers TC (2000) Vinblastine-induced phosphorylation of Bcl-2 and Bcl-XL is mediated by JNK and occurs in parallel with inactivation of the Raf-1/MEK/ERK cascade. *J Biol Chem* **275:**29980–29985.

Figueroa-Masot XA, Hetman M, Higgins MJ, Kokot N, and Xia Z (2001) Paclitaxel induces apoptosis in cortical neurons by a mechanism independent of Bcl-2 phosphorylation. *J Neurosci* **21:**4657–4667.

Hanada M, Delia D, Aiello A, Stadtmauer E, and Reed JC (1993) Bcl-2 gene hypomethylation and high-level expression in B-Cell chronic lymphocytic leukemia. *Blood* **82:**1820–1828.

Hayakawa J, Depatie C, Ohmichi M, and Mercola D (2003) The activation of c-Jun NH_2-terminal kinase (JNK) by DNA-damaging agents serves to promote drug resistance via activating transcription factor 2 (ATF2)-dependent enhanced DNA repair. *J Biol Chem* **278:**20582–20592.

Ito T, Deng X, Carr B, and May WS (1997) Bcl-2 phosphorylation required for anti-apoptosis function. *J Biol Chem* **272:**11671–11673.

Kaufmann SH and Vaux DL (2003) Alterations in the apoptotic machinery and their potential role in anticancer drug resistance. *Oncogene* **22:**7414–7430.

LaCour TG, Guo C, Ma S, Jeong JU, Boyd MR, Matsunaga S, Fusetani N, and Fuchs PL (1999) On topography and functionality in the B-D rings of cephalostatin cytotoxins. *Bioorg Med Chem Lett* **9:**2587–2592.

MacCorkle-Chosnek RA, VanHooser A, Goodrich DW, Brinkley BR, and Tan TH (2001) Cell cycle regulation of c-Jun N-terminal kinase activity at the centrosomes. *Biochem Biophys Res Commun* **289:**173–180.

May WS, Tyler PG, Ito T, Armstrong DK, Qatsha KA, and Davidson NE (1994) Interleukin-3 and Bryostatin-1 mediate hyperphosphorylation of BCL2α in association with suppression of apoptosis. *J Biol Chem* **269:**26865–26870.

Minden A and Karin M (1997) Regulation and function of the JNK subgroup of MAP kinases. *Biochim Biophys Acta* **1333:**F85–F104.

Mollinedo F and Gajate C (2003) Microtubules, microtubule-interfering agents and apoptosis. *Apoptosis* **8:**413–450.

Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, and Riccardi C (1991) A Rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139:**271–279.

Pettit GR (1994) Antineoplastic agents. 317. Marine animal and terrestrial plant anticancer constituents. *Pure Appl Chem* **66:**2271–2281.

Pettit GR, Inoue M, Kamano Y, Herald DL, Arm C, Dufresne C, Christie ND, Schmidt JM, Doubek KL, and Krupa TS (1988) Isolation and structure of powerful cell growth inhibitor cephalostatin 1. *J Am Chem Soc* **110:**2006–2007.

Pratesi G, Perego P, and Zunino F (2001) Role of Bcl-2 and its post-transcriptional modification in response to antitumor therapy. *Biochem Pharmacol* **61:**381–386.

Pratesi G, Polizzi D, Perego P, Dal Bo L, and Zunino F (2000) Bcl-2 phosphorylation in a human breast carcinoma xenograft: a common event in response to effective DNA-damaging drugs. *Biochem Pharmacol* **60:**77–82.

Reed JC (1999) Dysregulation of apoptosis in cancer. *J Clin Oncol* **17:**2941–2953.

Reed JC, Cuddy M, Slabiak T, Croce CM, and Nowell PC (1988) Oncogenic potential of Bcl-2 demonstrated by gene transfer. *Nature (Lond)* **336:**259–261.

Ruvolo PP, Deng X, and May WS (2001) Phosphorylation of Bcl2 and regulation of apoptosis. *Leukemia* **15:**515–522.

Saleem A, Datta R, Yuan ZM, Kharbanda S, and Kufe D (1995) Involvement of stress-activated protein kinase in the cellular response to 1-beta-D-arabinofuranosylcytosine and other DNA-damaging agents. *Cell Growth Differ* **6:**1651–1658.

Sawada M, Nakashima S, Banno Y, Yamakawa H, Hayashi K, Takenaka K, Nishimura Y, Sakai N, and Nozawa Y (2000) Ordering of ceramide formation, caspase activation and Bax/Bcl-2 expression during etoposide-induced apoptosis in C6 glioma cells. *Cell Death Differ* **7:**761–772.

Scatena CD, Stewart ZA, Mays D, Tang LJ, Keefer CJ, Leach SD, and Pietenpol JA (1998) Mitotic phosphorylation of Bcl-2 during normal cell cycle progression and paclitaxel-induced growth arrest. *J Biol Chem* **273:**30777–30784.

Srivastava RK, Mi QS, Hardwick JM, and Longo DL (1999) Deletion of the loop region of Bcl-2 completely blocks paclitaxel-induced apoptosis. *Proc Natl Acad Sci USA* **96:**3775–3780.

Strasser A, Harris AW, and Cory S (1993) E mu-Bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. *Oncogene* **8:**1–9.

Tang C, Willingham MC, Reed JC, Miyashita T, Ray S, Ponnathpur V, Huang Y, Mahoney ME, Bullock G, and Bhalla K (1994) High levels of p26BCL-2 oncoprotein retard paclitaxel-induced apoptosis in human pre-B leukemia cells. *Leukemia* **8:**1960–1969.

Tseng CJ, Wang YJ, Liang YC, Jeng JH, Lee WS, Lin JK, Chen CH, Liu IC, and Ho YS (2002) Microtubule damaging agents induce apoptosis in HL 60 cells and G2/M cell cycle arrest in HT 29 cells. *Toxicology* **175:**123–142.

Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, and Ron D (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science (Wash DC)* **287:**664–666.

Wang TH, Popp DM, Wang HS, Saitoh M, Mural JG, Henley DC, Ichijo H, and Wimalasena J (1999) Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. *J Biol Chem* **274:**8208–8216.

Yamamoto K, Ichijo H, and Korsmeyer SJ (1999) BCL-2 Is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G_2/M. *Mol Cell Biol* **19:**8469–8478.

Address correspondence to: Dr. Verena M. Dirsch, Department of Pharmacy, Center of Drug Research, University of Munich, Butenandtstraβe 5-13, D-81377 Munich, Germany. E-mail: verena.dirsch@cup.uni-muenchen.de