Induction of endoplasmic reticulum stress by ellipticine plant alkaloids

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Abstract

Anticancer drugs often show complex mechanisms of action, including effects on multiple cellular targets. Detailed understanding of these intricate effects is important for the understanding of cytotoxicity. In this study, we examined apoptosis induction by ellipticines, a class of cytotoxic plant alkaloids known to inhibit topoisomerase II. The potent ellipticine derivative 6-propanamine ellipticine (6-PA-ELL) induced rapid apoptosis in MDA-MB-231 breast cancer cells, preceded by a conformational change in Bak and cytochrome c release. Experiments using knock-out mouse embryo fibroblasts established that Bak was of particular importance for cytotoxicity. 6-PA-ELL increased the expression of the endoplasmic reticulum chaperones GRP78/Bip and GRP94, suggesting induction of endoplasmic reticulum stress. Induction of GRP78 expression was dependent on the endoplasmic reticulum stress response element (ERSE) of the GRP78 promoter. Examination of different ellipticine derivatives revealed a correlation between pro-apoptotic activity and the ability to induce GRP78 expression. Furthermore, 6-PA-ELL was found to induce splicing of the mRNA encoding the XBP1 transcription factor, characteristic of endoplasmic reticulum stress, and to induce activation of the endoplasmic reticulum-specific caspase-12 in mouse colon cancer cells. We finally demonstrate that 6-PA-ELL induces apoptotic signaling also in enucleated cells, consistent with the existence of a cytoplasmic target for this compound. Our data suggest that induction of endoplasmic reticulum stress may contribute to the cytotoxicity of ellipticines. [Mol Cancer Ther. 2004;3(4):489–497]

Introduction

Some anticancer drugs in clinical use and various investigational agents have been reported to affect more than one cellular target. Etoposide induces DNA damage at low concentrations, but has direct effects on mitochondria at higher concentrations (1). Taxol directly affects mitochondrial function and interferes with the Ca^{2+} signal cascade (2) and cisplatin induces endoplasmic reticulum stress (3). These pleiotropic effects are not surprising, considering that some of these compounds are used in micromolar concentrations.

Nuclear DNA damage and triggering of death receptors are well-established apoptotic stimuli. Cellular organelles such as the endoplasmic reticulum and the lysosomes also possess the capacity to sense stress and to transmit signals to mitochondria, leading to mitochondrial pore formation and apoptosis (reviewed in Ref. 4). Apoptosis via the endoplasmic reticulum may be elicited by accumulation of unfolded proteins induced by inhibitors of protein glycosylation (5, 6). The endoplasmic reticulum senses stress through chaperones such as GRP78 and GRP94, Ca^{2+}-binding proteins, and Ca^{2+} release channels, which might transmit endoplasmic reticulum Ca^{2+} responses to mitochondria (7). The endoplasmic reticulum contains Bcl-2-family proteins, and Bcl-2 exerts part of its cytoprotective effect within the endoplasmic reticulum (8, 9). Lysosomal cathepsins have been implicated in apoptosis induced by tumor necrosis factor (TNF) and other stimuli (10–12). Finally, some anticancer drugs, such as lonidamine, arsenite, and betulinic acid, act directly on mitochondria (13).

Ellipticine [5,11-dimethyl-6H-pyrido(4,3-b)carbazole] is a plant alkaloid with antitumor activity (14). Ellipticine analogues are active against brain tumor cell lines (15–17) and have showed promising results in treatment of metastatic breast cancer (18). The principal mechanism of cytotoxicity is believed to be inhibition of topoisomerase II-β (19). In addition, ellipticine has been reported to bind to proteins (20), to induce the generation of cytotoxic free radicals (21), to inhibit cytochrome P4501A1 (22), and to uncouple oxidative phosphorylation (23, 24). Interestingly, it was recently reported that ellipticine has the ability to activate the transcription function of mutant p53 (25).

The reports of complex biological activities by ellipticines raise the question of whether these compounds induce multifaceted biological responses. We here show that apoptotic activity of ellipticine derivatives correlates to the ability to induce expression of the endoplasmic reticulum chaperone GRP78, and that the potent ellipticine derivative 6-propanamine-ellipticine (6-PA-ELL) induces splicing of XBP1 mRNA. Our findings show that ellipticine derivatives can induce endoplasmic reticulum stress, and suggest that endoplasmic reticulum stress is a contributing factor to the cytotoxic activity of this class of topoisomerase II inhibitors.

Materials and Methods

Materials

Ellipticine compounds were obtained from the Developmental Therapeutics Program at the National Cancer Research Institute and Sigma-Aldrich.

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Institute (http://www.dtp.nci.nih.gov): NSC 176328, 6-PA-ELL; NSC 176327, 9-methoxy-6-PA-ELL; NSC 162907, 6-(5-hexen-1-yl)ellipticine; NSC 98949, 9-methyllumicine; NSC 338258, 9-dimethyl amino-ethoxy-ellipticine; NSC 69187, 9-methoxy-ellipticine; NSC 152731, 6H-pyrido[4,3-b]carbazole, 5,11-dimethyl-6-(oxiranylmethyl)-NSC 360319, β-D-ribofuranoside, (5,6-dimethyl-6H-pyrido[3,4-b]carba- azol-11-yl)ethyl; NSC 335142, 6H-pyrido[4,3-b]carba- azole-1-carboxamide, 5,11-dimethyl-, monohydrochloride. Other reagents: z-VAD-fmk (Enzyme Systems Products, Livermore, CA); E-64d (10 μM, Sigma Aldrich, Sweden); and thapsigargin (Karolinska Hospital Pharmacy, Stockholm, Sweden); carbonyl cyanide m-chlorophenylhydrazone (CCCP; Sigma); Rhodamine 123 (Sigma); cisplatin and doxorubicin (Karolinska Hospital Pharmacy, Stockholm, Sweden); BMH (Pierce, Rockford, IL); ellipticine (Sigma); and thapsigargin (Molecular Probes, Leiden, The Netherlands).

Cell Culture
MDA-MB-231 and MCF-7 human breast carcinoma (generously provided by Dr. Bert Vogelstein), mouse CT51 colon carcinoma cells (26) (kindly provided by Dr. Björn Öbrink), and SV40 immortalized mouse embryo fibroblast (MEF) lines defective in Bak and Bax (kindly provided by Dr. Stanley Korsmeyer) were maintained at 37°C in DMEM supplemented with 10% FCS, 10 mM Tris (pH 7.2), and homogenized on ice. All further steps were conducted at +4°C. Homogenates were centrifuged at 3400 rpm in an Eppendorf centrifuge for 10 min. The supernatant was recentrifuged (13,000 rpm, 30 min) and the pellet was resuspended in 60 μl Buffer B (250 mM sucrose, 10 mM HEPES, 5 mM Na-succinate, 25 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 4 mM MgCl₂). The sample was divided: 40 μl were treated with BMH cross-linker (10 mM, Pierce) and 20 μl as a control with DMSO for 30 min at room temperature as described (29). Samples were analyzed by SDS-PAGE and Western blotting.

Isolation of Mitochondria and Cross-Linking of Bak
Cells were resuspended in 200 μl Buffer A [250 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 5 mM HEPES (pH 7.2), 0.1 mM phenylmethylsulfonyl fluoride] and lysed in a glass homogenizer on ice. All further steps were conducted at +4°C. Homogenates were centrifuged at 3400 rpm in an Eppendorf centrifuge for 10 min. The supernatant was recentrifuged (13,000 rpm, 30 min) and the pellet was resuspended in 60 μl Buffer B (250 mM sucrose, 10 mM HEPES, 5 mM Na-succinate, 25 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 4 mM MgCl₂). The sample was divided: 40 μl were treated with BMH cross-linker (10 μM, Pierce) and 20 μl as a control with DMSO for 30 min at room temperature as described (29). Samples were analyzed by SDS-PAGE and Western blotting.

Isolation of Mitochondria and Measurements of ΔΨ
Cells were resuspended in 500 μl 250 mM sucrose in 10 mM Tris (pH 7.2), and homogenized on ice. All further steps were conducted at +4°C. After centrifugation at 600 × g for 5 min, the supernatant was collected and centrifuged at 10,000 rpm for 10 min. The pellet was suspended in 200 μl 250 mM sucrose, 10 mM Tris (pH 7.2). To assess changes in mitochondrial membrane potential, Rhodamine 123 was added to 5 μM. Changes in fluorescence induced by 6-PA-ELL (5 μM), CCCP (100 μM), or buffer only were monitored by flow cytometry.

Western Blot Analysis
Cell extract proteins were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane for Western blotting. The following antibodies were used: anti-Bak (1:1000; Upstate Technologies, Lake Placid, NY); antimouse pro-caspase-12 (1:40; kindly provided by Dr. J. Yuan); anti-GRP78 (1:1000; BD Biosciences Pharmingen, San Diego, CA); anti-GRP94 (1:1000; Calbiochem, San Diego, CA); and antitubulin (1:1000; Sigma Aldrich). Tubulin was used as an internal standard for loading.

Flow Cytometric Analysis
Activating conformational changes leading to exposure of otherwise inaccessible NH2-terminal epitopes of Bak and Bax were detected by flow cytometry as previously described (30). One antibody recognizing amino acids 1–52 of Bak (Oncogene Research Products, Boston, MA) and one recognizing amino acids 12–24 of Bax (PharMingen) were used. CK18 cleavage was assessed using the monoclonal antibody M30 (31). Cells or cytoplasts were fixed with 0.25% paraformaldehyde for 5 min, washed twice in PBS, and incubated for 30 min with primary antibody in the presence of digitonin for permeabilization, followed by incubation with the secondary FITC-conjugat- ted antibody (DAKO, Glostrup, Denmark) for 1 h in the absence of digitonin (32, 33). Analysis of GRP78 levels was similarly performed using an antibody from BD Biosciences (used at a 1:50 dilution).
Cytochrome c Release

Cells were incubated for 30 min in medium containing 10 mM Mitotracker Red CMXRos (Molecular Probes) and cytometer centrifuged onto glass slides. Cells were fixed in 3% paraformaldehyde for 20 min and permeabilized using 0.2% Triton X-100 in PBS for 10 min. Fixed cells were incubated with anti-cytochrome c antibody (1:300; 65918A; PharMingen) for 1 h, followed by incubation with a FITC-conjugated anti-mouse antibody (DAKO). Slides were mounted in Vectashield containing DAPI and images recorded in a Zeiss Axioplan 2 fluorescence microscope.

RT-PCR

Total RNA was isolated from cells by the guanidine-HCl method. RNA was reverse transcribed and cDNA amplified using a GeneAmp kit from PE Biosystems (San Francisco, CA). XBP1 primers have been described by Yoshida et al. (34) (nucleotides 412–431; 834–853). XBP1 amplification products were separated by electrophoresis in 2% agarose gels.

Reporter Assays

MDA-MB-231 cells were transfected by the calcium phosphate co-precipitation technique. The GRP78-LUC and GRP78mut-LUC reporters were kindly provided by Dr. Kazutoshi Mori (35). We used 1 μg of pGL3-GRP78(−132)-LUC (GRP78-LUC) or GRP78mut-LUC, and 0.1 μg pRL-SV40 plasmid (expressing the Renilla luciferase, Promega, Madison, WI) for each transfection. In the GRP78mut-LUC reporter, the ERSE sequences are mutated (35). At 24 h after transfection, agents were added and cells were harvested at the indicated time points.

Results

Induction of Apoptosis by Ellipticines in Human MDA-MB-231 Breast Cancer Cells

An assay measuring total accumulation of caspase-cleaved CK18 fragments (CK18-Asp396) in cells and culture media (28) was used to examine ellipticine-induced apoptosis in MDA-MB-231 breast carcinoma cells. Of 10 ellipticines tested, 6-PA-ELL (NSC176328; 5 μM) and 9-methoxy-6-PA-ELL (NSC176327; 2 μM) elicited the strongest responses (Fig. 1A). Compounds NSC162907, NSC360319, and 6-PA-ELL activation were three additional compounds showed intermediate activity.

Involvement of Bak in 6-PA-ELL-Induced Apoptosis

Conclusion: 6-PA-ELL induces rapid apoptosis in MDA-MB-231 cells.

Cytotoxic Activity of 6-PA-ELL on Human and Mouse Carcinoma Cell Lines

MDA-MB-231 is a highly malignant and metastatic breast cancer cell line (36). The non-metastatic breast cancer cell line MCF-7 was less sensitive to 6-PA-ELL (Table 1). HCT116 human colon cancer cells (p53wt) were approximately 2-fold more sensitive than a HCT116 derivative with a disrupted p53 locus (27) (Table 1). Mouse CT51 colon cancer cells showed a similar level of sensitivity to 6-PA-ELL as the human cancer cell lines.

Induction of CK18-Asp396 cleavage by 6-PA-ELL was observed at 4.5 h of treatment with 5 μM 6-PA-ELL (Fig. 1D). Release of cytochrome c was induced by 6-PA-ELL. Cytochrome c staining was localized to mitochondria in untreated cells [Fig. 2A; green staining overlapped with Mitotracker Red staining (not shown)]. In contrast, after 4.5 h treatment, the majority of the cells showed a diffuse cytoplasmic staining indicative of released cytochrome c from mitochondria (Fig. 2B). We conclude that 6-PA-ELL induces rapid apoptosis in MDA-MB-231 cells.

During apoptosis, Bak and Bax undergo conformational changes leading to exposure of otherwise inaccessible NH2-terminal epitopes (32, 33, 38), believed to represent activation of these proteins. Using a monoclonal antibody recognizing the NH2 terminus of Bak, we observed an increase in Bak modulation already after 2–3 h of 6-PA-ELL treatment of MDA-MB-231 cells (Fig. 3, B and C). Bak conformation can also be studied using the cysteine-reactive molecular cross-linker BMH (29). Bak has a molecular weight of 25,000. In viable cells, Bak can be intramolecularly cross-linked by BMH to a faster migrating species (M, 21,000) believed to represent an inactive form (29). The M, 21,000 band was reduced in cells exposed to 6-PA-ELL for 5 h, and was undetectable after 7.5 h (Fig. 3D).

As shown in Fig. 3E, modulation of Bax was observed at 18 h of 6-PA-ELL treatment, but not after 5 h. Because CK18 cleavage could be detected at 4 h in the majority of the cells (Fig. 3F), we conclude that Bak, but not Bax, is modulated before the onset of the execution phase of 6-PA-ELL-induced apoptosis, in accordance with a greater dependence on Bak than on Bax.
6-PA-ELL Does Not Act Directly on Mitochondria to Induce Loss of $\Delta \psi$

A number of experimental anticancer drugs induce apoptosis by acting directly on mitochondrial membranes (13); uncoupling agents such as the protonophore CCCP induce apoptosis (39). Ellipticines accumulate in mitochondria (23) and uncouple oxidative phosphorylation (24). We tested whether 6-PA-ELL has a direct effect on the mitochondrial transmembrane potential in MDA-MB-231 cells by incubating isolated mitochondria with the cationic lipophilic dye Rhodamine 123. A rapid increase in Rhodamine 123 fluorescence, indicating decreased quenching of the dye due to release from the mitochondrial matrix, was observed after addition of CCCP. By contrast, a slow increase in fluorescence, similar to that observed in the control, was observed after addition of $\text{5 M} \ 6$-PA-ELL (Fig. 4A).

Some apoptotic stimuli depend on the activity of lysosomal enzymes such as cathepsin B, and apoptosis may be blocked by inhibitors of lysosomal enzymes (11). We examined the effect of the broad-spectrum inhibitor E-64d, and did not observe any inhibition of apoptosis induction (Fig. 4B).

6-PA-ELL Induces Endoplasmic Reticulum Chaperone Proteins and Splicing of XBP1 mRNA

Endoplasmic reticulum stress induces expression of the endoplasmic reticulum-specific chaperones GRP78 and GRP94 (7). Induction of GRP78 and GRP94 expression was observed in 6-PA-ELL-treated MDA-MB-231 cells using Western blotting (Fig. 5A). Thapsigargin, an inhibitor of the endoplasmic reticulum Ca$^{2+}$-ATPase, also induced GRP78 and GRP94. Induction of GRP78 was observed using flow cytometry (Fig. 5B) and in HCT116 cells and 224 melanoma cells (not shown). In addition to thapsigargin, DTT induced GRP78 in MDA-MB-231 cells. Importantly, both of the

Figure 2. Cytochrome $c$ is released from mitochondria in 6-PA-ELL-treated cells. MDA-MB-231 cells were treated with 5 \( \mu \text{M} \) 6-PA-ELL, fixed and stained for cytochrome $c$. A, untreated cells. B, cells treated with 6-PA-ELL for 4.5 h. Note mitochondrial localization of cytochrome $c$ in untreated cells. After 4.5 h of treatment, cells showed cytoplasmic localization of cytochrome $c$. 

Figure 1. Induction of apoptosis by ellipticine analogues. A, MDA-MB-231 cells were treated with different ellipticines and the levels of the apoptosis-specific CK18-Asp396 epitope in media and cell extracts were assessed by ELISA after 18 h (for compounds/NSC numbers, see Materials and Methods). All compounds were used at 5 \( \mu \text{M} \) except NSC176327 which was used at 2 \( \mu \text{M} \) (rapid membrane blebbing and high side scattering in flow cytometry were observed at higher concentrations, suggesting general toxicity). B, MDA-MB-231 cells were treated with 5 \( \mu \text{M} \) 6-PA-ELL or 5 \( \mu \text{M} \) 6-PA-ELL + 50 \( \mu \text{M} \) z-VAD-fmk for 18 h and the levels of the CK18-Asp396 epitope were determined by ELISA. C, caspase-specific cleavage of CK18-Asp396 was measured at the indicated time points. MDA-MB-231 cells were treated with 2 \( \mu \text{M} \) 6-PA-ELL (open circles), 5 \( \mu \text{M} \) 6-PA-ELL (filled circles), 1 \( \mu \text{g} / \text{ml} \) doxorubicin (open squares), or 2 \( \mu \text{g} / \text{ml} \) doxorubicin (filled squares). D, nuclear morphology of untreated MDA-MB-231 cells (left) and 6-PA-ELL-treated cells which show nuclear fragmentation typical of apoptosis (right). Cells were treated for 4.5 h with 5 \( \mu \text{M} \) 6-PA-ELL, fixed and stained with ethidium bromide.
pro-apoptotic ellipticines [NCS176328 (6-PA-ELL) and NCS176327 (9-methoxy-6-PA-ELL)] induced expression of GRP78, whereas GRP78 was not induced by three ellipticines that did not induce apoptosis (Fig. 5B). Induction of GRP78 was observed at time points preceding apoptosis; a 2-fold increase was observed within 3 h (Fig. 5C).

During endoplasmic reticulum stress, the mRNA encoding the transcription factor XBP1 is spliced by the endoribonuclease IRE1 (34, 40). Splicing induces a frame shift and translation of a 376-amino acid form of XBP1 with enhanced activity as transcriptional activator (34) (Fig. 5D). 6-PA-ELL and thapsigargin were found to induce splicing of XBP1 mRNA in HCT116 cells (Fig. 5D).

**6-PA-ELL Induction of GRP78 Depends on the Endoplasmic Reticulum Stress Response Element**

The GRP78 promoter contains a cis-acting endoplasmic reticulum stress response element (ERSE). To determine whether induction of GRP78 expression by 6-PA-ELL is dependent on the ERSE, MDA-MB-231 cells were transfected with GRP78-promoter reporter constructs. As shown in Fig. 5E, 6-PA-ELL induced increased activity of a GRP78-LUC reporter (filled bars), but did not induce expression of a GRP78mut-LUC reporter with deletions of the ERSEs (GRP78mut-LUC; open bars). These results show that 6-PA-ELL-induced GRP78 expression is mediated by the ERSE.

**Involvement of Caspase-12**

In murine cells, endoplasmic reticulum stress results in the activation of the endoplasmic reticulum-resident pro-caspase-12 (41). 6-PA-ELL was cytotoxic to normal mouse fibroblasts (Fig. 3A) and to mouse colon cancer CT51 cells.

### Table 1. Effect of 6-PA-ELL (NSC176328) on human and mouse tumor cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Type</th>
<th>LD₉₀ (μM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>Breast cancer (p53mut; ER−b)</td>
<td>0.06</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast cancer (p53wt; ER+b)</td>
<td>0.09</td>
</tr>
<tr>
<td>HCT116 p53+/+</td>
<td>Colon cancer</td>
<td>0.06</td>
</tr>
<tr>
<td>HCT116 p53−/−</td>
<td>Colon cancer</td>
<td>0.11</td>
</tr>
<tr>
<td>CT51</td>
<td>Mouse colon cancer</td>
<td>0.15</td>
</tr>
</tbody>
</table>

*Dose required to elicit a 90% reduction in colony formation ability. Cells were treated with 6-propanamine-ellipticine for 4 h, and clonogenic outgrowth was determined.

bER, estrogen receptor.

Figure 3. 6-PA-ELL-induced apoptosis involves Bak. A, mouse embryo fibroblast (MEF) cell lines were treated with different doses of doxorubicin (DOX), 6-PA-ELL, or cisplatin (CISPL). The viability of Bak−/− and Bak+/− MEF at doses of each compound, which produced a 90% loss of viability of a wild-type MEF cell line, are shown. The numbers of viable cells were determined 24 h after treatment, and data are based on three independent experiments. Note that Bak−/− MEF cells show a viability of 66 ± 2.6% at a dose of 6-PA-ELL which generates a viability of 10% on wild-type MEF cells (i.e., an LD₉₀ dose). B–D, conformational modulation of Bak. B, MDA-MB-231 cells were treated with 5 μM 6-PA-ELL for 3 h. Cells were fixed, permeabilized, and incubated with an antibody recognizing an epitope in the unstructured NH₂-terminal loop of Bak followed by analysis by flow cytometry (30). C, conformational modulation of Bak at different times of treatment with 5 μM 6-PA-ELL. D, MDA-MB-231 cells were treated with 5 μM 6-PA-ELL for the indicated times. Mitochondria were isolated and incubated with the cross-linker BMH (upper panel). Proteins were solubilized and subjected to Western blot analysis for Bak. E, conformational modulation of Bax in 6-PA-ELL-treated MDA-MB-231 cells. Cells were fixed after 5 and 18 h of treatment, incubated with an antibody against the NH₂ terminus of Bax and analyzed by flow cytometry (untreated cells shown). F, caspase-mediated cleavage of CK18 in MDA-MB-231 after 4 h of 6-PA-ELL treatment. The intracellular levels of the CK18-Asp396 epitope were examined by staining with the epitope-specific M30 antibody and analysis by flow cytometry. Note that the CK18-Asp396 epitope is exposed in most cells at 4 h, when no conformational activation of Bax can be detected.
Table 1). 6-PA-ELL and 9-methoxy-6-PA-ELL induced cleavage of caspase-12 in CT51 cells (Fig. 6A). A Mr 60,000 protein is detected by caspase-12 antibodies in human cells, including MDA-MB-231 (42, 43) (Fig. 6B). 6-PA-ELL treatment reduced the intensity of this band. Caspase-12 is cleaved by calpain during endoplasmic reticulum stress (44), and the calpain inhibitor calpeptin inhibited 6-PA-ELL-induced caspase cleavage (Fig. 6B). Interestingly, 6-PA-ELL-induced apoptosis was inhibited by two different calpain inhibitors (Fig. 6C), suggesting a role of calpain in the apoptosis process.

Modulation of Bak and Caspase Cleavage Does Not Depend on the Cell Nucleus

An endoplasmic reticulum stress-mediated cytotoxic insult would occur in parallel with DNA damage. To determine whether 6-PA-ELL induces apoptotic signaling independent of damage to nuclear DNA, MDA-MB-231 cells were enucleated by isopyknic density centrifugation in the presence of cytochalasin B. Fractions containing nucleus-free cytoplasts and some contaminating intact cells were treated with 6-PA-ELL for 4 h. Cell preparations were stained with propidium iodide to enable the identification of enucleated cells by electronic gating during

Figure 4. Examination of potential mitochondrial and lysosomal targets. A, 6-PA-ELL does not induce loss of mitochondrial 

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mitochondrial membrane potential. Mitochondria were isolated from MDA-MB-231 cells. The cationic lipophilic dye Rhodamine 123 was added and the fluorescence measured (Ii). Compounds or buffer were added at (II), and increases in fluorescence (due to decreased quenching of Rhodamine 123 after release from mitochondria) were measured. Open squares, CCCP; open circles, 6-PA-ELL; closed circles, buffer. Note that the effect of 5 μM 6-PA-ELL does not differ from the control, whereas CCCP induces rapid release of Rhodamine 123, characteristic of depolarization. B, a broad-spectrum lysosomal protease inhibitor does not inhibit 6-PA-ELL-induced apoptosis. MDA-MB-231 cells were incubated with 5 μM 6-PA-ELL in the presence or absence of 30 nM E-64d. The levels of caspase-cleaved CK18-Asp396 were examined by ELISA at 18 h.

(0)

0 2 4 6

0 1 2 3

FOLD INDUCTION OF 6-PA-ELL

Figure 5. Induction of ER stress by pro-apoptotic ellipticines. A, 6-PA-ELL treatment induces expression of the ER chaperones GRP78 and GRP94. MDA-MB-231 cells were incubated with 5 μM 6-PA-ELL for the times indicated or with 300 nM thapsigargin for 12 h. Total cell extracts were analyzed by Western blotting. B, MDA-MB-231 cells were incubated with the indicated ellipticines for 5 h (at 5 μM; NSC176327 at 2 μM), 300 nM thapsigargin for 5 h, or 5 mM DTT for 5 h. The levels of GRP78 were determined by flow cytometry. The levels of GRP78 were determined by flow cytometry (see Materials and Methods for NSC numbers/compounds). C, time course of induction of GRP78 by 6-PA-ELL, measured by flow cytometry. D, induction of GRP78 by 6-PA-ELL for 8 h or thapsigargin for 16 h. The 416-bp form appearing in treated cells represents the spliced form of the mRNA. PCR primers were identical to those described in Ref. (34). E, induction of the GRP78 promoter by 6-PA-ELL. MDA-MB-231 cells were transfected with a GRP78-LUC reporter (closed bars), or a mutant reporter where the ERSE elements in the GRP78 promoter are deleted (open bars) (35). A control plasmid expressing the Renilla luciferase was cotransfected as an internal standard. Agents were used at the same concentrations and times as in B. Results are from four independent experiments. The difference between 6-PA-ELL-treated cells transfected with GRP78-LUC and GRP78mut-LUC was statistically significant (P = 0.042).
Many anticancer drugs are chemically reactive and used in cytotoxic compounds is not uncommon. It is conceivable that induction of endoplasmic reticulum stress may be of importance to the cytotoxic activity of ellipticines. However, it is possible that endoplasmic reticulum active compounds are highly cell permeable, or that that these compounds share activities required for both topoisomerase II inhibition and endoplasmic reticulum stress. Increased expression of the endoplasmic reticulum chaperone GRP78 was detected within a few hours in 6-PA-ELL-treated cells, before release of cytochrome c from mitochondria and caspase cleavage of CK18. GRP78 then accumulated in the cells. Endoplasmic reticulum stress is therefore likely to be an early event, preceding apoptosis. 6-PA-ELL induction of pro-caspase-12 cleavage is consistent with the hypothesis that apoptotic signaling is triggered by endoplasmic reticulum stress. These findings suggest that endoplasmic reticulum stress contributes to the cytotoxicity of 6-PA-ELL, but the relative importance of endoplasmic reticulum stress induction is uncertain. We were able to demonstrate, however, that 6-PA-ELL induced Bak activation and CK18 caspase cleavage in enucleated cells, directly demonstrating a non-nuclear apoptotic sensor. The cytoplasmic target may be important for the anticancer activity of the compound, and/or may be involved in producing toxic side effects in normal tissues.

Discussion

Many anticancer drugs are chemically reactive and used in micromolar concentrations. Not surprisingly, in addition to their postulated main mechanisms of action, various side activities have been attributed to such drugs. It is as yet unclear to which extent such additional effects contribute to the cytotoxicity. Etoposide and paclitaxel have direct effects on mitochondria (1, 2), and cisplatin has been shown to induce endoplasmic reticulum stress (3). We have here studied the ellipticines, a class of plant alkaloids known to inhibit topoisomerase II (19), but also reported to activate the transcription function of mutant p53 (25) and to directly affect mitochondria (24). We demonstrate that the ellipticine derivative 6-PA-ELL induces endoplasmic reticulum stress, a previously not described activity of this class of compounds.

Known endoplasmic reticulum stresses include calcium store depletion, inhibition of glycosylation, reduction of disulfide bonds, and overexpression of mutant proteins (45–47). Ellipticine interacts with proteins (20, 48), and bind to and prevent folding of proteins in the endoplasmic reticulum. We have previously observed that cisplatin induces GRP78 expression and caspase-12 cleavage in human melanoma cells (3), and have identified other cytotoxic drugs in the NCI mechanistic set which induce cleavage of mouse caspase-12 in concentrations of 5 μM, suggesting that induction of endoplasmic reticulum stress by cytotoxic compounds is not uncommon. It is conceivable that the protein folding compartment of the endoplasmic reticulum may be quite sensitive to disturbances, or that the Ca²⁺ stores of the endoplasmic reticulum may be released by some of these drugs.

The association between the ability of different ellipticines to induce endoplasmic reticulum stress and apoptosis suggests that endoplasmic reticulum stress may be of importance for the cytotoxic activity of ellipticines. However, it is possible that endoplasmic reticulum active compounds are highly cell permeable, or that these compounds share activities required for both topoisomerase II inhibition and endoplasmic reticulum stress. Increased expression of the endoplasmic reticulum chaperone GRP78 was detected within a few hours in 6-PA-ELL-treated cells, before release of cytochrome c from mitochondria and caspase cleavage of CK18. GRP78 then accumulated in the cells. Endoplasmic reticulum stress is therefore likely to be an early event, preceding apoptosis. 6-PA-ELL induction of pro-caspase-12 cleavage is consistent with the hypothesis that apoptotic signaling is triggered by endoplasmic reticulum stress. These findings suggest that endoplasmic reticulum stress contributes to the cytotoxicity of 6-PA-ELL, but the relative importance of endoplasmic reticulum stress induction is uncertain. We were able to demonstrate, however, that 6-PA-ELL induced Bak activation and CK18 caspase cleavage in enucleated cells, directly demonstrating a non-nuclear apoptotic sensor. The cytoplasmic target may be important for the anticancer activity of the compound, and/or may be involved in producing toxic side effects in normal tissues.

The endoplasmic reticulum chaperones GRP78 and GRP94 are generally regarded as reliable markers for endoplasmic reticulum stress (7, 49, 50). The transcription factor ATF6 is an endoplasmic reticulum transmembrane protein which binds GRP78 in unstressed cells, but translocates and is cleaved in the Golgi following endoplasmic reticulum stress induction (51). ATF6 then binds the ERES of the GRP78 and GRP94 promoters. Studies of induction of

[Unpublished observations.]
the GRP78 promoter by 6-PA-ELL in MDA-MB-231 showed that induction was dependent on the ERSE, providing direct evidence for an 6-PA-ELL-induced endoplasmic reticulum stress response. Increased levels of endoplasmic reticulum chaperones have been associated with resistance to chemotherapeutic agents (52, 53). It was recently reported that cell lines made to overexpress GRP78 show resistance to both topoisomerase I and II inhibitors (54), an effect attributed to binding of GRP78 to caspase-7 (54). These findings suggest that the endoplasmic reticulum is indeed involved in the response to chemotherapeutic agents.

In summary, we report induction of endoplasmic reticulum stress as a novel property of a derivative of the alkaloid ellipticine, 6-PA-ELL. The ellipticine induced rapid apoptosis in MDA-MB-231 breast cancer cells with a strong dependence on Bak. Together with our previous finding of the GRP78 promoter by 6-PA-ELL in MDA-MB-231 or 224 cells were enucleated as described in Materials and Methods, and fractions containing nucleus-free cytoplasts and contaminating intact cells were treated with 5 μM 6-PA-ELL for 4 h. Cells/cytoplasts were stained with propidium iodide to facilitate the identification and electronic gating of enucleated cells. A, quantification of the CK18-Asp396 epitope by flow cytometry. B, assessment of Bak modulation in enucleated MDA-MB-231 cells using an antibody recognizing the NH₂ terminus of Bak, as described in Fig. 3B.

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