

Late Activation of Apoptotic Pathways Plays a Negligible Role in Mediating the Cytotoxic Effects of Discodermolide and Etoposide B in Non-Small Cell Lung Cancer Cells

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ABSTRACT

Discodermolide and etoposide B are promising novel chemotherapeutic agents that induce cell death through potent stabilization of microtubules. In this study, we investigated the cellular and molecular events underlying the cytotoxicity of these drugs in non-small cell lung carcinoma (NSCLC) cell lines, focusing on apoptotic characteristics.

IC₅₀ concentrations of either drug effectively disrupted the microtubule cytoskeleton of H460 cells and induced cell cycle disturbances with early accumulation in the G₂-M phase and development of a hypodiploid cell population in both H460 and SW1573 cells. These events were followed by abnormal chromosome segregation during mitosis and subsequent appearance of multinucleated cells. At later time points, the cells displayed several apoptotic features, such as nuclear condensation and fragmentation as well as Annexin V staining, cleavage of poly(ADP-ribose) polymerase and the activation of caspases.

To examine the contribution of apoptotic pathways to the cytotoxic effects of these agents, the involvement of the mitochondria and death receptor routes was studied. At 48 h after treatment, both agents disrupted mitochondria of H460 cells, as indicated by cytochrome *c* release. Nonetheless, H460 cells stably overexpressing antiapoptotic Bcl-2 or Bcl-x_L did not show any protective effect from cell death induced by either drug. Possible death receptor dependency was investigated in H460 cells stably overexpressing dominant-negative FADD, which failed to reduce the cytotoxic effects of discodermolide and etoposide B. To study the role of caspases more directly, the effect of stable overexpression of the caspase-8 inhibitor cytokine response modifier A was studied in H460 cells. Furthermore, the effect of the pancaspase inhibitor z-Val-Ala-Asp-fluoromethyl ketone was investigated in a panel of lung carcinoma cell lines. Interestingly, caspase inhibition did not rescue cells from discodermolide or etoposide B-induced cell death. In conclusion, these results demonstrate that despite several apoptotic features detected at relatively late time points after drug exposure, apoptosis is not the dominant mode of cell death and induced low but efficacious concentrations of discodermolide and etoposide B.

INTRODUCTION

Microtubule-stabilizing agents represent an important class of chemotherapeutic drugs, of which paclitaxel was the first to be described. Since its introduction into the clinic in 1993, paclitaxel has established itself as one of the most active antineoplastic agents against a wide spectrum of malignancies, including lung, ovarian, and breast cancer (1, 2).

Recently, several novel natural cytotoxic microtubule-stabilizing compounds have been described, including the marine sponge product discodermolide and the etoposides produced by the myxobacteria

Sorangium cellulosum (3–5). Several etoposide analogues can be distinguished, of which etoposide B is one of the most potent (3, 6). Although discodermolide and etoposides are structurally unrelated to the taxanes, they have the same mechanism of action and stabilize the microtubules even more potently than paclitaxel, leading to mitotic arrest and subsequent cell death (3, 7). Both drugs competitively inhibit the binding of paclitaxel to the microtubules, indicating identical or overlapping binding sites (3, 8), and several molecular modeling assays have demonstrated that discodermolide and etoposides share a common pharmacophore with paclitaxel (9–12). Interestingly, their activity remains intact in paclitaxel-resistant cell lines with MDR³ phenotypes and certain β -tubulin mutations (3, 8, 13, 14). *In vivo* studies confirmed that desoxyetoposide B, one of the most potent etoposides, was curative in human tumor xenografts that were refractory to paclitaxel (15, 16). Clinical Phase I and II studies with etoposides are currently ongoing (17, 18).

Successful treatment with chemotherapeutic agents is largely dependent on their ability to trigger cell death in tumor cells and activation of apoptosis is at least partially involved in this process (19). The apoptotic cascade can be initiated via two major pathways, involving either the release of cytochrome *c* from the mitochondria (mitochondria pathway) or activation of death receptors in response to ligand binding (death receptor pathway; Refs. 20, 21). Upon triggering of either pathway, caspases, the final executioners of apoptosis, are activated, causing degradation of cellular proteins and disassembly of the cell, leading to typical morphological changes such as chromatin condensation, nuclear shrinkage, and the formation of apoptotic bodies (22–24). The majority of chemotherapeutic agents triggers the mitochondria pathway, but the death receptors have also been reported to be involved in chemotherapy-induced apoptosis (25, 26).

Discodermolide and etoposide B have been described to trigger apoptosis in several cell lines, as established by changes in cell membrane integrity, DNA fragmentation, and morphological abnormalities (27, 28), but the molecular pathways underlying this process are poorly described. Despite the activation of apoptotic pathways by many anticancer drugs, recent evidence suggests that there are forms of chemotherapy-induced cell death that cannot be readily classified as apoptosis or necrosis. Therefore, the contribution of apoptosis to the cytotoxic effects of chemotherapeutic agents is currently debated (29, 30). Recent studies demonstrate involvement of caspase-independent pathways in cell death induced by several cytotoxic agents (31). The outcome of the cellular response appears to be dependent on the type and dose of chemotherapeutic stress within the cellular context (32).

In the present study, we have investigated the contribution of the major apoptotic pathways to the cytotoxic effects of discodermolide

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³ The abbreviations used are: MDR, multidrug resistance; SCLC, small cell lung cancer; NSCLC, non-SCLC; 7-AAD, 7-actino-aminomycin D; CrmA, cytokine response modifier A; FADD, Fas-associated death domain; DN, dominant negative; zVAD-fmk, z-Val-Ala-Asp-fluoromethyl ketone; PI, propidium iodide; mAb, monoclonal antibody; PARP, poly(ADP-ribose) polymerase; PCD, programmed cell death.

and epothilone B in non-small cell lung cancer cell lines. Activation of the apoptotic machinery could be demonstrated, but only at relatively late time points. Moreover, inhibition of the major apoptotic routes and direct blockade of caspases failed to protect against the cytotoxic effects of discodermolide or epothilone B. These findings indicate that the apoptotic machinery is merely coactivated and not instrumental in cell death, implying that alternative, caspase-independent forms of cell death are responsible for the cytotoxic effects of discodermolide and epothilone B in NSCLC cells.

MATERIALS AND METHODS

Cell Lines and Transfectants. The human NSCLC cell lines NCI-H460 (H460), SW1573, and A549, the SCLC cell line GLC4 and Jurkat-T-leukemia cells were used. Cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS (Life Technologies, Inc., Breda, the Netherlands), 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μ g/ml streptomycin. Stably transfected H460 cells expressing Bcl-2, Bcl-x_L, CrmA, and FADD-DN were cultured in medium containing a final concentration of 1.5 μ g/ml puromycin (Bcl-2, Bcl-x_L, and CrmA) or 200 μ g/ml neomycin (FADD-DN), as described previously (33).

Drugs and Growth-Inhibition Assay. Discodermolide and epothilone B were kindly provided as pure substances by Dr. M. Wartmann (Novartis Pharma AG, Basel, Switzerland) and dissolved in DMSO. Their cytotoxic activities were assessed after 72 h of drug treatment by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay (Sigma Chemical Co., St. Louis, MO) as described previously (34, 35). Values were expressed as a percentage of untreated controls, from which IC₅₀ and IC₈₀ concentrations were calculated. All subsequent experiments were performed with IC₈₀ concentrations of each drug freshly diluted in culture medium. The broad-spectrum caspase inhibitor zVAD-fmk (Enzyme System Products, Livermore, CA) was diluted in DMSO and added to the cells at a final concentration of 50 μ M 1 h before the addition of the drugs.

Immunofluorescence Microscopy. Immunofluorescence stainings were performed as described previously (36). We used monoclonal anti- β -tubulin antibody (PharMingen, San Diego, CA; 1:100 dilution), secondary antimouse-FITC antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and Hoechst 33342 (Sigma). Slides were mounted in Vecta shield (Vector Laboratories, Burlingame, CA) and analyzed with a Leica DM IRBE fluorescence microscope.

DNA Labeling and Flow Cytometric Analysis. The extent of apoptosis was determined by flow cytometry, using either PI (Sigma) staining of hypodiploid DNA or Annexin V (Nexins Research, Kattendijke, the Netherlands) and 7-AAD (PharMingen) double staining, as described previously (33, 37). The percentage of specific apoptosis was calculated by subtracting the percentage of spontaneous apoptosis of the relevant controls from the total percentage of apoptosis.

Protein Extraction and Western Blotting. Preparation of cytosolic and total cell extracts as well as Western blot analysis was performed as described previously (34). For immunodetection, the following antibodies were used: anti-Bcl-2 mAb (Dako, Santa Barbara, CA), anti-caspase-8 mAb (Immunotech, Marseille, France), rabbit polyclonal anti-PARP antibody (Boehringer Mannheim, Mannheim, Germany), antimitochondrial heat shock protein 70 mAb (Affinity Bioreagents, Golden, CO), anti-cytochrome *c* mAb (PharMingen), anti-AU1 mAb (Covance, Richmond, CA), and horseradish peroxidase-conjugated secondary antibody (Amersham, Braunschweig, Germany). ECL (Amersham) was used for detection, and protein-loading equivalence was related to the expression of β -actin.

Fluorimetric Assay for Caspase Activity. Caspase-3-like enzyme activity was assayed in cellular extracts using a caspase-3 activity kit (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions. Fluorescence was detected using Spectra Fluor equipped with a 400-nm excitation and a 505-nm emission filter (Tecan, Salzburg, Austria). Relative percent activity, as measured by DEVD-AFC cleavage, was determined by comparing the levels of treated cells with untreated controls.

Statistics. Quantitative experiments were analyzed by Student's *t* test. All *P*s resulted from the use of two-sided tests and were considered significant when <0.05.

RESULTS

Discodermolide and Epothilone B Potently Induce Cell Death That Displays Several Characteristics of Apoptosis. To explore the cytotoxicity of discodermolide and epothilone B, we started our study with growth inhibition assays to determine IC₅₀ and IC₈₀ values in the NSCLC cell lines H460, SW1573, and A549, in the SCLC GLC4 cell line, as well as in Jurkat-T-leukemia cells (Table 1). Consistent with previous reports (reviewed in Ref. 38), both drugs were cytotoxic at low nanomolar concentrations, epothilone B being 6–20 times more potent than discodermolide.

Analysis of the cellular tubulin network by immunofluorescence microscopy revealed that IC₈₀ concentrations of each drug effectively disrupted the normal microtubule cytoskeleton of H460 cells, causing aberrant spindle formation during mitosis and the appearance of multipolar aster spindles (Fig. 1A), as described previously (3, 8). In time course experiments, we observed rapid induction of mitotic anomalies in epothilone B-treated cells, followed by abnormal chromosome segregation and the appearance of cells with multiple nuclei of various sizes (Fig. 1B). These abnormalities were also observed upon treatment with other microtubule-stabilizing agents such as paclitaxel (39, 40). The percentage of multinucleated cells increased to 48% at 24 h after treatment, followed by an increasing number of cells displaying apoptotic features, such as chromatin condensation, nuclear fragmentation, and apoptotic bodies (Fig. 1, B and C). Similar results were obtained after exposing the cells to discodermolide (data not shown).

In parallel to the analysis of morphology, H460 and SW1573 cells treated with discodermolide and epothilone B for 8–48 h were evaluated by PI staining-based fluorescence-activated cell sorter analysis to determine the effect of these drugs on the cell cycle. As shown in Fig. 1D, epothilone B caused an early accumulation of cells in the G₂-M phase and concomitant induction of a cell population with sub-G₁ DNA content. This hypodiploid population further increased from 19% at 8 h to 34% at 48 h (Fig. 1E). Although a sub-G₁ DNA content is indicative for apoptotic cells, this method is not exclusively specific for apoptosis (41). To further characterize the apoptotic features of discodermolide and epothilone B-induced cell death, we performed Annexin V-FITC/7-AAD double staining assays that measure the amount of phosphatidylserine externalization on the plasma membrane as an indicator of early apoptosis. Interestingly, this assay did not reveal a substantial number of Annexin V-positive cells before 48 h of treatment (Fig. 1E), suggesting that the cells with a sub-G₁ DNA content that were detected at earlier time points represent a cell fraction that does not display the classical characteristics of apoptotic cells. There were no significant differences in responses between the two drugs in either H460 or SW1573 cells (data not shown). Taken together, these results demonstrate that discodermolide and epothilone B potently induce cell death in NSCLC cells, which is accompanied by the appearance of mitotic anomalies and multinucleated cells shortly after drug exposure, whereas at later time points several

Table 1 Drug concentration responsible for 50 and 80% growth inhibition (IC₅₀ and IC₈₀)

The values (nM) were determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay and represent the mean \pm SD of four experiments.

Cell line	Discodermolide		Epothilone B	
	IC ₅₀	IC ₈₀	IC ₅₀	IC ₈₀
H460	3.2 \pm 0.6	12 \pm 1.7	0.44 \pm 0.05	1.9 \pm 0.05
A549	4.3 \pm 0.7	26 \pm 7	0.25 \pm 0.05	1.2 \pm 0.1
SW1573	2.6 \pm 0.6	19 \pm 4	0.50 \pm 0.1	2.8 \pm 0.7
GLC4	17.0 \pm 3	48 \pm 5	0.40 \pm 0.05	2.1 \pm 0.06
Jurkat	6.0 \pm 1.1	30 \pm 8	0.19 \pm 0.05	1.3 \pm 0.1

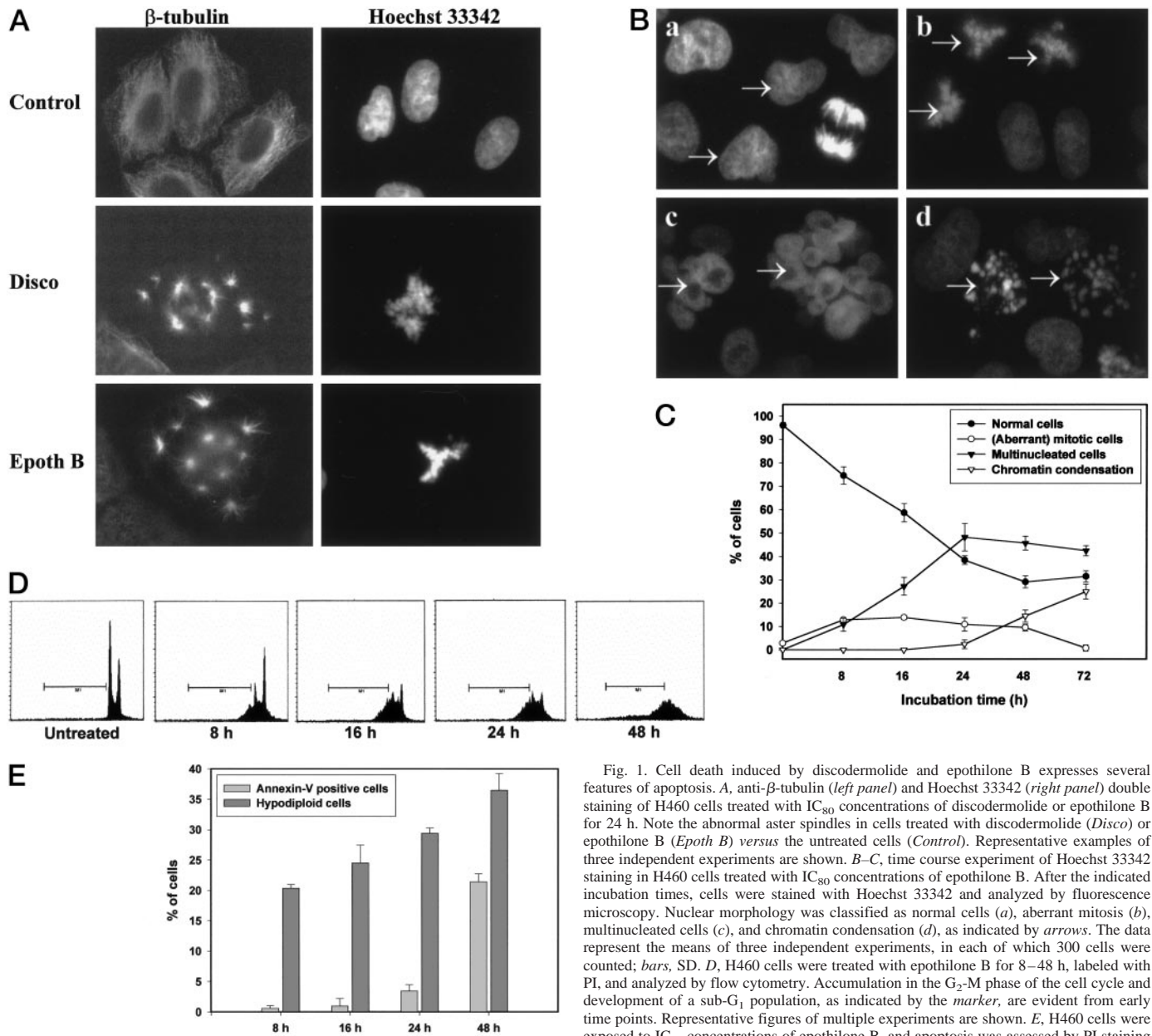


Fig. 1. Cell death induced by discodermolide and epothilone B expresses several features of apoptosis. *A*, anti- β -tubulin (left panel) and Hoechst 33342 (right panel) double staining of H460 cells treated with IC_{50} concentrations of discodermolide or epothilone B for 24 h. Note the abnormal aster spindles in cells treated with discodermolide (*Disco*) or epothilone B (*Epoth B*) versus the untreated cells (*Control*). Representative examples of three independent experiments are shown. *B–C*, time course experiment of Hoechst 33342 staining in H460 cells treated with IC_{80} concentrations of epothilone B. After the indicated incubation times, cells were stained with Hoechst 33342 and analyzed by fluorescence microscopy. Nuclear morphology was classified as normal cells (*a*), aberrant mitosis (*b*), multinucleated cells (*c*), and chromatin condensation (*d*), as indicated by arrows. The data represent the means of three independent experiments, in each of which 300 cells were counted; bars, SD. *D*, H460 cells were treated with epothilone B for 8–48 h, labeled with PI, and analyzed by flow cytometry. Accumulation in the G₂-M phase of the cell cycle and development of a sub-G₁ population, as indicated by the marker, are evident from early time points. Representative figures of multiple experiments are shown. *E*, H460 cells were exposed to IC_{80} concentrations of epothilone B, and apoptosis was assessed by PI staining as well as Annexin V-FITC/7-AAD double staining at the indicated time points. The results represent the means of three experiments; bars, SD.

apoptotic features become evident, such as Annexin V staining and nuclear condensation and fragmentation.

Discodermolide and Epothilone B Trigger Late Activation of Caspases. To further examine the role of apoptotic pathways in discodermolide and epothilone B-induced cytotoxicity, we assessed the activation of caspases. In time course experiments, cleavage of caspase-8, shown previously to be the apical caspase in NSCLC cells treated with DNA-damaging agents (33), was detected in H460 and SW1573 cells only after 48 h of incubation with discodermolide or epothilone B (data not shown). The downstream effector protease, caspase-3, was also activated upon treatment with discodermolide and epothilone B, as established by DEVD-AFC cleavage in H460 and SW1573 cells after 48 h incubation (Fig. 2A). As an additional indicator of caspase activation, cleavage of the caspase substrate PARP was determined by Western blotting. PARP cleavage, as indicated by the appearance of an M_r 89,000 fragment, became weakly evident at 48 h after treatment with discodermolide or epothilone B in

H460 cells (Fig. 2B), whereas it was not detected before 72 h of incubation in SW1573 cells (data not shown). These results show that caspases are activated only after prolonged incubation with discodermolide or epothilone B.

The Mitochondria and Death Receptor Pathways Are Not Instrumental in Discodermolide- or Epothilone B-induced Cell Death. To further assess the role of apoptosis in the cytotoxic effects of discodermolide and epothilone B, we investigated the contribution of the two major apoptotic pathways more directly. Involvement of the mitochondria pathway was evaluated by investigating the release of cytochrome *c* that could only be demonstrated after 48 h of treatment with discodermolide or epothilone B (Fig. 3A). Next, we exploited H460 cells stably overexpressing Bcl-2 and Bcl-x_L (Fig. 3B), which effectively prevented the release of cytochrome *c* upon exposure to chemotherapeutic agents in previous studies (33, 37). Although these antiapoptotic proteins protected H460 cells from the cytotoxic effects of DNA-damaging agents (data not shown; see also

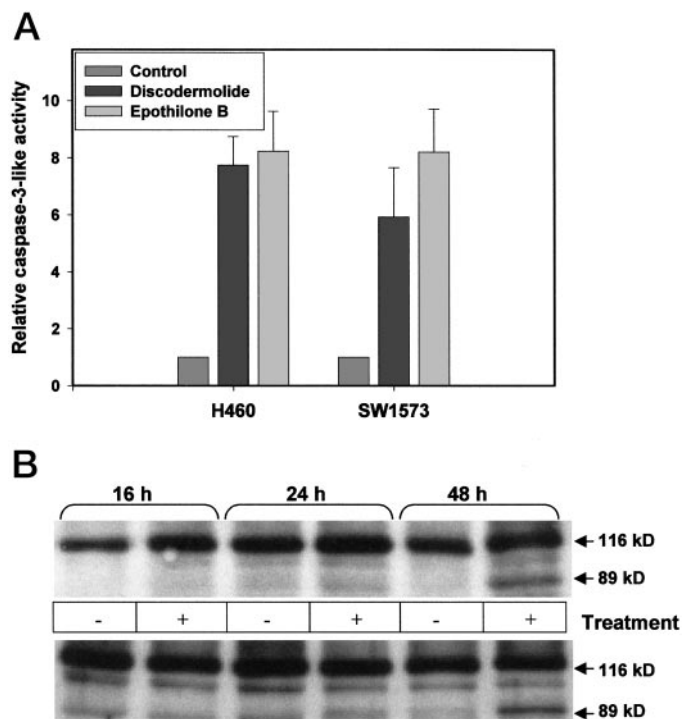


Fig. 2. Prolonged incubation with discodermolide and epothilone B induces caspase activation. *A*, caspase-3-like protease activity upon 48 h exposure to discodermolide and epothilone B in H460 and SW1573 cells, as measured by DEVD-AFC cleavage. The changes in activity are shown relative to activity in control cells, which is set as 1. Results are depicted as the means of at least three independent experiments; bars, SD. *B*, Western blot analysis showing cleavage of PARP in H460 cells upon treatment with discodermolide (upper panel) and epothilone B (lower panel), respectively.

Ref. 33), they did not prevent the development of a hypodiploid population upon exposure to discodermolide or epothilone B (Fig. 3C; *P*s range from 0.16 to 0.77). This was confirmed in growth-inhibition assays in which H460 cells overexpressing Bcl-2 (Fig. 3D) or Bcl-x_L (data not shown) were equally sensitive to the cytotoxic effects of discodermolide or epothilone B as the parental cell line. Moreover, the morphological changes induced by discodermolide and epothilone B were similar in both cell lines (Fig. 3E), and neither drug induced phosphorylation of Bcl-2 or Bcl-x_L (data not shown). These results suggest that the mitochondrial pathway does not substantially contribute to the cytotoxic effects of discodermolide and epothilone B, although it becomes activated as a late event.

The role of death receptors was tested using H460 cells stably overexpressing FADD-DN (Fig. 3B), a truncated form of FADD that interferes with the function of the normal variant as an adapter molecule between the death receptor and caspase-8. This cell line was effectively protected against Fas-induced apoptosis (data not shown; see also Ref. 33) but was equally sensitive to the cytotoxic effects of discodermolide and epothilone B as the parental cell line (Fig. 3F; *P* = 0.50 and 0.62, respectively). In summary, these data suggest that neither the mitochondria nor the death receptor pathways are instrumental in discodermolide and epothilone B-induced cell death.

Blockade of Caspases Does Not Influence the Cytotoxicity of Discodermolide or Epothilone B. To confirm that the apoptotic machinery is dispensable for the cytotoxic effects of discodermolide and epothilone B in NSCLC cells, we studied the effect of direct blockade of caspases on drug sensitivity. We examined H460 cells overexpressing the cowpox viral protein CrmA, a selective inhibitor of caspase-1 and -8 found previously to protect strongly against cisplatin-induced apoptosis (33). Despite the late activation of caspase-8 by discodermolide and epothilone B, stable expression of

CrmA failed to reduce the cytotoxic effects of these drugs (Fig. 4A; *P* = 0.25 and 0.46, respectively).

Next, we evaluated the effect of the broad-spectrum caspase inhibitor zVAD-fmk on discodermolide- and epothilone B-induced cell death. Pretreatment with 50 μM zVAD-fmk effectively prevented activation of caspase-3 (Fig. 4B) but did not significantly reduce the cytotoxic effects of discodermolide or epothilone B in H460, A549, and SW1573 cells (Fig. 4, C and D; *P*s range from 0.08 to 0.44), as determined by the development of cells with a hypodiploid DNA content. In the SCLC cell line GLC4, zVAD-fmk reduced the percentage of hypodiploid cells from 35 to 25% (*P* = 0.01) and from 27 to 18% (*P* = 0.008) after 48 h of incubation with discodermolide or epothilone B, respectively (Fig. 4, C and D). Interestingly, in the apoptosis-prone Jurkat-T-leukemia cell line (31), zVAD-fmk also provided no more than a 30% protection against epothilone B-induced cell death (*P* = 0.04), whereas the percentage of hypodiploid cells was only reduced from 29 to 27% upon exposure to discodermolide (*P* = 0.38). Apart from the only modest protective effect of caspase inhibition on the appearance of hypodiploid cells, zVAD-fmk did not prevent the morphological changes induced by discodermolide or epothilone B in H460 cells (Fig. 4E). We thus conclude that discodermolide and epothilone B can kill cells through a mechanism independent of caspase activation that may not be exclusively specific for NSCLC cells.

DISCUSSION

The molecular mechanisms underlying cell death induced by the promising microtubule-interacting agents discodermolide and epothilone B are still largely unknown. Although discodermolide and epothilone B have been described to induce several characteristics of apoptosis, such as changes in cell membrane integrity, DNA fragmentation, and morphological abnormalities (27, 28), it is not known to what extent activation of the apoptotic machinery is essential for the cell killing potential of these drugs. In the present study, we investigated the contribution of the apoptotic cascade to the cytotoxic effects of discodermolide and epothilone B. Despite late activation of the apoptotic machinery, neither blockade of the mitochondria or death receptor pathways nor direct inhibition of caspases substantially reduced the cytotoxic effects of either drug, indicating that alternative, caspase-independent mechanisms are responsible for the cell-killing effects of discodermolide and epothilone B.

As summarized in Table 2, nanomolar concentrations of discodermolide and epothilone B rapidly disrupted the microtubule cytoskeleton, leading to morphological abnormalities during mitosis, followed by abnormal exit from mitosis and nuclear fragmentation, which gave the cells a multinucleated appearance, similar to the nuclear morphology observed in several cell lines after incubation with low concentrations of paclitaxel (39, 40, 42). The subsequent increase of cells with apoptotic features such as nuclear condensation, nuclear shrinkage, apoptotic bodies, phosphatidylserine externalization, cytochrome *c* release, caspase activation, and PARP cleavage (Table 2) suggests that the apoptotic cascade is involved in mediating the cytotoxic effects of discodermolide and epothilone B.

Despite these apoptotic features, discodermolide and epothilone B were found to trigger cell death independent of the two major apoptotic routes. In contrast to the mitochondria dependency of many chemotherapeutic agents (33, 43), discodermolide and epothilone B seem to bypass the mitochondria route, because overexpression of the antiapoptotic proteins Bcl-2 and Bcl-x_L did not protect against cell death. Inhibition of the death receptor pathway by overexpression of FADD-DN did not affect the cytotoxicity of these drugs either. The ability of anticancer drugs to trigger cell death independent of the

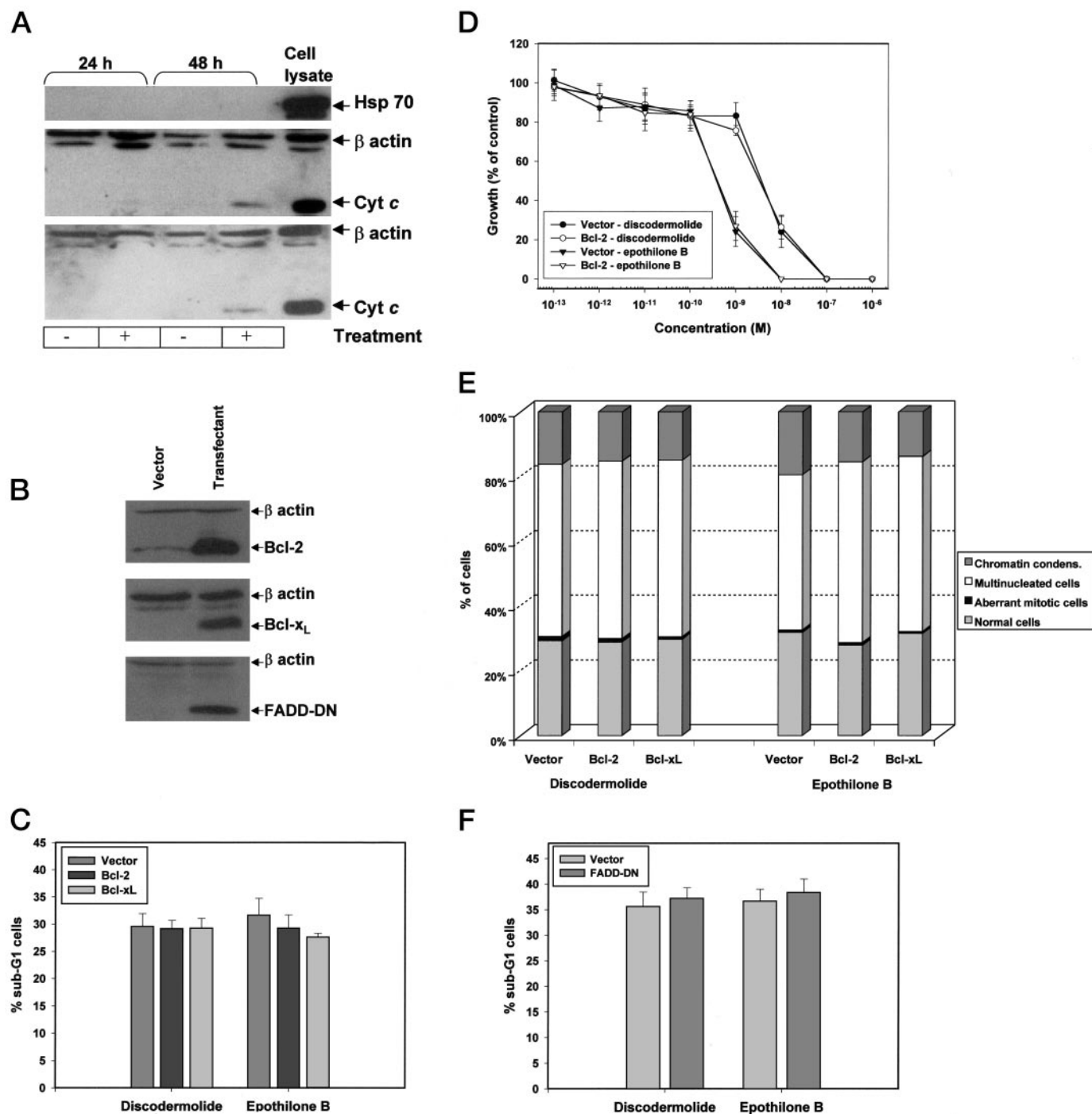


Fig. 3. The apoptotic pathways are not essential for discodermolide- or epothilone B-induced cell death. *A*, cytochrome *c* (*Cyt c*) release from mitochondria was determined by Western blotting in cytosolic extracts at 24 and 48 h after treatment with IC₅₀ concentrations of discodermolide (*upper panel*) and epothilone B (*lower panel*). Heat shock protein 70 (*Hsp 70*) was used as a control for the purity of the cytosolic extracts in comparison to a whole-cell lysate, whereas β -actin was applied as a control for protein loading. *B*, Western blots showing the expression of Bcl-2 (*upper panel*), Bcl-x_L (*middle panel*), and truncated, AU1-tagged FADD (*FADD-DN*; *lower panel*) in H460 cells compared with cells transfected with the empty vector. β -Actin was used as a control for loading. *C*, effect of stable overexpression of Bcl-2, Bcl-x_L, and the empty vector in H460 cells treated with discodermolide and epothilone B. After 48 h of exposure, the percentage of cells with a sub-G₁ DNA content was assessed by PI staining. The results represent the means of at least three independent experiments; *bars*, SD. *D*, growth inhibition assay of discodermolide and epothilone B in H460 cells overexpressing Bcl-2 and the empty vector. The results were obtained 72 h after treatment and represent the means of three independent experiments; *bars*, SD. *E*, morphological changes (as defined in Fig. 1*B*) induced by 72 h exposure to discodermolide and epothilone B in H460 cells overexpressing Bcl-2, Bcl-x_L, or the empty vector. Results represent the means of three independent experiments, in each of which 300 cells were counted. All SDs were <10%. *F*, effect of stable expression of FADD-DN or the empty vector in H460 cells treated with discodermolide or epothilone B for 48 h. The percentage of cells with a sub-G₁ DNA content was determined by PI staining, and the results are depicted as the means of at least three independent experiments; *bars*, SD.

mitochondria and death receptor pathways is not unprecedented and has also been shown for other agents, such as the sesquiterpene lactone helenalin (44). Moreover, we have found recently that the death receptor and mitochondria pathways contribute only partially to

the early and late stages of paclitaxel-induced cell death, respectively (37).

The absence of a role for the two major apoptotic routes and the facts that most apoptotic features occurred only at very late time

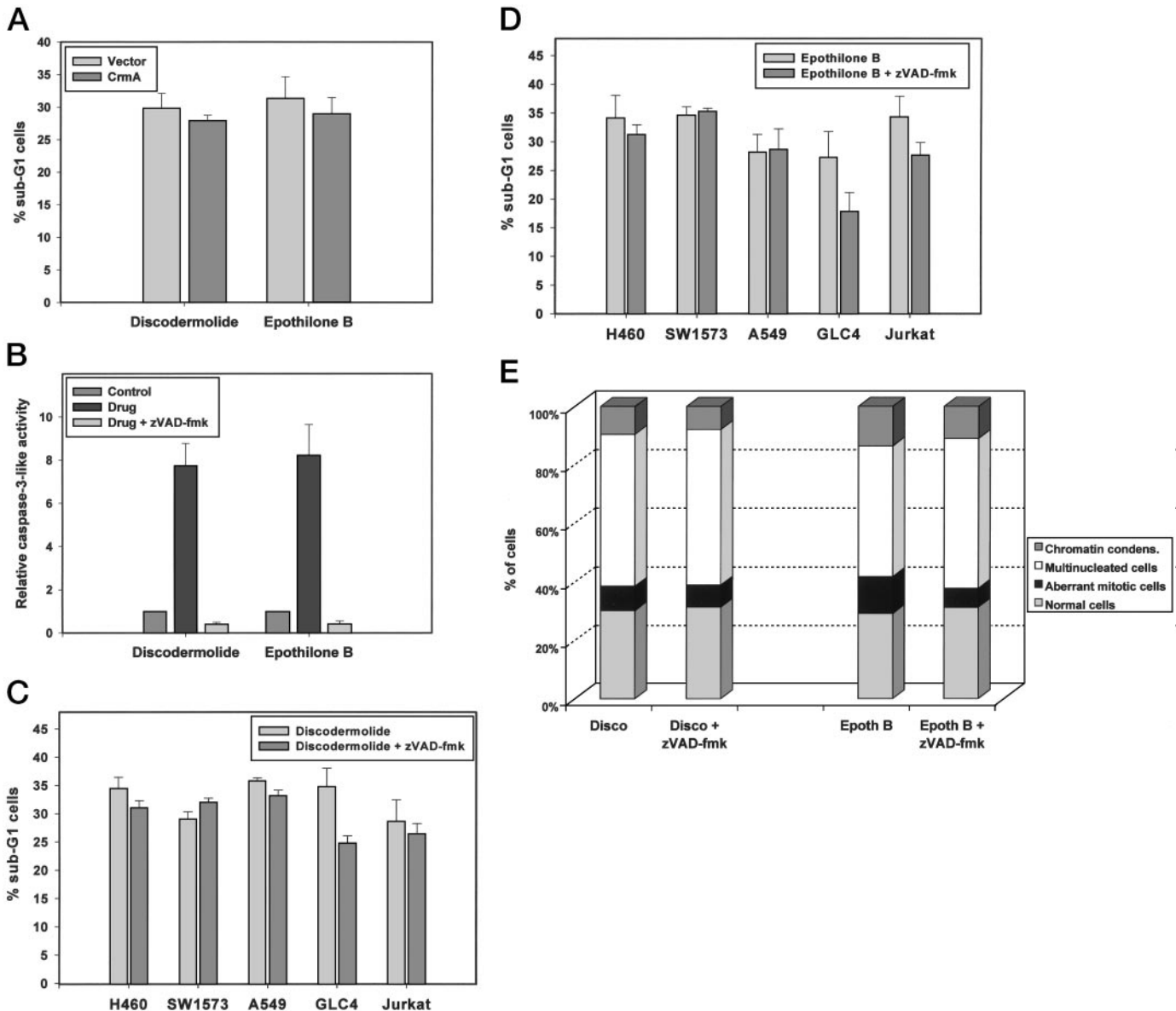


Fig. 4. Caspase activation is not required for discodermolide- or epothilone B-induced cell death. *A*, H460 cells stably transfected with the caspase-8 inhibitor CrmA or the empty vector were treated with IC₈₀ concentrations of discodermolide or epothilone B. After 48 h, the percentage of cells with a sub-G₁ DNA content was determined by PI staining. *B*, effect of zVAD-fmk (50 μM) on caspase-3-like protease activity induced by 48 h incubation with discodermolide or epothilone B. *C* and *D*, indicated cell lines were treated for 48 h with discodermolide (*C*) or epothilone B (*D*) and compared with cells pretreated with 50 μM zVAD-fmk. The fraction of cells with a sub-G₁ DNA content was assessed by PI staining. Results represent the means of at least three independent experiments; bars, SD. *E*, effect of zVAD-fmk (50 μM) on the morphological changes (as defined in Fig. 1B) induced by 48 h of incubation with discodermolide (*Disco*) and epothilone B (*Epoth B*) in H460 cells. Results represent the means of at least three independent experiments, in each of which 300 cells were counted. All SDs were <10%. *condens.*, condensation.

points or were rather nonspecific, such as the development of a hypodiploid population (41), raise the possibility that the observed activation of the apoptotic machinery occurred only as a bystander effect and is not relevant for the cytotoxic effects of discodermolide and epothilone B. In support of this view, neither expression of the

caspase-8 inhibitor CrmA nor preincubation with the broad-spectrum caspase blocker zVAD-fmk decreased the effects of these compounds. Our findings imply that caspase-independent routes are involved in the cytotoxic effects of discodermolide and epothilone B, as has been demonstrated recently in several cell lines upon exposure to paclitaxel or docetaxel (45–47). The activation of caspase-dependent or -independent death pathways seems to rely on yet uncharacterized cell-specific factors (31) and may also be related to the applied drug concentration, because it has been reported that the mechanism of paclitaxel-induced cytotoxicity is concentration dependent (39, 48, 49). This may also hold true for discodermolide and epothilone B that were used at low but effective doses in the current study.

The role of apoptosis *versus* necrosis on drug-induced cell death in tumors is still a controversial issue (29, 30, 50–52). In this context, the existence of alternative death pathways (as described

Table 2 Overview of the observed characteristics of discodermolide- and epothilone B-induced cell death in NSCLC cells

	Early events (<24 h)	Late events (>24 h)
Cellular	G ₂ -M arrest/aberrant mitosis Multinucleated cells Hypodiploid DNA content	Nuclear condensation, apoptotic bodies Cytochrome <i>c</i> release
Molecular	Disruption of microtubule cytoskeleton	Phosphatidylserine externalization PARP cleavage Caspase-3 and -8 activation

here) supports the view that other forms of cell death, which cannot be strictly classified as apoptosis or necrosis, may account for the effect of certain chemotherapeutic agents (31). Various patterns of cell death have been described thus far (53–56) that were recently categorized by Leist and Jäätelä (57) in apoptosis, apoptosis-like PCD, necrosis-like PCD, and accidental cell death/cell lysis. In this model, caspases are the main players in apoptosis, whereas other proteases such as cathepsins, calpains, and serine proteases account for other types of PCD. Whether similar mechanisms are involved in discodermolide- and epothilone B-induced cell death remains to be demonstrated. Further studies to unravel the signaling pathways triggered by discodermolide and epothilone B are currently being carried out in our laboratory. Understanding the caspase-independent pathways triggered by agents such as discodermolide and epothilone B might contribute to the development of new, mechanistic-based therapeutic strategies to circumvent chemoresistance by triggering, for instance, caspase-independent death routes in apoptosis-resistant tumors.

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Late Activation of Apoptotic Pathways Plays a Negligible Role in Mediating the Cytotoxic Effects of Discodermolide and Etoposide in Non-Small Cell Lung Cancer Cells

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