Endoplasmic Reticulum Stress in the Proapoptotic Action of Edelfosine in Solid Tumor Cells

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Abstract

The endoplasmic reticulum (ER) has been posited as a potential anticancer target. The synthetic antitumor alkyl-lysophospholipid analogue edelfosine accumulates in the ER of solid tumor cells. This ER accumulation of the drug leads to the inhibition of phosphatidylcholine and protein synthesis, G2-M arrest, depletion of ER-stored Ca2+, Bax up-regulation and activation, transcriptional factor growth arrest and DNA damage–inducible gene 153 up-regulation, caspase-4 and caspase-8 activation, and eventually to apoptosis. Edelfosine prompted ER stress apoptotic signaling, but not the survival unfolded protein response. Edelfosine also induced persistent c-Jun NH2-terminal kinase (JNK) activation. Gene transfer–mediated overexpression of apoptosis signal–regulating kinase 1, which plays a crucial role in ER stress, enhanced edelfosine-induced JNK activation and apoptosis. Inhibition of JNK, caspase-4, or caspase-8 activation diminished edelfosine-induced apoptosis. Edelfosine treatment led to the generation of the p20 caspase-8 cleavage fragment of BAP31, directing proapoptotic signals between the ER and the mitochondria. bak−/−/bak−/− double-knockout cells fail to undergo edelfosine-induced ER-stored Ca2+ release and apoptosis. Wild-type and bak−/−/bak−/− cells showed similar patterns of phosphatidylcholine and protein synthesis inhibition, despite their differences in drug sensitivity. Thus, edelfosine-induced apoptosis is dependent on Bak/Bak-mediated ER-stored Ca2+ release, but phosphatidylcholine and protein synthesis inhibition is not critical. Transfection-enforced expression of Bcl-XL, which localizes specifically in mitochondria, prevented apoptosis without inhibiting ER-stored Ca2+ release. These data reveal that edelfosine induces an ER stress response in solid tumor cells, providing novel insights into the edelfosine-mediated proapoptotic action. Our data also indicate that mitochondria are indispensable for this edelfosine-induced cell death initiated by ER stress.

Introduction

Synthetic alkyl-lysophospholipid analogues (ALP), from which 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphocholine (ET-18-OCH3, edelfosine) is their prototypic compound, represent a promising class of oral anticancer drugs that do not target the DNA (1–3). Recent in vitro and in vivo data (4, 5) have prompted renewed interest in the study of their antitumor properties and underlying mechanisms. The antitumor action of edelfosine relies on its ability to induce apoptosis in a wide spectrum of tumor cells (1, 6), involving c-Jun NH2-terminal kinase (JNK) persistent activation (7) and mitochondria (8). Edelfosine induces a rapid apoptotic response in leukemic cells through cooclustering of Fas/CD95 death receptor and lipid rafts, independently of Fas/CD95 ligand (5, 9, 10). Edelfosine targets mainly lipid rafts in hematologic malignancies and the endoplasmic reticulum (ER) in solid tumor cells (10, 11), hence showing different targets in distinct tumor cell types.

ER has been recently shown to modulate cell death. When the functions of the ER are severely impaired, the organism protects itself by getting rid of the damaged cells through apoptosis. ER is composed of a system of connected membranous tubules and vesicles along which protein and phospholipid synthesis occur and intracellular calcium levels are regulated. Thus, disruption in Ca2+ homeostasis, inhibition of protein glycosylation, accumulation of misfolded proteins, and inhibition of phosphatidylcholine could all challenge the function of the ER-Golgi network resulting in ER stress, and excessive ER stress leads to apoptosis (12, 13). The ER stress response includes: induction of the transcription factor CCAAT/enhancer binding protein–homologous protein (CHOP), a.k.a. growth arrest and DNA damage–inducible gene 153 (GADD153), activation of JNK through the apoptosis signal–regulating kinase 1 (ASK1), and activation of the ER-localized caspase-12 in murine systems or caspase-4 in human cells (12–15).

Some anticancer chemotherapeutic agents have been shown to induce ER stress, including the proteasome inhibitor bortezomib (16), the Hsp90 inhibitors, geldanamycin and 17-allyl-amidogeldanamycin (17), cisplatin (18), and cannabinoids (19). However, these agents target ER-mediated processes rather than the ER itself, leading indirectly to ER stress. We have recently found that edelfosine accumulates predominantly at the ER in a number of solid tumor cells (11). Now, we report here that the accumulation of edelfosine at the ER of solid tumor cells alters ER functions, leading to ER stress and eventually to apoptosis.

Materials and Methods

Reagents. Edelfosine (INKEYSA) was prepared as a 1 mmol/L stock solution as previously described (1). Caspase-4 inhibitor z-LEVD-fmk was synthesized by Calbiochem.
from Alexis Biochemicals. Caspase-8 inhibitor z-IETD-fmk and JNK inhibitor SP600125 were from Calbiochem. Fura-2 acetoxyethyl ester (fura-2/AM) was from Molecular Probes. Cycloheximide, tunicamycin, and histamine were from Sigma.

Cell culture and bcl-xL transfection. Human cells were grown in DMEM (HeLa) or RPMI 1640 (A549) supplemented with 10% heat-inactivated FCS, 2 mmol/L of L-glutamine, 100 units/ml penicillin, and 100 µg/ml of streptomycin at 37°C in 5% CO2 humidified air. HeLa cells (1–2 × 10⁷) were transfected with 2 µg of pSFFV-bcl-xL or pSFFV-Neo expression vector (1), using Lipofectin reagent (Life Technologies). Transfected clones were selected by growth in the presence of 500 µg/ml of G418 (Sigma) and monitored by Western blotting using the 21-anti 29 kDa Bcl-xL monoclonal antibody (BD Biosciences Pharmingen).

Wild-type and Δbax/bak−/− double-knockout (DKO) SV40-transformed mouse embryo fibroblasts (MEFs), as well as single knockout Δbax/− or Δbak/−/− MEFs, kindly provided by S.J. Korsmeyer (Dana-Farber Cancer Institute, Boston, MA; ref. 20), were cultured in DMEM supplemented with 10% FCS, 2 mmol/L of L-glutamine, 0.1 mmol/L of nonessential amino acids, 50 µmol/L of β-mercaptoethanol, 100 units/ml of penicillin, and 100 µg/ml of streptomycin.

ASK1 transfection. HeLa cells (5 × 10⁴) were seeded and transfected with 6 µg of ASK1 wild-type (pcDNA3.1-ASK1), kindly provided by H. Ichijo (University of Tokyo, Japan), or with empty pcDNA3.1 vector (Invitrogen), using the cationic polymer transfection reagent jetPEI (Qiogene). Transfected clones were selected by growth in the presence of 500 µg/ml of G418 and monitored by Western blotting using an anti–155 kDa ASK1 rabbit polyclonal antibody (Cell Signaling Technology). Signal was developed using an enhanced chemiluminescence detection kit (Amersham).

Flow cytometry detection of intracellular proteins. Cells were fixed and permeabilized by means of the Fix & Perm cell permeabilization kit (CALTAG Laboratories) following the manufacturer's instructions. Cells were then incubated with monoclonal antibodies against the active forms of Bax (clone 6A7; BD Biosciences Pharmingen) and Bak (clone Ab-1; Calbiochem) at 1:50 dilution in PBS for 15 min at room temperature, washed with PBS, stained with 15 min with Cy2-conjugated antimouse IgG at 1:50 dilution (Jackson ImmunoResearch Laboratories, Inc.), and analyzed in a Becton Dickinson fluorescence-activated cell sorting (FACS)Calibur flow cytometer. Data are shown as the mean fluorescence intensity, which was corrected for unspecified staining by subtracting the fluorescence of cells stained with negative controls. P3X63 myeloma cell supernatant, kindly provided by F. Sánchez-Madrid (Hospital de La Princesa, Madrid, Spain), and an isotype-matched nonrelevant IgG monoclonal antibody were used as negative controls, leading to virtually identical background values.

Single cell calcium measurement. Cells were loaded with the cell-permeable fura-2/AM by incubation in standard medium (145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, 10 mmol/L Hepes; pH 7.4) for 10 min at room temperature. Cells were then washed with standard medium for 10 min at room temperature and mounted in a cell chamber on the stage of a Zeiss Axiovert 200 microscope under continuous perfusion. Single cell fluorescence was excited at 340 and 380 nm using a Cairn monochromator (100 ms excitation at each wavelength every 2 s) and images of the fluorescence emitted above 500 nm obtained with a 40× Fluor objective were recorded by a Hamamatsu ORCA-ER camera (Hamamatsu, Japan). Single cell fluorescence records were rationed and calibrated into cytoplasmic free Ca²⁺ concentration values off-line as described previously (23) using Metallor program (Universal Imaging). Experiments were done at 37°C using an on-line heater from Harvar Apparatus. Cells were perfused with standard medium containing 1 mmol/L of CaCl₂ or with Ca²⁺-lacking medium (0 mmol/L of Ca²⁺) that contained 0.5 mmol/L of EGTA in substitution of Ca²⁺. Edelfosine and histamine were added to the perfusion medium at the indicated final concentrations from stock solutions. Aqueous stock solutions of 10 and 1 mmol/L edelfosine were used. Different solutions were changed by means of a multichannel perfusion system.

Results

Edelfosine inhibits phosphatidylcholine and protein synthesis. We have recently found that the edelfosine fluorescent analogue PTE-edelfosine, which has been previously shown to preserve the apoptotic features of the parental edelfosine in a number of different cell types, is located in the ER of distinct cancer cell lines derived from human malignant solid tumors,
Figure 1. Effects of edelfosine on cell cycle, apoptosis, and ER homeostasis. A, cells were treated with 10 μmol/L of edelfosine for the indicated times and the proportion of cells in each phase of cell cycle was quantitated by flow cytometry. The sub-G₁ region represents apoptotic cells. Untreated control cells were run in parallel. Columns, means; bars, SE (n = 4). B, left, [¹⁴C]choline-labeled cells were grown in the absence (C) or presence of 10 μmol/L of edelfosine (EDLF) for the indicated times. Image shows phosphatidylcholine corresponding TLC spots visualized by phosphorimaging and is representative of three independent experiments. Right, percentage of [¹⁴C]choline (Cho) incorporation into phosphatidylcholine (PtdCho) in HeLa cells treated with 10 μmol/L of edelfosine for 6 h (EDLF) with respect to untreated control HeLa cells (Cont; 100%). Columns, means; bars, SE (n = 3). C, left, HeLa and A549 cells were preincubated for 30 min in the absence (Cont) or presence of edelfosine (EDLF) or cycloheximide (CHX), and then [³⁵S]methionine was added to the culture medium for the indicated times. Percentages of newly synthesized labeled proteins in drug-treated cells with respect to untreated samples (100%). Columns, means; bars, SE (n = 3). Right, HeLa cells were incubated in the absence (C) or presence of 10 μmol/L of edelfosine for the indicated times and analyzed for eIF2α phosphorylation by immunoblotting, using a specific anti–phosphorylated eIF2α antibody. Immunoblotting for eIF2α was used as a control for protein loading. Representative of three independent experiments. D, left, cytosolic free calcium concentration was measured in fura-2/AM–loaded HeLa cells. Calcium release from intracellular stores was elicited by 1-min incubation with 100 μmol/L of histamine (His) in 0 Ca²⁺ medium. When indicated (arrow), medium containing 10 μmol/L of edelfosine was perfused for 15 min (middle). Bottom, cells incubated for 60 min in standard medium containing 10 μmol/L of edelfosine. During the experiment, cells were perfused with 0 Ca²⁺ medium and calcium release from intracellular stores was elicited by the addition of 100 μmol/L of histamine. Top, cells in the absence of edelfosine (control). Mean values of >100 cells from four different experiments. Right, plot of increments in calcium release from intracellular stores versus incubation time with 10 μmol/L of edelfosine in HeLa cells. Best fit to first-order exponential decay is represented.
including human cervix epitheloid carcinoma HeLa and human lung carcinoma A549 cells (11). Owing to the accumulation of edelfosine in the ER, we investigated whether an alteration in ER homeostasis could be involved in the antitumor action of this drug.

Edelfosine induced apoptosis in HeLa and A549 cells after a rather long incubation time following a cell cycle arrest in G2-M (Fig. 1A). Because ER is responsible for the synthesis of proteins and lipids, we first analyzed the effect of the drug on both phosphatidylcholine and protein synthesis. Cells were incubated with [14C]choline for 30 min, followed by exhaustive washing, and incubation in the absence or presence of edelfosine for different times, thus avoiding any effects that edelfosine might have on the uptake of choline into the cells (24). We found that edelfosine inhibited [14C]choline incorporation into the phosphatidylcholine pool in both HeLa and A549 cells (Fig. 1B). These results are in agreement with previous studies showing inhibition of phosphatidylcholine biosynthesis by this drug at the phosphocholine cytidylyltransferase (CCT) step (25). Edelfosine also inhibited protein synthesis in both HeLa and A549 cells (Fig. 1C). Cycloheximide, used at a concentration that prevented protein synthesis (Fig. 1C), induced a slight apoptotic response which was increased following its combination with edelfosine.

Figure 2. ER stress in edelfosine-treated HeLa cells. A, top, cells were untreated (C) or treated with 10 μmol/L edelfosine for the indicated times and chop/gadd153 gene expression was assessed by semiquantitative RT-PCR. Cells treated with 25 μmol/L of tunicamycin (Tm) for 3 h were used as a positive control. PCR amplification of β-actin was used as an internal control. Representative of three experiments. Bottom, cells untreated (C) or treated with 10 μmol/L edelfosine for the indicated times were analyzed by Western blot (WB) using specific antibodies for the indicated proteins. B, cells were preincubated without or with 20 μmol/L of z-LEVD-fmk or 100 μmol/L of z-IETD-fmk for 1 h, and then incubated in the absence or presence of 10 μmol/L of edelfosine (EDLF) for 24 h, and analyzed by flow cytometry to evaluate apoptosis (top) or by immunoblotting using specific antibodies (bottom). Untreated control cells (CONT) were run in parallel. C, cells untreated (C) or treated with 10 μmol/L of edelfosine for the indicated times were analyzed by immunoblotting using specific antibodies for the indicated proteins. D, conformational changes in Bax and Bak were determined as described in Materials and Methods in untreated control cells and following treatment with 10 μmol/L of edelfosine for the indicated times. β-Actin was used as a loading control in Western blot analyses. B (top) and D, columns, means; bars, SE (n = 3). Blots are representative of three independent experiments.
Edelfosine-induced apoptosis is mediated by ASK1-JNK activation in HeLa cells. A, cells were pretreated with 20 μmol/L of SP600125 (SP) for 1 h, and then incubated in the absence or presence of 10 μmol/L of edelfosine (EDLF) for 16 h, and analyzed for apoptosis and JNK activation. The migration position of GST-c-Jun-1-223 is indicated. Untreated control cells (C) were run in parallel. Columns, means; bars, SE (n = 3). B, ASK1 protein expression was analyzed by SDS-PAGE of 100 μg of cell extract protein and immunoblotting. β-Actin was used as a control for protein loading. C, cells were untreated (C) or treated with 10 μmol/L of edelfosine for different times and assayed for JNK activation. The migration position of GST-c-Jun-1-223 is indicated. Representative of three experiments. D, cells were treated with 10 μmol/L of edelfosine (EDLF) for 24 h and analyzed for apoptosis. Untreated control cells (Cont) were run in parallel. Columns, means; bars, SE (n = 3).

Edelfosine induces the release of ER-stored Ca2+. Because ER is the main Ca2+ cellular store, we analyzed the effect of edelfosine on ER calcium release. Treatment of HeLa cells with edelfosine for 15 min reduced histamine-induced calcium release from intracellular stores by ~ 45% (integral of calcium wave; Fig. 1D, left). A 60-min pretreatment with edelfosine completely prevented histamine-induced Ca2+ release (Fig. 1D, right), indicating that the ER-Ca2+ stores had been depleted during drug treatment. Short drug treatments (≤15 min) were carried out in the microscope chamber while recording calcium changes (upper and medium records) in Ca2+-free (0 Ca2+) medium containing 0.5 mmol/L of EGTA to prevent any possible effect of edelfosine on calcium entry. Some experiments were done in the presence of 0.2 mmol/L of Mn2+ as a calcium surrogate rendering identical results (data not shown), thus, ruling out calcium entry in these conditions. When we did longer drug treatments (>30 min), cells were incubated in standard medium with 10 μmol/L of edelfosine to avoid Ca2+ store depletion due to protracted incubations in 0 Ca2+ medium. Then, cells were mounted in the microscope chamber to measure cytosolic Ca2+ as above. In these cases, cells were initially perfused with standard medium and then with 0 Ca2+ medium to stimulate the cells with histamine (Fig. 1D, bottom left). We also observed a small increase in the cytosolic calcium baseline with long drug pretreatments, 15 to 60 min (Fig. 1D, left; data not shown), indicating that Ca2+ stores are being emptied. In time course assays, we found that edelfosine caused a gradual calcium discharge from the ER up to its total depletion in HeLa cells. In Fig. 1D (right), we represent the amplitude of the calcium peak with histamine as cytoplasmic-free Ca2+ concentration (nmol/L) versus the time of edelfosine treatment, showing an exponential decay of histamine-induced calcium release when drug incubation times are increased. These data indicate that edelfosine affects Ca2+ homeostasis.

Edelfosine induces ER stress. We next examined the effects of edelfosine on a number of ER stress–associated markers. RT-PCR and immunoblotting analyses showed that edelfosine induced the expression of transcription factor CHOP/GADD153 at both mRNA and protein levels (Fig. 2A). The N-glycosylation inhibitor tunicamycin was used as a positive control for ER stress (27).

Because ER is not only responsible for protein synthesis, but also for initial posttranslational protein modification, folding and export, disturbances in the ER lead to the accumulation of misfolded proteins. Cells trigger a specific survival signaling in response to ER stress, known as the unfolded protein response (UPR; ref. 13), which mainly involves the expression of Grp78/BiP protein levels (Fig. 2A). Thus, these results suggest that edelfosine induces an apoptotic signal without unleashing the typical UPR. In this regard, inhibition of phosphatidylinositol synthesis has been reported to induce CHOP/ GADD153 without any change in Grp78/BiP protein levels (28).

Caspase-12 mediates ER stress–induced apoptosis in murine cells, but human cells do not possess a functional caspase-12 gene (29). Because human caspase-4 is a potential homologue of murine caspase-12, we analyzed whether caspase-4 was involved in edelfosine-induced cell death. Caspase-4 was cleaved to its active form after edelfosine treatment. This effect was dependent on ASK1-JNK activation, because caspase-4 cleavage was not observed in cells pretreated with SP600125 (Fig. 3A).

Figure 3. Edelfosine-induced apoptosis is mediated by ASK1-JNK activation in HeLa cells. A, cells were pretreated with 20 μmol/L of SP600125 (SP) for 1 h, and then incubated in the absence or presence of 10 μmol/L of edelfosine (EDLF) for 16 h, and analyzed for apoptosis and JNK activation. The migration position of GST-c-Jun-1-223 is indicated. Untreated control cells (C) were run in parallel. Columns, means; bars, SE (n = 3). B, ASK1 protein expression was analyzed by SDS-PAGE of 100 μg of cell extract protein and immunoblotting. β-Actin was used as a control for protein loading. C, cells were untreated (C) or treated with 10 μmol/L of edelfosine for different times and assayed for JNK activation. The migration position of GST-c-Jun-1-223 is indicated. Representative of three experiments. D, cells were treated with 10 μmol/L of edelfosine (EDLF) for 24 h and analyzed for apoptosis. Untreated control cells (Cont) were run in parallel. Columns, means; bars, SE (n = 3).
form in HeLa cells following protracted incubation times with edelfosine and after the triggering of apoptosis (see Figs. 1A and 2A). However, caspase-8 and caspase-3 were activated before the onset of the apoptotic response (Fig. 2A). BAP31, an integral membrane protein of the ER that is a caspase-8 substrate (30), was cleaved into a p20 fragment following edelfosine treatment (Fig. 2A). The p20-BAP31 protein has been shown to direct proapoptotic signals between ER and mitochondria (31).

We also found that preincubation of HeLa cells with the caspase-4 inhibitor z-LEVD-fmk and the caspase-8 inhibitor z-IETD-fmk diminished edelfosine-induced apoptosis by 42% and 65%, respectively (Fig. 2B, top), when used at concentrations that

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**Figure 4.** Resistance of DKO cells to edelfosine-induced apoptosis. A, wild-type (WT) and DKO cells were treated for 24 h with edelfosine at the indicated concentrations. The proportion of cells in each phase of the cell cycle were quantitated by flow cytometry, the sub-G1 region representing apoptotic cells. Untreated control cells were run in parallel. Columns, means; bars, SE (n = 3). B, left, [14C]choline-labeled cells were grown in the absence (C) or presence of 20 μmol/L of edelfosine (EDLF) for the indicated times. Image shows phosphatidylcholine-corresponding TLC spots visualized by phosphorimaging, and is representative of three independent experiments. Right, percentage of [14C]choline (Cho) incorporation into phosphatidylcholine (PtdCho) in DKO cells treated with 20 μmol/L of edelfosine for 6 h (EDLF) with respect to untreated control DKO cells (Cont; 100%). Columns, means; bars, SE (n = 3). C, cells were preincubated for 30 min in the absence (Cont) or presence of 20 μmol/L edelfosine (EDLF) or 75 μmol/L of cycloheximide (CHX), and then [35S]methionine was added to the culture medium for the indicated times. Percentage of newly synthesized labeled proteins in drug-treated cells with respect to untreated samples (100%). Columns, means; bars, SE (n = 3). D, cytosolic calcium was measured in fura-2/AM–loaded wild-type (left) or DKO (right) cells. Calcium release from intracellular stores was elicited by 100 μmol/L of histamine in 0 Ca2+ medium in control conditions (top) or in the presence of 1 μmol/L of edelfosine (bottom) after 15-min incubation. Inset, the same record as the bottom left values in an expanded calcium scale.
prevented caspase activation (Fig. 2B, bottom). Caspase-8 inhibition almost completely abrogated the p20-BAP31 fragment generation (Fig. 2B, bottom right). Our data suggest that caspase-4 contributes to the apoptotic response mediated by ER, but it does not participate in the early triggering of apoptosis signaling. ER stress has been shown to induce the activation of Bax and Bak leading to conformational changes that expose previously NH2-terminus–occluded regions in their corresponding nonactive forms (32). Activation of these proapoptotic Bcl-2 family members can be detected by using anti-Bax (clone 6A7) and anti-Bak (clone Ab-1) monoclonal antibodies that recognize the NH2-terminal epitopes of human Bax and Bak, respectively, and thereby their corresponding active forms. These antibodies have been previously used to show the activation of Bax and Bak in response to distinct apoptotic stimuli (32, 33). Interestingly, edelfosine treatment induced an increase in the Bax protein expression level, without affecting the...
protein levels of Bak and of the antiapoptotic Bcl-2 family member Bcl-X\textsubscript{L} (Fig. 2C). We also found that treatment of HeLa cells with edelfosine promoted a significant activation of Bax, whereas Bak was weakly activated (Fig. 2D).

**ASK1-mediated JNK activation in edelfosine-induced apoptosis.** Previous studies indicate that ASK1-mediated JNK activation is crucial for ER-induced apoptosis (14). Edelfosine has been reported to induce persistent JNK activation before the onset of apoptosis (7). Pretreatment of HeLa cells with the specific JNK inhibitor SP600125 reduced edelfosine-induced JNK activation and apoptosis (Fig. 3A). We stably transfected HeLa cells with wild-type ASK1 (Fig. 3B) and found that ASK1 overexpression enhanced edelfosine-induced JNK activation (Fig. 3C) and cell death (Fig. 3D). These data are in concordance with the notion that ASK1/JNK signaling is involved in ER stress–induced apoptosis.

**Absence of Bax and Bak inhibits edelfosine-induced apoptosis.** Although the function of Bcl-2 family proteins is best characterized at the mitochondrion, some of these proteins also localize to the ER affecting its membrane permeability and regulating apoptosis (32). ER stress responses induce conformational changes and oligomerization of Bax and Bak on the ER, initiating apoptosis (32), and \(\text{bax}^{-/}\text{bak}^{-/}\) DKO MEFs are resistant to ER stress–mediated apoptosis (20). DKO cells were completely resistant to edelfosine even when used at high concentrations (Fig. 4A; Supplementary Fig. S2A and B) and showed an accumulation of cells in G\textsubscript{2}-M phase following edelfosine treatment (Fig. 4A; Supplementary Fig. S2B). Edelfosine induced apoptosis at a similar extent in wild-type MEFs and MEFs individually deficient in either Bax or Bak, requiring the absence of both Bax and Bak to prevent cell death (Supplementary Fig. S2A). This is in agreement with the redundant roles of Bax and Bak on apoptosis, entailing the knockdown of both proteins to abrogate ER stress–mediated apoptosis (34).

Phosphatidylcholine was weakly and similarly inhibited in either wild-type or DKO cells (Fig. 4B). Edelfosine inhibited protein biosynthesis in both wild-type and DKO cells (Fig. 4C). These data suggest that inhibition of phosphatidylcholine and protein biosynthesis is not critical for the apoptotic response triggered by edelfosine.

Overexpression of Bax and Bak promotes Ca\textsuperscript{2+} mobilization from the ER to the mitochondria during apoptosis (35). Bax and Bak have been shown to alter Ca\textsuperscript{2+} homeostasis in response to ER stress (34). On these grounds, we analyzed the ER-stored Ca\textsuperscript{2+} depletion in wild-type versus DKO cells following edelfosine treatment. We found that incubation of wild-type MEFs with edelfosine led to an almost total depletion of ER-stored Ca\textsuperscript{2+} after 15 min of incubation (Fig. 4D, left). Cells were perfused with 0 Ca\textsuperscript{2+} medium containing 1 \(\mu\)mol/L of edelfosine and then stimulated with 100 \(\mu\)mol/L of histamine. Calcium release in this condition was 73% smaller than in control edelfosine-free conditions. Edelfosine was used at 1 \(\mu\)mol/L because MEFs were detached at higher drug concentrations. Calcium was gradually increased in the cytosol, being slowly released from the Ca\textsuperscript{2+} stores upon edelfosine incubation (see inset in Fig. 4D, left). However, the absence of Bax and Bak in DKO cells blocked edelfosine-induced ER-stored Ca\textsuperscript{2+} depletion, and thereby, Ca\textsuperscript{2+} could be released by histamine (Fig. 4D, right). Edelfosine failed to promote Ca\textsuperscript{2+} release in DKO cells even after 60 min of incubation (data not shown). These data suggest that edelfosine-induced apoptosis is dependent on Bax/Bak-mediated Ca\textsuperscript{2+} release from ER.

**Bcl-X\textsubscript{L} overexpression protects cells from edelfosine-induced apoptosis.** To study the role of mitochondria in edelfosine-induced cell death, we stably transfected HeLa cells with the expression vector pSFFV-bcl-x\textsubscript{L}, containing the human bcl-x\textsubscript{L} open reading frame, or with control pSFFV-Neo plasmid. HeLa cells transfected with the pSFFV-Neo plasmid behaved similarly to wild-type HeLa cells (data not shown). HeLa cells expressed endogenous Bcl-X\textsubscript{L}, but Western blot analysis showed a much higher expression of Bcl-X\textsubscript{L} protein in bcl-x\textsubscript{L}–transfected HeLa cells (Supplementary Fig. S3A). Gene transfer–mediated overexpression of Bcl-X\textsubscript{L} prevented apoptosis in edelfosine-treated HeLa cells (Fig. 5A). Bcl-X\textsubscript{L}–transfected HeLa cells were accumulated in the G\textsubscript{2}-M phase following edelfosine treatment (Fig. 5A), and after 4 days of incubation, most of the cells were arrested in G\textsubscript{2}-M phase without induction of apoptosis (Supplementary Fig. S3B). Edelfosine inhibited both phosphatidylcholine and protein synthesis in Bcl-X\textsubscript{L}–transfected HeLa cells (Fig. 5B and C). Edelfosine caused a total calcium discharge from the ER of Bcl-X\textsubscript{L}–transfected HeLa cells (Fig. 5D), an effect that is similar to that observed in wild-type HeLa cells (Fig. 1D). The middle and bottom traces of Fig. 5D show that the basal calcium levels are higher in edelfosine-treated cells than in untreated control cells (300 versus 100 nmol/L Ca\textsuperscript{2+}), which is compatible with Ca\textsuperscript{2+} being released from the ER. Because Bcl-X\textsubscript{L} protein specifically localizes and acts on mitochondria (36), our results suggest that the apoptotic signals triggered by edelfosine at the ER converge on the mitochondria.

**Discussion**

Here, we report for the first time that the ALP prototype edelfosine affects ER functions which lead to ER stress and apoptosis in human solid tumor cells. Edelfosine promotes a number of changes in ER-regulated homeostatic processes leading to the triggering of sudy ER-derived apoptotic events that eventually converge on the mitochondria.

Edelfosine inhibits phosphatidylcholine synthesis in solid tumor cells. Previous reports have shown that phosphatidylcholine biosynthesis is blocked by edelfosine through the inhibition of CCT (25). CCT catalyzes the rate-limiting step in de novo phosphatidylcholine synthesis in higher animal cells, and its activity is modulated by the stored curvature strain energy in lipid membranes (37). Class II lipids, like diacylglycerol and unsaturated phosphatidylethanolamines, tend to form aggregates in which the polar/apolar interface bends toward the polar environment leading to a curvature disposition. The flattening of these lipids at the cell membrane leads to a stored curvature elastic strain that is released by the partitioning of CCT into the membrane, and thereby, class II lipids activate CCT. By contrast, class I lipids, such as ALPs, are characterized by their ability to form aggregates in which the polar/apolar interface curves away from the polar region and inhibit CCT (37, 38). However, the similar patterns of phosphatidylcholine inhibition observed in both sensitive HeLa cells and drug-resistant Bcl-X\textsubscript{L}–transfected cells as well as in wild-type and drug-resistant \(\text{bax}^{-/}\text{bak}^{-/}\) DKO cells suggest that phosphatidylcholine inhibition is not essential for the apoptotic cell death induced by edelfosine. This is in agreement with recent evidence showing that the inhibition of phosphatidylcholine synthesis is not the primary pathway in the induction of apoptosis by the ALP hexadecylphosphocholine, as assessed by comparison of hexadecylphosphocholine-induced apoptosis in CHO cells versus...
CHO-MT58 cells that contain a genetic defect in CCT and phosphatidylcholine synthesis (39). Likewise, farnesol-induced apoptosis has also been shown to be uncoupled from farnesol-mediated inhibition of phosphatidylcholine biosynthesis (40). Edelfosine has also been reported to inhibit phosphatidylcholine synthesis at a similar rate in both sensitive MCF-7 and resilient A549 tumor cell lines, A549 cell proliferation being unaffected (41). Hexadecylphosphocholine-induced apoptosis is prevented by the addition of either lysophosphatidylethanolamine or lysophosphatidylcholine, despite the fact that only lysophosphatidylcholine is able to attenuate the effect of the drug on phosphatidylcholine synthesis (39). This suggests that a more general disturbance in membrane structure, rather than a specific alteration in phosphatidylcholine metabolism, underlies ALP-induced apoptosis (39). In this regard, edelfosine accumulates in lipid rafts (10, 42), modifying membrane lipid composition and protein function (43). Thus, ALPs could induce a general disturbance in the membrane structure or bilayer packing, which may lead to membrane leakage or destabilization.

The results reported here indicate that edelfosine inhibits protein synthesis, a hallmark of ER stress. UPR is triggered as a cellular survival response to ER stress. The balance between Grp78/BiP, a UPR hallmark, and CHOP/GADD153 expression is considered to lead to either cell survival or cell death in ER stress. Less susceptibility to cell death upon activation of the UPR may contribute to the tumor progression of solid tumors. Here, we found that edelfosine induced CHOP/GADD153 expression, whereas Grp78/BiP protein levels remained unaffected, tipping the balance in favor of an ER stress–induced cell death. Our study shows that edelfosine induces a number of ER stress markers, including CHOP/GADD153 up-regulation. Interestingly, Fas/CD95 death receptor is involved in the antitumor action of edelfosine in tumor cells (5, 9, 10, 21), and Fas/CD95-induced apoptosis has been reported to be mediated by CHOP/GADD153 (44). Thus, a putative link between Fas/CD95 and ER could be envisaged in the action of edelfosine in cancer cells. In addition, the accumulation of edelfosine in lipid rafts (10, 42), together with the edelfosine-induced coclustering of Fas/CD95 in lipid rafts in leukemic cells (5, 9, 10, 45), and recent evidence suggesting the existence of lipid rafts in the ER (46, 47), might link the involvement of lipid rafts and ER in the antitumor action of edelfosine.

Our data suggest that edelfosine-induced JNK activation is mediated by ASK1. Caspase-4 was activated after the onset of apoptosis in edelfosine-treated HeLa cells, suggesting that caspase-4 enhances the apoptotic response, but it is not required for the triggering of cell death. These data square with recent evidence showing that caspase-4 activation detected in ER stress–induced apoptosis is not the result of an initiating event, but it occurs further downstream in the apoptotic cascade (48). However, caspase-8 and caspase-3 were activated before the onset of apoptosis following edelfosine treatment. Caspase-8 cleavage of BAP31 at the ER leads to the generation of a p20 fragment that directs proapoptotic signals into the ER and its concomitant uptake into the mitochondria, which prompts mitochondrial fission and cytochrome c release to the cytosol (31). Our current data show that edelfosine induces the cleavage of BAP31 with the formation of proapoptotic p20 fragment and causes a gradual ER-stored Ca\textsuperscript{2+} release up to its total depletion in HeLa cells. Edelfosine-induced apoptosis and ER-stored Ca\textsuperscript{2+} discharge were totally prevented in DKO cells. These data suggest that edelfosine-induced apoptosis is dependent on Bax/Bak-mediated Ca\textsuperscript{2+} release. Thus, our results indicate that Ca\textsuperscript{2+} signaling is essential for the mechanism of action of edelfosine, likely regulating the link between ER and mitochondria, and in this way, influencing the sensitivity of mitochondria to other stress signals. Overexpression of Bcl-X\textsubscript{L}, which specifically localizes and acts on the mitochondria (36), blocks the apoptotic response induced by edelfosine without affecting G\textsubscript{2}-M arrest or Ca\textsuperscript{2+} discharge from the ER. This indicates that the
apoptotic signals set off by edelfosine at the ER converge on the mitochondria. Our data from wild-type and DKO cells indicate that edelfosine inhibition of phosphatidylcholine and protein biosynthesis is not critical for the apoptotic response induced by edelfosine and is independent of the mechanism related with Bax/Bak. Edelfosine treatment leads to a conformational change of Bax. This conformational change has been coupled to its activation and precedes Bax translocation to the mitochondria and/or ER, which may lead to a lesion of the organelle membrane integrity (32, 49). In addition, edelfosine increased the protein levels of Bax, whereas the protein levels of the antiapoptotic Bcl-XL and proapoptotic Bak were not altered. Thus, the accumulation of edelfosine in the ER leads to Bax activation and to higher levels of Bax protein level, suggesting a major role for Bax in the edelfosine-induced apoptotic response. Our previous (1) and present data indicate that edelfosine does not modify the expression of antiapoptotic Bcl-2 and Bcl-XL genes, whereas Bax is up-regulated. Thus, the ratio of antiapoptotic to proapoptotic molecules such as Bcl-2 or Bcl-XL/Bax, which constitutes a rheostat that sets the threshold of susceptibility to apoptosis, is shifted in favor of apoptosis induction following edelfosine treatment. The apparent lack of significant activation of Bak in drug-treated cells, despite the fact that cells deficient in both bax/bak are required for edelfosine resistance, might be due to either a faulty experimental approach to detect the appropriate activating Bak conformational change or to a rather different and complex regulation of Bak in apoptosis that involves the preexistence and alteration of several protein complexes (50). Figure 6 depicts a model for the involvement of ER in edelfosine-induced apoptosis in solid tumor cells based on our current data. Unlike several anticancer drugs that induce ER stress in an indirect way, edelfosine accumulates in the ER of solid tumor cells (11). This drug accumulation leads first to the inhibition of phosphatidylcholine and protein synthesis by inhibiting CCT and promoting eIF2α phosphorylation. These events promote G2-M arrest, but fail to induce per se an apoptotic response. In addition, edelfosine induces Bax/Bak-mediated ER-stored Ca2+ depletion, Bax up-regulation and activation, CHOP/GADD153 up-regulation, caspase-3 and caspase-8 activation, p20-BAP31 fragment generation, and ASK1-dependent JNK-persistent activation. The fact that prevention of caspase-3, caspase-4, or JNK activation inhibits only in part edelfosine-induced apoptosis indicates that these processes are triggered in a rather parallel way. In contrast, Bcl-XL overexpression totally blocks cell death, indicating that these parallel apoptotic pathways converge on the mitochondria as the ultimate step to rendering an apoptotic outcome. Our data on the ER Ca2+ release exerted by edelfosine in wild-type cells, but not in bax−/−; bak−/− doubly deficient cells, suggests that Bax and Bak play an important role in the proapoptotic mechanism of the drug, involving the release of the ER Ca2+ pool with subsequent sensitization of mitochondria. Taken together, our findings indicate that accumulation of edelfosine in the ER results in profound alterations of the organelle functions, followed by transcriptional and biochemical changes that eventually lead to apoptosis through a mitochondrial-mediated process.

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**References**


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