Evidence That the Apoptotic Actions of Etoposide Are Independent of c-Jun/Activating Protein-1-Mediated Transregulation

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ABSTRACT

We recently demonstrated that physiological induction of apoptosis by cytotoxic sphingolipid messengers proceeds via activating protein-1 (AP1)-dependent and AP1-independent mechanisms in U937 human monoblastic leukemia cells. Here we examine involvement of the stress-activated protein kinase (SAPK) cascade and AP1 in the initiation of apoptosis in U937 cells by podophyllotoxin-derived inhibitors of topoisomerase II. Induction of apoptotic cell death and DNA damage by treatment of U937 cells with etoposide (100 μM) was associated with phosphorylation and activation of the c-Jun NH2-terminal kinase (JNK1) SAPK enzymes p46 and p54-JNK2 and transient increases in expression of the transcription factor c-Jun, a primary JNK substrate. These responses were accompanied by a modest, but sustained, recruitment of the mitogen-activated protein kinases p42-extracellular signal receptor-activated kinase (ERK)1 and p44-extracellular signal receptor-activated kinase 2. The capacity of etoposide to promote double-stranded DNA degradation and cell death was unaffected by manipulations that interfere with SAPK signaling outflow through c-Jun/AP1, including: 1) pharmacological inhibition of AP1 activity by diferuloylmethane and 2) molecular ablation of normal c-Jun function by the Jun dominant-negative mutant TAM-67. Cytotoxicity of the structurally related compound teniposide was similarly unaffected. In parallel trials, the lethal actions of ceramide (but not of sphingosine) were markedly diminished by pretreatment with diferuloylmethane or expression of TAM-67, confirming the effectiveness of these interventions in suppression of SAPK/AP1-dependent apoptosis. The involvement of AP1 in the proapoptotic actions of other inhibitors of topoisomerase II activity was also evaluated. Induction of cell death by the anthracyclines daunorubicin, daunorubicin, and idarubicin was found to be insensitive to pretreatment with diferuloylmethane or expression of TAM-67. Collectively, the present data indicate that induction of apoptosis by etoposide and related inhibitors of topoisomerase II is mediated through a cell death pathway that does not require SAPK-dependent recruitment of AP1. These findings additionally suggest that activation of the SAPK represents a consequence, rather than an underlying cause, of etoposide-induced apoptosis in myeloid leukemia cells.

The epipodophyllotoxins represent an effective class of anticancer agents used in the treatment of a variety of malignant disorders (Liu et al., 1989; Beck et al., 1994, Perez et al., 1994, Pommier et al., 1994, 1995). Among several compounds, the actions of etoposide have been the most intensively examined (Pommier et al., 1995). Numerous studies have demonstrated that etoposide inhibits the religation activity of topoisomerase II without affecting the initial DNA scission function of the enzyme (Tewey et al., 1984). Acute exposure to etoposide thus promotes extensive double-stranded degradation of genomic DNA, reflected by 1) the formation of large [50-kilobase pair (kbp) and/or 300-kbp] DNA fragments corresponding to rosette and loop structures within static chromatin (Oberhammer et al., 1993) and 2) subsequent degradation of these fragments into oligonucleosomal fragments (ranging from 0.1–1.8 kbp in size, corresponding to free oligonucleosomes) (Filipski et al., 1990; Oberhammer et al., 1993; Testolin et al., 1997). Induction of double-stranded DNA damage by inhibitors of topoisomerase

ABBREVIATIONS: VP-16, etoposide; VM-26, teniposide; DFM, diferuloylmethane; ERK, extracellular signal receptor-activated kinase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; DXR, doxorubicin; DNR, daunorubicin; IDR, idarubicin; ara-C, 1-[β-c-arabinofuranosyl]cytosine; MEKK2, MAP/ERK kinase kinase-1.
II is temporally associated with the onset of apoptotic cell death in most neoplastic cell types (Filipski et al., 1990; Oberhammer et al., 1993; Pommier et al., 1995). Although it has been suggested that the cytotoxicity of these agents results directly from chromatinolysis (Oberhammer et al., 1993), the primary mechanism underlying the lethal actions of etoposide and related compounds remains to be identified (Pommier et al., 1995).

The stress-activated protein kinase (SAPK) cascade is engaged by numerous cellular stresses and lethal insults (Kyriakis et al., 1994; Sanchez et al., 1994; Yan et al., 1994; Osborn and Chambers, 1996; reviewed by Ip and Davis, 1998). Recruitment of this signaling system entails activation of one or more isoforms of c-Jun NH2-terminal kinase (JNK), NH2-terminal phosphorylation of c-Jun, and consequent induction of activating protein-1 (AP1)-mediated transactivation (Kyriakis et al., 1994). The SAPK cascade has also been implicated in the apoptotic responses of several myeloid leukemia cell lines (e.g., U937, HL-60, and K562) to various antineoplastic agents (e.g., anthracyclines, deoxyctitidine analogs, and ionizing radiation). Acute exposure to etoposide potently activates JNK, resulting in in situ phosphorylation of c-Jun (Seimiya et al., 1997). In addition, etoposide treatment increases expression of c-jun/c-Jun (Rubin et al., 1991; Ritke et al., 1993, 1994a, b; Perez et al., 1994; Eliot et al., 1995) and c-fos/c-Fos (Perez et al., 1994; Eliot et al., 1995), with attendant induction of AP1-dependent transactivation (Rubin et al., 1991; Ritke et al., 1994a; Perez et al., 1994). Given the evidence identifying the AP1 complex as a principal target of the SAPK cascade, it has been inferred that etoposide-mediated lethality stems from downstream activation of AP1 (Rubin et al., 1991). The functional role of SAPK-AP1 signaling in etoposide action remains uncertain, however, because transient ablation of JNK expression by treatment with antisense oligonucleotides reportedly eliminates SAPK responses to etoposide but only partially reduces cytotoxicity (Seimiya et al., 1997).

The present investigations sought to define more rigorously the contribution of SAPK-related AP1 activity to the apoptotic actions of etoposide in U937 human monoblastic leukemia cells. Although etoposide and the structurally related compound teniposide promoted SAPK activation and increased c-Jun expression, these events did not appear to underlie drug-induced cell death, insofar as apoptotic responses to these agents were not affected by 1) molecular ablation of normal AP1 function by the c-Jun dominant-negative mutant TAM-67 or 2) pharmacological blockade of c-Jun/AP1 binding by the curcumin-derived phytoxpin diferuloylmethane (DFM). Moreover, whereas the cytotoxicity of sphingosine was similarly unaffected by these interventions, ceramide lethality was sharply attenuated. Although induction of apoptosis by the anthracycline antibiotics doxorubicin, daunorubicin, and idarubicin was associated with delayed recruitment of the SAPK cascade, the lethal effects of these agents were also insensitive to interference with AP1 by either DFM or TAM-67. Our findings therefore suggest that the apoptotic responses of U937 cells to etoposide, teniposide, and other topoisomerase II inhibitors do not derive from the SAPK/JNK cascade and c-Jun/AP1. In addition, these observations indirectly suggest that induction of apoptosis by topoisomerase II inhibitors proceeds along a proapoptotic signaling pathway that is independent of ceramide.

**Materials and Methods**

**Drugs and Reagents.** Crystalline preparations of etoposide [4’-demethyllepidodipodophyllotoxin-β-9-ethylidene glucoside (VP-16); Sigma, St. Louis, MO] and teniposide [4’-demethyllepidodipodophyllotoxin-β-d-thelylidene glucoside (VM-26); Bristol-Myers, New York] were dissolved in sterile dimethyl sulfoxide immediately before use; doxorubicin (DXR; Sigma), daunorubicin (DNR; Sigma), and idarubicin (IDR; provided by Dr. D. A. Gewirtz) were also prepared in dimethyl sulfoxide. Synthetic preparations of D-erythro-ceramide and D-erythro-sphingosine (BIOMOL, Plymouth Meeting, PA) were initially dissolved in ethanol; organic sphingolipid stocks were complexed with delipidated albumin as described previously (Jarvis et al., 1997). The curcumin extract DFM [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione; BIOMOL] was dissolved in ethanol immediately before use. All test reagents were prewarmed and presented at final concentrations in complete medium at 37°C; the vehicles used were without effect in HL-60 cells during exposure intervals of up to 24 hr.

**Cell Culture.** The human monoblastic leukemia cell line U937 was derived from a patient with diffuse histiocytic lymphoma (Sundstrom et al., 1976). U937 cells were previously transfected by electroporation with pMexM metallothionine-inducible vectors without or with the insertion for the c-Jun deletion mutant TAM-67 (Brown et al., 1994), giving rise to the stable sublines U937/136-4 and U937/101-2-1 (designated U937/TAM), respectively (Jarvis et al., 1994b, 1997); U937/136-4 cells were tested in parallel with wild-type parental cells (referred to as U937/WT), and consistently exhibited identical biological responses. All cell lines were grown in complete RPMI-1640 medium (phenol red-free formulation, supplemented with 10% sodium pyruvate, nonessential amino acids, l-glutamine, penicillin, streptomycin (all from Life Technologies, Grand Island, NY), and 10% heat-inactivated fetal bovine serum) and maintained under a fully humidified atmosphere of 95% room air, 5% CO2 at 37°C; transfected cell lines were grown in the presence of G418 (400 µg/ml; Life Technologies). Cultures were passaged twice weekly. Cell densities were determined by Coulter counter, and cell viability was assessed by trypan blue exclusion.

**Test Exposures.** All experimental incubations were performed as described (Jarvis et al., 1994b, 1996). Cells in logarithmic-phase growth were pelleted, rinsed twice in complete medium, resuspended at a density of 4 x 106 cells/ml, and maintained as indicated above. Cells were exposed to test agents for appropriate intervals in complete medium; loss of cells under these conditions because of either washing or cell adherence was negligible (≤5%). Test incubations were terminated with gentle pelleting of the cells by centrifugation at 400g for 10 min at 4°C; in some instances, aliquots of the medium were retained for direct assay of released DNA. After determination of cell density, the cells were pelleted and prepared as outlined below for spectrofluorophotometric assays of DNA damage, assay of cloning efficiency, examination of cellular morphology, Northern and Western analyses, or assay of SAPK and mitogen-activated protein kinase (MAPK) activities.

**Quantitative Analyses of DNA Damage.** The formation and release of DNA fragments and the corresponding breakage of bulk DNA were assessed by bisbenzimide spectrofluorophotometry as described (Jarvis et al., 1994b, 1996, 1997). To measure DNA fragments, pelleted cells (4 x 106 cells/pellet) and medium aliquots were mixed with 5 mM Tris-HCl, 30 mM EGTA, 30 mM EDTA, 0.1% Triton X-100 (pH 8.0). Lysate and medium preparations were centrifuged at 30,000g for 40 min; nonsedimenting DNA fragments in the extracts were quantified by spectrofluorophotometry in the presence of Hoechst-33258 (1 µg/ml; λex = 365, λem = 460; Hoechst-Roussel Pharmaceuticals, Somerville, NJ). Values are expressed as nanograms per microgram DNA recovered or released.
from 10^6 cells. To measure corresponding loss of integrity of bulk DNA, pelleted cells (8.25 × 10^6 cells/pellet) were resuspended in cold PBS and subjected to timed alkaline denaturation in 0.1 N NaOH; denaturation was terminated by neutralization in 0.1 N HCl. Cells were then lysed by addition of 200 mM K_2HPO_4, 50 mM EDTA, and 0.16% N-lauroylsarcosine. Bulk DNA breakage was quantified by spectrofluorophotometry in the presence of Hoechst-33258 (λex = 350, λem = 450). Values are expressed as rad-equivalents.

**Determination of Clonogenicity.** Pelleted cells were rinsed extensively and prepared for soft-agar cloning as described (Jarvis et al., 1994b, 1996). Cells were resuspended in cold PBS and seeded in 35-mm culture plates at a fixed density (400 cells/ml/well) in complete RPMI-1640 medium containing 20% fetal calf serum, 10% 5637-conditioned media, and 0.3% Bacto agar. Cultures were maintained for 10 to 12 days before formation of colonies (defined as groups of ≥50 cells) was scored.

**Cytological Characterization of Apoptosis.** Pelleted cells were resuspended in PBS and fixed in cytotoxic preparatations according to established procedures (Jarvis et al., 1997). For visualization of apoptotic morphological alterations, fixed cells were stained with 20% Wright-Giemsa stain. At least 500-cell fields were scored for each treatment by conventional light microscopy by assessing the expression of cytoarchitectural characteristics of apoptosis (i.e., condensed nucleoplasm and cytoplasm, formation of membrane blebs, karyolytic degeneration of the nucleus into apoptotic bodies, and overall cell shrinkage). For visualization of apoptotic DNA damage, fixed cells were sequentially treated with ethanol/acetic acid (2:1, v/v) at 20°C for 5 min, stained for broken DNA by treatment with terminal deoxynucleotidyl transferase in the presence of fluorescein isothiocyanate-dUTP (Molecular Probes, Eugene, OR) at 37°C for 60 min, and counterstained for intact DNA with 0.01% propidium iodide in sodium citrate at 20°C for 10 min. At least three 100-cell fields were scored for each treatment by fluorescent microscopy by assessing increased direct fluorescence of end-labeled double-stranded DNA.

**Determination of SAPK and MAPK Activities.** Pelleted cells were rinsed in PBS, pelleted, and flash-frozen. For determination of SAPK and MAPK activities in vitro, cell pellets were lysed in 25 mM HEPES (pH 7.4) containing 5 mM EGTA and 5 mM EDTA, supplemented with protease inhibitors, phosphatase inhibitors, and adjusted to 0.05% sodium deoxycholate (w/v), 1% Triton X-100 (v/v) and 0.1% 2-mercaptoethanol (v/v) as described (Jarvis et al., 1997). Lysates were clarified by centrifugation at 5000 g for 5 min. Kinases were immunoprecipitated from clarified lysates with protein A/agarose-conjugated antibodies, and activities were determined as described (Jarvis et al., 1997). SAPK activities were assayed after immunoprecipitation of p54-JNK1/p46-JNK2 using glutathione S-transferase–c-Jun 1-169 as substrate. MAPK activity was assayed by the exposure interval. In vitro kinase assays demonstrated that the induction of apoptosis was associated with a robust activation of SAPK but a relatively attenuated activation of MAPK (Fig. 2). Etoposide potently stimulated p54-JNK1/p46-JNK2 activity. This response was discernible within 2 h of the exposure interval. In vitro kinase assays demonstrated that the induction of apoptosis was associated with a robust activation of SAPK but a relatively attenuated activation of MAPK (Fig. 2).

**Results**

Preliminary trials characterized the time course of etoposide-induced apoptosis in U937 human monoblastic leukemia cells (Fig. 1). U937 cells were exposed to etoposide for 0 to 4 h at a concentration previously determined to be maximally toxic (10 μM) in vitro. Etoposide treatment produced a time-dependent increase in the presence of apoptotic cells that was evident within 2 h and increased progressively throughout the exposure interval. In vitro kinase assays demonstrated that the induction of apoptosis was associated with a robust activation of SAPK but a relatively attenuated activation of MAPK (Fig. 2). Etoposide potently stimulated p54-JNK1/p46-JNK2 activity. This response was discernible within 2 h.
and became more pronounced over time (4- to 5-fold at 4 h). A considerably smaller increase in p42-ERK1/p44-ERK2 activity (~65%) was detected over the exposure interval. Corresponding in situ phosphorylation of SAPK, but not of MAPK, was revealed by Western analysis (Fig. 3). Etoposide produced a time-dependent increase in the presence of phosphorylated p54-JNK1 and p46-JNK2. JNK phosphorylation was evident within 1 to 2 h and increased progressively throughout the exposure, consistent with the stimulated activities of these enzymes described above. In contrast, a small amount of phosphorylated p44-ERK2 was evident throughout the exposure; the presence of this species was not discernibly increased in response to etoposide, however. Expression of the primary SAPK substrate c-Jun was also monitored (Fig. 4) in related studies; Western analyses demonstrated that recruitment of the SAPK cascade was accompanied by increased steady-state levels of c-Jun. Similar responses also were obtained using the structurally related compound teniposide (data not shown). Subsequent trials examined the importance of SAPK cascade outflow through AP1 in etoposide action. This was accomplished by monitoring etoposide-induced apoptosis in conjunction with impaired c-Jun/AP1 function.

The transactivation potential of c-Jun was suppressed by stable transfection of U937 cells with the c-Jun mutant protein TAM-67 (Brown et al., 1994; Freemerman et al., 1996). This deletion mutant possesses both the leucine-zipper site and c-terminal DNA-binding domain, but lacks residues 3 to 122 of the N-terminal transcriptional-activation domain; thus, TAM-67 possesses normal dimerization and AP1 consensus site-recognition functions but is devoid of transactivating activity, resulting in dominant-negative suppression of normal c-Jun transactivation (Brown et al., 1994). Alternatively, AP1 activity was directly inhibited by pretreatment with DFM, a bioactive component extracted from the phyto-
toxin curcumin (Pendurthi et al., 1997). Treatment with DFM inhibits binding of intact c-Jun/c-Jun and c-Jun/c-Fos AP1 complexes to AP1 consensus-site DNA-recognition sequences, thereby limiting AP1-dependent transactivation (Bierhaus et al., 1997).

Suppression of AP1-dependent apoptosis either by c-Jun dominant-negatives such as TAM-67 or by DFM in various cell types has been documented by several laboratories (Yamamoto, 1995; Sawai et al., 1995; Ham et al., 1995; Verheij et al., 1996; Jarvis et al., 1997). We have recently reported that JNK and c-Jun/AP1 subserves lethal signaling functions in the response to ceramide (Verheij et al., 1996; Jarvis et al., 1997), but not to sphingosine (Jarvis et al., 1997), demonstrating that physiological initiation of apoptosis can proceed along AP1-dependent and AP1-independent pathways in U937 cells. Control studies therefore examined the effects of molecular ablation of c-Jun or pharmacological inhibition of c-Jun/AP1 on the responses to ceramide and sphingosine. Consistent with earlier observations, the lethal actions of ceramide, but not those of sphingosine, were markedly attenuated by TAM-67 (Table 1) as well as by pretreatment with DFM (Table 2).

The induction of apoptosis by etoposide or teniposide also was tested under these conditions. Spectrofluorometric assessment of DNA damage demonstrated the accumulation of double-stranded DNA fragments (Fig. 5A) and double-stranded breakage of bulk DNA (Fig. 5B) after 4-h exposure to etoposide or teniposide at equitoxic levels (100 and 10 μM, respectively). DNA damage closely paralleled induction of apoptosis and loss of clonogenic potential (Fig. 6). Expression of TAM-67 failed to modify induction of DNA damage by either agent. Similarly, the manifestation of apoptotic morphology in response to etoposide and teniposide was completely insensitive to the presence of TAM-67. In related studies, U937 cells were pretreated for 12 h with DFM (500 nM) and then treated for 4 h with either etoposide (100 μM) or teniposide (10 μM) in the continued presence of DFM. DFM failed to modify the induction of cell death by either agent (data not shown). Neither TAM-67 expression nor DFM treatment was effective in altering the apoptotic responses to etoposide and teniposide at submaximal concentrations (e.g., 500 and 50 nM respectively; data not shown). Together, these findings indicated that the mechanism underlying the lethal influences of etoposide and teniposide is independent of normal AP1-mediated transactivation.

Other antineoplastic agents associated with topoisomerase II inhibition were evaluated in related studies. Treatment of U937 cells with the structurally interrelated anthracycline antibiotics DXR (Fig. 7A), DNR (Fig. 7B), and IDR (Fig. 7C) at a maximally cytotoxic concentration (10 μM) for 24 h elicited apoptotic cell death. In each instance, cell death exhibited a delayed onset as compared with etoposide and teniposide. Apoptotic morphology was discernible within 6 h of exposure to DXR and DNR with maximal toxicity (i.e., ≥80%) manifested only after ~18 h. The response to IDR followed a similar time course, although the maximal toxicity of this agent was less pronounced (i.e., 64% after 18 h). Anthracycline-induced cell death, like that triggered by epipodophyllotoxins, was associated with activation of the SAPK cascade. Interestingly, p54-JNK1/p46-JNK2 activity initially declined to subbasal levels in response to each of these agents and then rose sharply over time. In fact, stimulation of p54-JNK1/p46-JNK2 activity appeared to lag closely behind the induction of apoptosis, compatible with the notion that recruitment of the SAPK cascade occurs as a consequence of cell death. Consistent with this inference, anthracycline lethality was insensitive to pharmacological inhibition of c-Jun/AP1 by molecular ablation of c-Jun by expression of TAM-67 (Fig. 8) or treatment with DFM (data not shown), as noted in the case of the etoposide and teniposide exposures described above. The inability of TAM-67 to suppress the actions of DXR, DNR, and IDR was also noted at a submaximal drug concentrations (e.g., 1 μM; data not shown).

In many neoplastic cell types, the response to treatment with antineoplastic agents involves the recruitment of one or more cytoprotective signaling elements (e.g., conventional and novel isoforms of protein kinase C; cPKC, nPKC), interference with which can enhance cytotoxicity. Clinically relevant chemomodulators acting at different levels along the cPKC/nPKC → Raf-1 → MEK1 → ERK1/ERK2 pathway markedly potentiates the antileukemic capacity of the deoxyctydine analog 1-[β-D-arabinofuranosyl]cytosine (ara-C) by suppression of drug-induced MAPK recruitment. We have previously shown that induction of leukemic cell apoptosis by ara-C is sharply increased through pharmacological reductions of cPKC/nPKC (Jarvis et al., 1998), whether through down-regulation by bryostatin 1 or through inhibition by safingol; moreover, we have found that disruption of MEK/ERK signaling further downstream by flavonoid inhibitors of MEK1 (e.g., aminomethoxy flavone) are comparatively effective in potentiation of ara-C action (Jarvis et al., 1998). Final trials therefore examined whether etoposide action is also susceptible to these interventions. Etoposide-induced cell death was not discernibly enhanced by either chronic pretreatment with bryostatin or acute coexposure to safingol (Fig. 9A). Similarly, etoposide action was not modified in the

### TABLE 1

<table>
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<th>Treatment</th>
<th>U937/WT</th>
<th>U937/TAM-67</th>
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<tbody>
<tr>
<td>% vehicle control</td>
<td>% vehicle control</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>3 ± 1</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>Ceramide</td>
<td>39 ± 6</td>
<td>14 ± 1</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>94 ± 11</td>
<td>91 ± 9</td>
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* Increased versus vehicle (p < .01)
* Decreased versus vehicle (p < .01)

### TABLE 2

<table>
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<th>Control</th>
<th>DFM</th>
</tr>
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<tbody>
<tr>
<td>% vehicle control</td>
<td>% vehicle control</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>1 ± 1</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Ceramide</td>
<td>33 ± 4</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>Sphingosine</td>
<td>89 ± 3</td>
<td>90 ± 2</td>
</tr>
</tbody>
</table>

* Increased versus vehicle (p < .01)
* Decreased versus ceramide (p < .01)
presence of aminomethoxyflavone (Fig. 9B). The insensitivity of etoposide action to interference with PKC and/or the MAPK cascade suggest a fundamental difference between the cytotoxic mechanisms engaged by epipodophyllotoxins and ara-C in the induction of leukemic cell death.

**Discussion**

The present findings demonstrate that etoposide-induced apoptosis is associated with activation of SAPK-AP1 signaling but additionally suggest that recruitment of this system represents a consequence, rather than a cause, of drug-related cell death. Thus, the lethal actions of etoposide, teniposide, and other inhibitors of topoisomerase II appear to proceed along an AP1-independent process. Participation of both AP1-dependent and AP1-independent processes in the physiological initiation of apoptosis by sphingolipid messengers is supported by abundant evidence. For example, induction of apoptosis by tumor necrosis factor-α via activation of tumor necrosis factor R1 can involve acute generation of either ceramide (Jarvis et al., 1994a) or sphingosine (Ohta et al., 1994). Initiation of AP1-dependent apoptosis by ceramide or ceramide-coupled stresses is antagonized by experimental interference with normal c-Jun function, including 1) suppression of c-Jun expression by c-jun antisense oligonucleotides (Seimiya et al., 1997); 2) pharmacological inhibition of AP1 binding by curcumin (Sawai et al., 1995); and 3) dominant-negative blockade of c-Jun activity (Ham et al., 1995; Verheij et al., 1996; Jarvis et al., 1997). Direct comparison of SAPK and AP1 involvement in the induction of apoptosis in U937 cells by these cytotoxic messengers revealed that ceramide lethality is markedly attenuated by disruption of the SEK/JNK module or interference with AP1 (Jarvis et al., 1997), whereas sphingosine lethality is unaffected by such manipulations (Jarvis et al., 1997). The potential relevance of these findings for drug-induced cell death is underscored by reports from other laboratories indicating that ceramide generation is associated with cellular responses to a variety of cytotoxic agents, including inhibitors of topoisomerase II (reviewed by Jarvis and Grant, 1998).

Pharmacological inhibition of topoisomerase II function by epipodophyllotoxin derivatives elicits dramatic apoptotic responses in neoplastic cells (Pommier et al., 1995). The functional significance of the stress signaling pathway(s) engaged by agents such as etoposide and teniposide is unclear, however. Etoposide treatment increases expression of c-jun/c-Jun

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**Fig. 5.** Effect of TAM-67 expression on the apoptotic responses to etoposide and teniposide. Parental (U937/WT) and transfected (U937/TAM) cell lines were treated with dimethyl sulfoxide vehicle (VEH), etoposide (VP-16; 100 μM), or teniposide (VM-26; 10 μM) for 4 h. Apoptotic DNA damage was quantified by spectrophotometry as described in Materials and Methods. A, formation (hatched columns) and release (cross-hatched columns) of double-stranded DNA fragments. B, double-stranded breakage of bulk DNA (solid bars). Data shown are from a representative study performed three times with comparable results. All values reflect the mean ± S.E. of triplicate determinations.

**Fig. 6.** Effect of TAM-67 expression on the induction of DNA damage by etoposide and teniposide. Parental (U937/WT) and transfected (U937/TAM) cell lines were treated with dimethyl sulfoxide vehicle (VEH), etoposide (VP-16; 100 μM), or teniposide (VM-26; 10 μM) for 4 h. Apoptotic cell death was then assessed in fixed cells as before. Data shown are from a representative study performed three times with comparable results.
Rubin et al., 1991; Ritke et al., 1993, 1994a,b; Perez et al., 1994) and c-fos/c-Fos (Perez et al., 1994; Ritke et al., 1994b; Eliot et al., 1995) and AP1-dependent transactivation (Rubin et al., 1991; Ritke et al., 1994a; Perez et al., 1994). The documented role of the AP1 constituent c-Jun as a primary target for cytotoxic signaling through JNK (Kyriakis et al., 1994) raises the possibility that the lethal effects of etoposide are mediated through SAPK-dependent activation of AP1, although direct evidence supporting or refuting this hypothesis is presently lacking. In this regard, antisense blockade of p46-JNK1 expression reportedly blunts the apoptotic capacity of etoposide in U937 cells (Seimiya et al., 1997), potentially consistent with a requirement for this signaling pathway in etoposide-mediated lethality. On the other hand, ablation of c-fos by site-directed mutagenesis does not modify the apoptotic response to etoposide (Gajate et al., 1996), suggesting that heterodimeric AP1 (i.e., AP1 species composed of c-Fos/c-Jun) may not represent an essential element in the initiation of apoptosis by etoposide. Nonetheless, the possibility that homodimeric (i.e., c-Jun/c-Jun) forms of AP1 underlie etoposide-mediated lethality cannot be excluded, however. Recent characterization of apoptotic proteolysis indicates that various enzymes comprising specific pathways for survival signaling (both anti- and proapoptotic) are rapidly degraded during the final stages of cell death (Widmann et al., 1998). Among these activities, it has been noted that the initial kinase in the SAPK cascade, MAP/ERK kinase kinase-1 (MEKK1), undergoes proteolytic activation by the apoptotic protease caspase 1 during etoposide treatment (Widmann et al., 1998). This is of particular interest, because MEKK1 directly engages the SEK/JNK module in response to an array of upstream stress-induced kinases (e.g., p21-activated kinase and TGFβ-activated kinase), suggesting that MEKK1 integrates convergent cytotoxic signals for recruitment of the SAPK cascade in many settings. Proteolytic activation of this enzyme by one or more apoptotic proteases raises the alternative possibility that the MEKK1 → SEK1 → JNK1/2 → AP1 sequence can also be engaged as a relatively late (and therefore secondary) event in the final apoptotic process rather than as an essential component in the cell death program.
The present findings confirm the close temporal association between etoposide-related cytotoxicity and recruitment of SAPK/AP-1 but fail to support a direct role for these signaling elements in etoposide action. The lethal actions of etoposide and teniposide in U937 cells were associated with activation of SAPK (p46-JNK1/p54-JNK2), increased c-jun/c-Jun expression, and stimulation of AP1 transactivation potential. Despite this prominent SAPK cascade response, the cytotoxicity of these agents was not limited by pharmacologic manipulations or molecular interventions that interfere with normal AP1 function. This was demonstrated by the observation that U937 cells transfected with the c-Jun N-terminal deletion mutant TAM-67 were fully susceptible to the apoptotic actions of etoposide and teniposide (Brown et al., 1994; Freemerman et al., 1996; Jarvis et al., 1997). Confirmatory results were obtained when etoposide and teniposide were administered in the presence of the curcumin metabolite DFM, which inhibits targeting of AP1 (Pendurthi et al., 1997; Bierhaus et al., 1997). Taken together, the persistence of etoposide- and teniposide-induced apoptosis in conjunction with manipulations that impair AP1 function indicates that the cytotoxicity of these agents does not require AP1-dependent transactivation potential.

The results of the present study differ in some respects from other reports suggesting that direct interruption of proapoptotic signaling through the SAPK cascade (e.g., by antisense ablation of p46-JNK1) limits the cytotoxicity of etoposide in U937 cells (Seimiya et al., 1995). The requirement for this signaling pathway in etoposide-related apoptosis in an apparently AP1-independent mode of cell death may derive from the participation of one or more alternative target substrates for p46-JNK1/p54-JNK2). For example, involvement of the activating transcription factor has been documented in some settings (Gupta et al., 1995), although we have previously reported this protein is not expressed in our U937 cell subline basally or during the onset of apoptosis (Jarvis et al., 1997). The present findings also differ from those of Bose et al. (1995), which support a direct contribution of ceramide to DNR-induced apoptosis in P388 murine lymphoblastic leukemia cells. In contrast, we found that TAM-67 abrogated the initiation of cell death in U937 cells by synthetic ceramide, but not by several anthracyclines, an observation that argues, albeit indirectly, that ceramide is not the sole proapoptotic effector engaged as a consequence of topoisomerase II inhibition in all leukemic cell types. The present results exhibit both similarities to those obtained with other cytotoxic agents linked to c-Jun/AP1 activation, most notably ara-C, although in other respects, clear differences were noted. For example, treatment of myeloid leukemia cells with ara-C elicits the generation of ceramide (Strum et al., 1994), engages the SAPK cascade (Saleem et al., 1995; Jarvis et al., 1998), increases expression of c-jun/c-Jun (Kharbanda et al., 1990), and induces AP1 (Brach et al., 1992), events potentially consistent with a role for the JNK/AP1 system in ara-C action. Consistent with the etoposide responses noted in the present study, molecular ablation of c-Jun does not suppress the induction of apoptosis by ara-C (Grant et al., 1996), suggesting that activation of JNK1/JNK2 and downstream recruitment of c-Jun/AP1 represents a consequence, rather than an underlying cause, of ara-C-mediated lethality. Interestingly, interference with c-Jun/AP1 does limit the antiproliferative influence of low-dose ara-C treatment, apparently through inhibition of drug-induced leukemic cell differentiation (Grant et al., 1996). It is also noteworthy that, in marked contrast to results obtained using ara-C (Jarvis et al., 1998), several agents known to disrupt the cPKC/nPKC and MAPK. U937 cells were exposed to etoposide (VP-16; 50 μM) or teniposide (VP-26; 50 μM) for 4 h in conjunction with either chronic pretreatment with bryostatin (10 nM) or acute cotreatment to safingol (750 μM) (A), or coexposure to aminomethoxyflavone (B).

Fig. 9. Insensitivity of epipodophyllotoxin cytotoxicity to modulation of cPKC/nPKC and MAPK. U937 cells were exposed to etoposide (VP-16; 50 μM) or teniposide (VP-26; 50 μM) for 4 h in conjunction with either chronic pretreatment with bryostatin (10 nM) or acute cotreatment to safingol (750 μM) (A), or coexposure to aminomethoxyflavone (B).
actions of these agents are mediated through an AP1-independent pathway for apoptosis and suggest that recruitment of the SAPK/AP1 system during these responses represents a consequence, rather than an underlying cause, of the apoptotic actions of topoisomerase II inhibitors.

References

Gajate C, Alonso MT, Schimmang T and Mollinedo F (1996) c-Fos is not essential for DNA folding in higher order chromatin structures.