F16, a Mitochondriotoxic Compound, Triggers Apoptosis or Necrosis Depending on the Genetic Background of the Target Carcinoma Cell

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ABSTRACT

Mutations that lead to the emergence of resistance to apoptosis are commonly observed among tumor cells. Some of the proteins affected are integral parts of the apoptotic cascade such as pro- and antiapoptotic members of the Bcl-2 family. F16 is a small molecule that accumulates in mitochondria of a variety of tumor cells and interferes with their physiological function. Because this interference ultimately triggers apoptosis in many affected cell lines, we examined the effect of antiapoptotic Bcl-2 overexpression on the response of cells to F16. Our results showed that high levels of Bel-2 did not block the ability of F16 to induce cell death. However, unlike the apoptotic response that followed F16 treatment of cells with moderate Bcl-2 levels, cells resistant to a variety of apoptotic stimuli by virtue of Bcl-2 overexpression succumbed to F16 by necrosis. Thus, dual ability of the mitochondrial toxin compound F16 to induce apoptosis and necrosis may represent an added advantage by expanding its spectrum of action toward genetically altered tumor cells incapable of apoptosis.

INTRODUCTION

We have recently reported the identification of F16, a small molecule that exhibits selective cytotoxicity toward transformed cells (1). This compound is preferentially concentrated by mitochondria of several oncogene-initiated mouse mammary tumor cell lines and of human breast cancer cell lines. The basis for the selective accumulation of this delocalized lipophilic cation (DLC) lies in the higher mitochondrial membrane potential (ΔΨm) and, to some extent, plasma membrane potential of the transformed versus nontransformed cells (2). Even though the accumulation of DLCs has been explained in terms of a general mechanism, the toxicity imposed by each on the mitochondria of carcinoma cells results from inhibition of different and specific essential molecular targets and processes (3). We have shown that F16 is capable of inhibiting oxidative phosphorylation and inducing mitochondrial transmembrane depolarization (1). This event is followed by an increase in the permeability of the inner mitochondrial membrane [mitochondrial permeability transition (PT)], cytochrome c release, and apoptotic cell death (4, 5).

Most anticancer drugs, regardless of their molecular target, rely on intact apoptotic cascades to effectively induce malignant cell death. However, impaired apoptosis is commonly observed in tumors. Alterations in the cell death program confer emerging neoplastic cells with selective advantages to withstand extreme environmental conditions (low pO2, low pH, and nutrient deprivation) in rapidly growing tumors and metastatic outgrowths. Many cancer cells that exhibit impaired apoptosis experience changes in the balance between pro- and antiapoptotic proteins (i.e., like those that belong to the Bcl-2 family) as well as down-regulation of members of the caspase family of proteases or mutations in tumor suppressors such as p53 (6–8).

We have previously speculated that F16 might be capable of inducing cell death in cells resistant to apoptosis, and, thus, be a particularly effective antitumor agent. One distinctive feature of F16 is that its toxicity is a consequence of direct mitochondrial accumulation. F16 is capable of inducing mitochondrial PT both in cells and in isolated mitochondria. These effects are transiently blocked by cyclosporine A (1), an inhibitor of the voltage-dependent PT pore (4). Thus, we hypothesized that F16 cytotoxicity might not rely on the status of genes involved in the upstream apoptotic cascade. We focused our attention on alterations of proteins that participate directly in the mitochondrial apoptotic pathway. Of those, we decided to examine the role of proteins that, like members of the Bcl-2 family, have been implicated in the control of mitochondrial membrane permeability either by direct pore formation or by regulation of the PT pore opening (9–12). We have previously shown that Bax/Bak (DKO) cells, although resistant to apoptosis in response to various stimuli (13), were sensitive to F16 treatment (1). In the present study, we sought to determine whether overexpression of the Bcl-2 oncogene could induce resistance to F16.

Abrupt expression of antiapoptotic Bcl-2 has been identified in many hematological malignancies and solid tumors (14, 15). In transfection studies, high levels of Bcl-2 protect cells against a variety of apoptotic stimuli (16). In fact, overexpression of Bcl-2 has been linked to chemoresistance and radioresistance of cancer cells (17–20). How Bcl-2 protects cells from apoptosis is still the subject of intensive research and remains to be determined. The cytoprotective role of Bcl-2 has been associated with various mechanisms of action. For example, it has been reported that Bcl-2 is capable of blocking apoptosis by inhibiting the opening of the voltage-dependent PT pore and thus preventing mitochondrial membrane permeabilization (21). Bcl-2 has also been shown to prevent apoptosis by acting as an antioxidant and inhibiting cytochrome c release and downstream caspase activation (22–24).

Our results showed that overexpression of Bcl-2 in F16-sensitive cells did not prevent F16-induced cytotoxicity. Bcl-2 is capable of blocking cytochrome c release, caspase activation, and apoptosis induced by F16 and several other stimuli. However, the mitochondrial injury imposed by F16, due to its direct accumulation within the organellar matrix, leads to necrosis of the treated Bcl-2-overexpressing cells. Altogether, our data suggest that the death program triggered by F16 depends on the genetic background of the target cells. Cells capable of extensive mitochondrial accumulation of F16 succumb to apoptosis by activation of the mitochondrial pathway. Alternatively, cells harboring alterations that render them resistant to apoptosis may die from necrosis resulting from an energetic, ionic, and redox catastrophe.

MATERIALS AND METHODS

Reagents. Anti-Neu was purchased from Upstate Biotechnology Inc., anti-Bcl-2 from Transduction Laboratories, anti-caspase-3 from Cell Signaling, and anti-β-actin from Sigma. F16 was obtained from Asinex, and rhodamine 123 (Rh123) from Molecular Probes. Staurosporine (ICN), thapsigargin (Sigma), and etoposide (Sigma) were used where indicated. 4,5-Dihydroxy-1,3-benzene disulfonic acid (Tiron) was purchased from Sigma, and the broad spectrum caspase inhibitor zVAD-fmk from Calbiochem.

Cell Culture and Generation of Stable Cell Lines. Unless indicated, cells were grown in DMEM and 10% fetal bovine serum at 37°C/5% CO2. Mouse
mammary epithelial Eph4 cells stably expressing the neu⁷ oncogene (Eph4-H-A6) were established as described previously (1). Neu-initiated tumor cell lines SMF and NF324-1C have been established in our laboratory from tumors dissected from Neu-transgenic mice. pcDNA3 (Invitrogen) carrying the bcl-2 gene was transfected into EpH4, EpH4-A6, and SMF cells using Fugene reagent according to the manufacturer’s protocol (Roche). Selection of stable clones was done in medium containing 1.2 mg/ml G418 (Life Technologies, Inc.).

**Effect of F16 on Cell Proliferation.** Cells (1 × 10⁵) were seeded in each well of 6-well plates. On the following day, F16 or staurosporine was added to the medium (3 and 4 μM, respectively), whereas control cells were left untreated. Cells were harvested and counted whenever indicated. Counts were done in triplicate for untreated and treated cells, and the entire experiment was repeated three times.

**Cell Lysate Preparation and Immunoblotting.** Cells were washed with PBS and lysed in buffer [40 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 10 mM sodium PP, 2% NP40, 10 mM NaF, 2 mM EDTA, and 5 mM Na₂VO₃] containing Complete protease inhibitor mixture (Roche). The insoluble material was separated by centrifugation at 4°C. Protein concentration in the supernatants was determined by Bradford assay (Bio-Rad). For each sample, aliquots containing equivalent amounts of protein were separated by SDS-PAGE and transferred to Polyvyscreen membranes (NEN) in Towbin’s buffer (25 mM Tris, 190 mM glycine, 20% methanol, and 0.005% SDS). Membranes were blocked with 1.5% BSA in Tris-Buffered Saline Tween-20 (TBST) [150 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.3% Tween 20]. The primary antibodies (1 μg/ml) and secondary antibodies were in TBST/0.2% BSA. Membranes were washed with TBST buffer. Detection was done using horseradish peroxidase conjugated to antirabbit IgG or to antimouse IgG (Amersham Biosciences) and the enhanced chemiluminescent substrate (Fierce).

**Electron Microscopy.** Cells were grown in 35-mm dishes and treated as indicated. Cells were subjected to formic acid (1.25%)/glutaraldehyde (2.5%) fixation and treated as described previously (1). Ultrathin sections were analyzed using a JEOL 1200EX microscope.

**Oligonucleosomal DNA Fragmentation.** Cells were left untreated or treated for 48 h with 0.3, 1, and 3 μM F16 or with 4 μM staurosporine as a control. DNA was isolated, in each case, from 1 × 10⁶ cells using SuicideTrack (Oncogene) following recommended protocol. Samples were resolved in 1.5% agarose gel and ethidium bromide stained.

**Flow Cytometry.** For detection of apoptosis after various treatments, cells were stained for 15 min with annexin V-phycocerythrin conjugate and the vital dye 7-amino-actinomycin D (7-AAD) following manufacturer’s procedure (PharMingen). For determination of Δψₗ, among various cell lines, the potentialmetric probe tetramethylrhodamine methyl ester (TMRM; Molecular Probes) was used. To normalize the TMRM fluorescent signal according to mitochondrial mass, cells were incubated with nonyl-acridine orange (Molecular Probes). Cells were stained and analyzed as detailed previously (1).

The ability of cell lines to accumulate the fluorescent DLCs F16 (absorbance, 420 nm; emission, 520 nm) and Rh123 (absorbance, 507 nm; emission, 520 nm) and fluorescein isothiocyanate (FITC) was compared by flow cytometry. Cells were stained and analyzed as detailed previously (1). Neu-initiated tumor cell lines SMF and NF324-1C have been established in our laboratory from tumors dissected from Neu-transgenic mice. pcDNA3 (Invitrogen) carrying the bcl-2 gene was transfected into EpH4, EpH4-A6, and SMF cells using Fugene reagent according to the manufacturer’s protocol (Roche). Selection of stable clones was done in medium containing 1.2 mg/ml G418 (Life Technologies, Inc.).

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The ability of cell lines to accumulate the fluorescent DLCs F16 (absorbance, 420 nm; emission, 520 nm) and Rh123 (absorbance, 520 nm; emission, 520 nm; Molecular Probes) was compared by flow cytometry. Cells were incubated for 1 h in medium containing 1 μM F16 or 500 nM Rh123. Next, cells were harvested, washed with PBS, and resuspended in growth medium, and immediately analyzed.

Changes in the levels of superoxide anion were followed using the fluorescent probe dihydorhodamine (DHE; Molecular Probes) as detailed previously (1). EpH4-A6, EpH4-A6.C13, and EpH4-A6.C18 cells were grown in the absence or in the presence of 3 μM F16 for 15, 24, 48, and 72 h. EpH4-A6.C18 cells were incubated for 42 h in medium containing 1 μM F16 as a control. DHE was added to the cell suspension (10⁶ cells/ml) to a final 2 μM in each case. Cells were incubated at 37°C for 15 min and immediately subjected to flow cytometric analysis using the Epics Altra (Beckman-Coulter).

The effect of buffered ATP levels and caspase inhibition on cell death upon F16 treatment was determined by annexin V/7-AAD staining as previously indicated. Cells were subjected to various treatments as follows. Cells were either left untreated or treated with 3 μM F16 for 24 or 48 h in glucose-free DMEM medium supplemented with 5% diazylated fetal bovine serum (90 mg/dl glucose; Invitrogen), 2 mM glucose, and 1 mM pyruvate (to sustain mitochondrial function) and F16-insensitive EpH4-EV (transfected with the empty vector) cell lines (1). Clones stably overexpressing Bcl-2 were isolated (Fig. 1A). EpH4-A6.C13, EpH4-A6.C18 clones derived from EpH4-A6 (A6) cells, and an EpH4-D6 clone derived from EpH4-EV cells exhibited between 8–10-fold higher Bcl-2 protein levels than the corresponding parental cells from which they were derived. As previously mentioned, high levels of Bcl-2 have been shown to effectively protect cells against a variety of apoptotic stimuli (30, 31). To functionally evaluate Bcl-2 overexpression, we compared the effect of the potent protein kinase C inhibitor staurosporine, the endoplasmic reticulum Ca²⁺ adenosine triphosphate pump inhibitor thapsigargin, and the topoisomerase II inhibitor etoposide on parental EpH4-A6 cells and derived Bcl-2-overexpressing EpH4-A6.C13 and EpH4-A6.C18 cells. Phosphatidylinerse is normally restricted to the inner leaflet of the plasma membrane. Its appearance on the outer side of the cell surface is an early event in the apoptotic response. To detect the externalization of phosphatidylinerse, we monitored by flow cytometry cells stained with annexin V in conjunction with the vital dye 7-AAD (Fig. 1B). Treatment of EpH4-A6 cells with the apoptosis-
inducing compounds for 24 h resulted in a detectable early apoptotic cell subpopulation (annexin V positive, 7-AAD negative cells). Under the same conditions, Bcl-2-overexpressing EpH4-A6.C3 cells appeared primarily viable, nonapoptotic (annexin V negative/7-ADD negative). The Bcl-2-overexpressing clone EpH4-A6.C18 behaved in a similar way. These results confirmed that transfected Bcl-2 rendered cells resistant, capable of long-term survival to various apoptosis inducers. In contrast to the observed apoptosis in F16-treated EpH4-A6 cells (annexin V positive/7-AAD negative), F16 treatment of Bcl-2-overexpressing cells resulted in the appearance of an annexin V negative/7-AAD positive, morphologically early necrotic cell population. Thus, high levels of Bcl-2 are not capable of protecting cells from F16-induced cytotoxicity.

Bcl-2 Overexpression Is Not Capable of Protecting F16-Sensitive Cells from the Small Molecule-Induced Cell Death. The ability of F16 to affect cell growth of EpH4-A6 and Bcl-2-overexpressing EpH4-A6.C13 and EpH4-A6.C18 was evaluated by increase in cell number during a time course experiment in the presence of 3 μM F16. As a control, cells were treated with the potent apoptosis inducer staurosporine.

The three cell lines showed a progressive reduction in the number of cells upon treatment with F16 for 24 and 48 h when compared with untreated cells, and only Bcl-2-overexpressing cells remained resistant to staurosporine (Fig. 2). Even though the Bcl-2-overexpressing clones exhibited a slower response, these cells were largely affected by the small molecule as evidenced by the drastic decline in the number of living cells observed after 48 h of incubation in F16-containing medium. Thus, these results are in agreement with the appearance of annexin V negative/7-AAD positive subpopulation during the flow cytometric analysis of F16-treated EpH4-A6.C13 cells (Fig. 1B). Altogether, these results confirm that antiapoptotic Bcl-2 is capable of blocking apoptosis in response to a variety of toxic agents but is unable to protect cells from F16-induced cell injury.

While performing the cell viability assays, we noticed that the parental and Bcl-2-overexpressing cells presented different morphological features upon F16 treatment. To examine in more detail these phenotypic differences, we examined untreated or F16-treated EpH4-A6 and Bcl-2-overexpressing EpH4-A6.C13 and EpH4-A6.C18 cells by electron microscopy. Upon F16 treatment, Bcl-2-overexpressing cells showed cellular swelling, characteristic of necrotic cells (Fig. 2B). In contrast, parental EpH4-A6 treated with F16 shrunk and exhibited chromatid condensation, characteristic of apoptosis. These observations together with the results obtained by fluorescence-activated cell sorter analysis suggested that Bcl-2-overexpressing cells undergo necrosis in response to F16.
A6.C13 (A6.C13), and EpH4-A6.C18 (A6.C18) cells were stained with the H9004 overexpressing cell lines. We have previously shown that accumulation of enhanced production of ROI below and above physiological levels, mitochondrial respiration results in a decrease in cellular ATP levels and an increase in reactive oxygen intermediates (ROI). A blockade in mitochondrial mass between cell lines. Redox Imbalance.

Mitochondrial Dysfunction and the Concomitant Energetic and Redox Imbalance.

Bcl-2 Overexpression Does Not Protect Cells from F16-Induced Cytoxicity. Bcl-2 overexpression has been reported to prevent mitochondria depolarization during apoptosis (24, 35). Therefore, we reasoned that because F16 inhibition of respiration and oxidative phosphorylation was related to its ability to depolarize mitochondria, the stabilizing effect of Bcl-2 on $\Delta \psi_m$ could partially compensate for F16-induced depolarization. This effect could in turn prevent or slow down F16-induced depletion of ATP levels and increase production of ROI and could explain the delayed onset of cell death observed in the Bcl-2-overexpressing cells. Cellular ATP levels in untreated or F16-treated EpH4-A6, EpH4-A6.C13, and EpH4-A6.C18 for 15, 24, and 48 h were assessed by detection of the chemiluminescent signal resulting from the luciferase-catalyzed, ATP-dependent oxidation of luciferin (Fig. 4A). Upon F16 incubation, a similar time-dependent decrease of the cellular ATP pool was observed in both parental EpH4-A6 and Bcl-2-overexpressing EpH4-A6.C13 and EpH4-A6.C18 cells. Superoxide anion production was followed in parallel by flow cytometric analysis of cells stained with DHE and Rh123, another DLC concentrated by cells. Superoxide anion production was measured as the ratio between the TMRM and the mitochondrial cardiolipin dye nonyl-acridine orange fluorescence, as a mean to normalize the TMRM uptake according to mitochondrial mass between cell lines. B. cells were incubated in medium containing F16 or Rh123 and subjected to flow cytometry as indicated in "Materials and Methods." For each determination, 10,000 cells were analyzed. The results obtained from a representative set of samples are presented as a histogram. a.u., arbitrary units.

former, leading to a decrease in F16 accumulation. Therefore, to test this possibility, cells were stained with the $\Delta \psi_m$ probe TMRM and analyzed by flow cytometry. F16-sensitive cells displayed a higher $\Delta \psi_m$ in comparison with F16-resistant cells (Fig. 3A). These results recapitulated the positive correlation between $\Delta \psi_m$ and mitochondrial accumulation of DLCs in transformed cells (1–3, 33). The accumulation of F16 as well as of Rh123, another DLC concentrated by cells with elevated $\Delta \psi_m$, was monitored (Fig. 3B). We took advantage of the fact that both compounds are fluorescent and that their accumulation in cells can be followed by flow cytometry. No significant difference in the levels of fluorescent signal was detected between parental and derived Bcl-2-overexpressing clones. These results ruled out a decrease in $\Delta \psi_m$ and/or an impairment in F16 uptake as factors that may contribute to the difference in the response of Bcl-2-overexpressing cells to F16.

Bcl-2 Overexpression Does Not Protect Cells from F16-Induced Mitochondrial Dysfunction and the Concomitant Energetic and Redox Imbalance. Mitochondria are a major source of cellular ATP and reactive oxygen intermediates (ROI). A blockade in mitochondrial respiration results in a decrease in cellular ATP levels and an enhanced production of ROI below and above physiological levels, respectively (34). We have previously shown that accumulation of F16 in mitochondria led to inhibition of oxidative phosphorylation. Treatment of cells with F16 resulted in the bionenergetic compromise of their mitochondria as a function of time.

Bcl-2 overexpression has been reported to prevent mitochondria depolarization during apoptosis (24, 35). Hence, we reasoned that because F16 inhibition of respiration and oxidative phosphorylation was related to its ability to depolarize mitochondria, the stabilizing effect of Bcl-2 on $\Delta \psi_m$ could partially compensate for F16-induced depolarization. This effect could in turn prevent or slow down F16-induced depletion of ATP levels and increase production of ROI and could explain the delayed onset of cell death observed in the Bcl-2-overexpressing cells. Cellular ATP levels in untreated or F16-treated EpH4-A6, EpH4-A6.C13, and EpH4-A6.C18 for 15, 24, and 48 h were assessed by detection of the chemiluminescent signal resulting from the luciferase-catalyzed, ATP-dependent oxidation of luciferin (Fig. 4A). Upon F16 incubation, a similar time-dependent decrease of the cellular ATP pool was observed in both parental EpH4-A6 and Bcl-2-overexpressing EpH4-A6.C13 and EpH4-A6.C18 cells. Superoxide anion production was followed in parallel by flow cytometric analysis of cells stained with DHE and Rh123, another DLC concentrated by cells. Superoxide anion production was measured as the ratio between the TMRM and the mitochondrial cardiolipin dye nonyl-acridine orange fluorescence, as a mean to normalize the TMRM uptake according to mitochondrial mass between cell lines. B. cells were incubated in medium containing F16 or Rh123 and subjected to flow cytometry as indicated in "Materials and Methods." For each determination, 10,000 cells were analyzed. The results obtained from a representative set of samples are presented as a histogram. a.u., arbitrary units.

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F16-Imposed Mitochondrial Injury Does Not Trigger Apoptosis in Bcl-2-Overexpressing Cells. As previously mentioned, an annexin V negative/7-AAD positive subpopulation, characteristic of necrotic cells, was detected among F16-treated EpH4-A6.C13 and EpH4-A6.C18 cells. These results along with the electron microscopy images and the decrease in the total number of cells in response to F16 treatment suggested that in the presence of the antiapoptotic Bcl-2, F16 induced necrosis instead of apoptosis.

To assess this issue, we examined three additional apoptosis markers. First, we looked at cytochrome c release from mitochondria into the cytoplasm, an early event in the apoptotic cascade. Immunofluorescence detection of cytochrome c was performed on EpH4-A6 and Bcl-2-overexpressing EpH4-A6.C13 18 h after addition of F16 or staurosporine, as a control, to the medium. F16 treatment promoted early release of cytochrome c in transformed EpH4-A6 cells, whereas no release of cytochrome c was observed in the Bcl-2-overexpressing clones (Fig. 5A). Unlike staurosporine, after prolonged incubation (36 h and beyond) of EpH4-A6.C13 cells in F16-containing medium, diffuse cytochrome c staining was observed in about 15% of cells. For those cells in which release of cytochrome c was evident, localization of manganese superoxide dismutase to the mitochondria was also lost (data not shown). This result indicates that the relocation observed was due to loss of mitochondrial structural integrity, and the release of cytochrome c, a consequence rather than a cause of cell death. Likewise, cytochrome c release from the mitochondria to the cytoplasm was also detected by cell fractionation followed by Western blot analysis in F16-treated EpH4-A6 cells (3 μM for 18 h) but not in the Bcl-2-overexpressing clones (Fig. 5B).

Activation of the caspase family of cysteine aspartyl proteases is a common mechanism involved in the induction of apoptosis (11). Caspase activation has been associated with degradation of a variety of cellular components and cell death. This group of enzymes is synthesized as inactive pro-caspases that become enzymatically active upon proteolytic maturation. To monitor the processing of the effector caspase-3, Western blot analysis was performed on whole-cell lysates from untreated cells or cells treated for 24 h with F16 or staurosporine as a control. The appearance of the cleaved (M₅ 18,000) active form of caspase-3 was observed in EpH4-A6 cells in response to both treatments (Fig. 5C). On the other hand, no cleaved caspase-3 was detectable in Bcl-2-overexpressing cells.

Finally, we looked at DNA fragmentation, one of the later stages of cellular apoptosis. Cells were incubated for 48 h in medium containing F16 at concentrations ranging from 0.3 to 3 μM. This treatment resulted in the characteristic apoptotic DNA laddering in the EpH4-A6 cells (Fig. 5D). Stauroporine treatment of F16-insensitive EpH4 cells triggered their apoptosis, indicating once again that EpH4 cells are fully capable of apoptotic response and that F16 induces apoptosis in a target-selective manner. In the case of the Bcl-2-overexpressing EpH4-A6.C13 cells, neither F16 nor staurosporine led to appearance of DNA ladder. Incubation of EpH4-A6.C13 cells in the presence of F16 for 48 h, even though sufficient to induce cell death as evidenced by annexin V/7-AAD staining or by the decline in cell number, did not result in oligonucleosomal DNA fragmentation.

In light of the inability of Bcl-2 to prevent cell death upon F16 treatment, we concluded that cell death in the absence of cytochrome c release, caspase-3 processing, or appearance of a DNA ladder was indeed consistent with necrosis of the treated cells.

F16-Induced Mitochondrial Dysfunction Triggers Apoptosis or Necrosis, Depending on the Genetic Background. Our experiments demonstrate that overexpression of Bcl-2 in EpH4-A6 cells does not prevent F16-induced cell death, but rather alters the way in which cells die. To further evaluate the role of caspase activation in death resulting from F16 treatment, EpH4-A6 and Bcl-2-overexpressing cells were left untreated or incubated in medium containing F16 in the presence of the pan-caspase inhibitor zVAD-fmk (Fig. 6). The results shown for clone EpH4-A6.C13 are representative of those for clones EpH4-A6.C18. Inhibition of caspase activity results in a reduction of the number of dead cells among EpH4-A6 cells exposed to F16 for a short period (24 h). After 48 h of incubation in F16-containing medium, the protective effect of caspase inhibition becomes less robust.
from F16-induced cytotoxicity.

Fig. 6. Effect of buffered ATP levels and inhibition of caspase activity on F16 treatment of Bcl-2-overexpressing cells. EpH4-A6 and Bcl-2-overexpressing EpH4-A6.C13 cells were left untreated or treated with 3 nmol/10^6 cells in medium without glucose supplementation). In addition, we included in this analysis a Neu-initiated tumor cell line that is inherently resistant to apoptosis although capable of F16 uptake into mitochondria. Bcl-2 protein levels in NF324-1C are comparable with those observed in other mammary epithelial cells (Fig. 7B). The underlying cause for their apoptosis resistance remains to be determined, however, these cells are sensitive to F16 (Fig. 7B). Like in Bcl-2-overexpressing SMF cells, cell death in NF324-1C in response to F16 occurred in the absence of apoptosis markers (Fig. 7, C and D). These cells exhibited progressive cellular swelling (sign of necrosis) upon F16 treatment in a time-dependent manner (data not shown). In all of the cell lines tested, whether apoptosis sensitive or apoptosis resistant, F16 treatment resulted in mitochondrial dysfunction as evidenced by the decrease in the cellular ATP levels (Fig. 7E).

DISCUSSION

In the present study, we examined the effect of Bcl-2 overexpression in transformed mammary epithelial EpH4-A6 and in adenocarcinoma SMF cells does not compromise the ability of F16 to trigger necrosis in these same cells. The analysis performed in NF324-1C additionally suggests that F16 is capable of triggering necrosis in cells with an impaired apoptosis response resulting from other genetic alterations in addition to Bcl-2 overexpression. Provided that F16 extensively accumulates in mitochondria of treated cells, the damage exerted to this organelle leads to loss of energetic and redox homeostasis and concomitant death in these cells.

evident. Of note, zVAD-fmk completely prevents cell death of EpH4-A6 cells in response to staurosporine (data not shown). However, there is a significant percentage of cell death in the presence of the caspase inhibitor among F16-treated EpH4-A6 cells. No DNA ladder was detectable in these cells (data not shown). These results suggest that pharmacological inhibition of caspase activity in EpH4-A6 can recapitulate the effect of Bcl-2-overexpression in these cells. Thus, when apoptosis is impaired (e.g., by Bcl-2 overexpression) or blocked (e.g., by caspase inhibition) in EpH4-A6 cells, necrosis in response to F16 is uncovered. On the other hand, inhibition of caspase activity in EpH4-A6.C13 cells (already apoptosis resistant) does not affect the percentage of dead cells at any time point.

From our previous results, we speculated that the necrosis observed in F16-treated EpH4-A6.C13 and EpH4-A6.C18 cells could be the consequence of the energetic breakdown in these cells. Indeed, the lag phase observed in the response of the Bcl-2-overexpressing cells to F16 treatment correlated with the incubation time required for cells to show a drastic depletion of their ATP pool. It has been reported that manipulations aimed to maintain a high concentration of cellular ATP favored the onset of apoptosis over necrosis (28, 36). Conversely, conditions leading to a depletion of the ATP pool could shift the balance in the opposite direction. To test whether the depletion of ATP and the increase in ROI were direct triggers of the necrosis induced by F16 in the Bcl-2-overexpressing cells, we examined the effect of buffering ATP and superoxide levels in F16-treated cells (Fig. 6). For that purpose, the medium was supplemented with glucose (28 mm final concentration) to stimulate glycolysis and to maintain the ATP concentration at a high level. The cellular ATP concentration in F16-treated EpH4-A6.C13 cells was maintained at 11 ± 0.8 nmol/10^6 cells in glucose-supplemented medium (in contrast to 2.8 ± 0.3 nmol/10^6 cells in medium without glucose supplementation). In addition, we used the superoxide scavenger Tiron to protect against superoxide anion burst. Our results showed that caspase-independent cell death of Bcl-2- overexpressing EpH4-A6.C13 cells upon F16 treatment was largely prevented in high-glucose medium (1.7-fold; Fig. 6). This protective effect was potentiated by combination with Tiron (2.5-fold). Similar results were obtained with the Bcl-2-overexpressing clone EpH4-A6.C18. Interestingly, in the Bcl-2-overexpressing cells grown in high-glucose- and Tiron-containing medium, neither did F16 lead to the emergence of apoptotic phenotypes (data not shown), nor did addition of the caspase inhibitor zVAD-fmk exhibit any added beneficial effect.

These data show that the manipulation of ATP levels alone, even though sufficient to partially protect cells from necrotic death upon F16 treatment, is not enough to shift the Bcl-2-overexpressing cells into the apoptotic pathway. It is possible that, in addition to ATP concentration, alterations in mitochondrial Ca^2+ levels play a key role in the commitment of cells to apoptosis or necrosis, as previously reported (37). Taken together, our results show that the limiting availability of ATP and the excess of ROI are important factors leading to necrotic death of Bcl-2 overexpressing cells in response to F16.

F16 Induces Necrosis in Various Cell Lines Resistant to Apoptosis. Next, we examined in other F16-sensitive cell systems the ability of the small molecule to bypass Bcl-2-imposed blockade in the apoptotic cascade, by triggering a necrotic response. For that purpose, we examined the effect of F16 on the Neu-initiated adenocarcinoma cell line SMF and on SMF stably overexpressing Bcl-2 (Fig. 7A). We have previously reported that SMF cells are sensitive to F16 (Ref. 1; Fig. 7B). Similarly to Bcl-2-overexpressing EpH4-A6 cells, Bcl-2-overexpressing SMF cells were resistant to the apoptosis inducer staurosporine, albeit sensitive to F16 (Fig. 7B). Unlike the activation of caspase-3 observed in SMF cells after F16 or staurosporin treatment, no proteolytic processing of caspase-3 was detectable in the SMF cells overexpressing Bcl-2 (Fig. 7C). Moreover, the pan-caspase inhibitor zVAD-fmk could not prevent cell death of Bcl-2-overexpressing SMF cells in response to F16 (data not shown). F16-induced cell death in SMF cells was accompanied by the emergence of a DNA ladder, characteristic of apoptosis (Fig. 7D). In contrast, no oligonucleosomal DNA fragmentation was observed in Bcl-2-overexpressing SMF cells treated with F16. In addition, we included in this analysis a Neu-initiated tumor cell line that is inherently resistant to apoptosis although capable of F16 uptake into mitochondria. Bcl-2 protein levels in NF324-1C are comparable with those observed in other mammary epithelial cells (Fig. 7B). The underlying cause for their apoptosis resistance remains to be determined, however, these cells are sensitive to F16 (Fig. 7B). Like in Bcl-2-overexpressing SMF cells, cell death in NF324-1C in response to F16 occurred in the absence of apoptosis markers (Fig. 7, C and D). These cells exhibited progressive cellular swelling (sign of necrosis) upon F16 treatment in a time-dependent manner (data not shown). In all of the cell lines tested, whether apoptosis sensitive or apoptosis resistant, F16 treatment resulted in mitochondrial dysfunction as evidenced by the decrease in the cellular ATP levels (Fig. 7E).
Fig. 7. F16 induces cell death in genetically altered cells resistant to apoptosis. A, Western blot analysis on whole-cell lysates from parental adenocarcinoma cells SMF and SMF stably overexpressing Bcl-2. These cells were established by transfection of bcl-2 cDNA as described in "Materials and Methods." Aliquots of lysates containing an equivalent amount of protein were probed with antibodies against Bcl-2, HER-2/Neu, and β-actin. For a comparison, lysates from Neu-transformed EpH4-A6 cells and tumor cell lines NF324-1C and SMF were also subjected to Bcl-2, Neu, and β-actin immunodetection. B, effect of F16 on proliferation and survival SMF. Bcl-2-overexpressing SMF, and NF324-1C cell lines. Cells were left untreated or treated with 3 mM F16, or with 4 µM staurosporine for 24 h (II) as a control and counted. The results are expressed as average ± SE fold reduction in cell number with respect to untreated cells for each time point. C, Western blot analysis of whole-cell lysates from cells left untreated or treated with F16 and staurosporine. Immunodetection was done with anti-caspase-3 and anti-β-actin as loading control. D, agarose gel electrophoresis followed by ethidium bromide staining of DNA isolated from cells left untreated or treated for 48 h with F16 or staurosporine as indicated. E, reduction of ATP levels in F16-treated cells with respect to untreated cells.

Bcl-2 DOES NOT PROTECT CELLS FROM F16-INDUCED CYTOTOXICITY

to inhibit apoptosis, and in a few circumstances necrosis, this protein is not capable of universally preventing cell death (38). Our results showed that stable expression of antiapoptotic Bcl-2 in F16-sensitive cells did not induce resistance to the compound, despite rescuing several other forms of apoptotic cell death. However, unlike the apoptosis triggered by F16 in several transformed cell lines expressing moderate levels of Bcl-2, cells overexpressing the protein died of necrosis upon F16 incubation. This shift in the balance between apoptosis and necrosis by Bcl-2 overexpression upon toxic stimulation has been described previously (37). Indeed, in our case, death of Bcl-2-overexpressing cells was cytochrome c and caspase independent, and hallmarks of apoptosis such as phosphatidyl serine externalization and DNA laddering were absent.

Apoptosis and necrosis are two forms of cell death that exhibit very distinctive biochemical and morphological characteristics. However, these two modalities of cell death, once featured as opposed processes, are currently viewed as "two extremes of a continuum of possible types of cell death" (38, 39). In fact, apoptosis and necrosis can occur simultaneously in cells exposed to the same stimulus. Furthermore, the same stimulus at different concentrations can commit the same cells to either the apoptotic or the necrotic pathway. During the apoptotic response, a highly orchestrated set of reactions is set into motion to efficiently execute the suicide of a mammalian cell. Several of these regulated cascades converge at the level of mitochondria, leading to mitochondria membrane permeabilization and the release into the cytoplasm of apoptogenetic factors that mediate cell death. Likewise, after toxin exposure, the poorly characterized necrotic response results in mitochondrial PT that translates into the breakdown of Δψm. It is now clear that mitochondria play a pivotal role in sensing and integrating signals during both apoptosis and necrosis.

F16, unlike other apoptosis inducers, acts exactly at the level of mitochondria, where both necrotic and apoptotic pathways intersect. F16 mitochondrial accumulation results in membrane depolarization and induction of PT, compromising the functional integrity of an organelle essential for cell survival. It has been proposed that, upon mitochondrial membrane permeabilization, the balance between the rate of the energetic depletion and oxidative burst, and the rate of caspase and nuclease activation determines the commitment of cells to one or the other form of cell death. High-energy equivalents are required for the complex set of catabolic reactions to proceed during the degradation phase of the apoptotic response. In contrast, conditions leading to a decrease in ATP levels result in cellular necrosis. Unlike the observed protective effect of caspase inhibition against F16 cytotoxicity in EpH4-A6 cells, our data indicate that cell death in F16-treated Bcl-2-overexpressing clones derived from these cells is prevented under conditions aimed to maintain a high concentration of ATP and to neutralize superoxide anion. Therefore, our results additionally show that the onset of necrotic death in Bcl-2-overexpressing cells coincides with F16-driven mitochondrial dysfunction.

Finally, it is important that an antiproliferative small molecule can still trigger cell death in a genetically altered environment that can resist apoptosis. Tumor cells are characterized by a high accumulation of genomic mutations (40). Alterations in proteins that are integral to the mitochondrial apoptotic pathway or tumor suppressors that affect the upstream apoptotic cascade are frequently observed in tumors. These types of mutations often render cells resistant to a wide range of antitumor drugs. In those cases, induction of necrosis might provide an alternative mechanism to stop tumor cell proliferation. Some authors have even argued that compounds capable of eliciting necrosis might have a wider spectrum of action because they would not rely on the actual status of genes that control the apoptotic response (41). Despite the crucial role of apoptosis in chemotherapy, necrotic elimination of cells significantly contributes to tumor clearance upon radiation treatment (42). It has also been noticed that in addition to apoptosis, commonly used anticancer drugs trigger necrosis in cancer cells (43).

In comparison with necrosis, apoptosis has been frequently regarded as a preferred and superior mechanism for tumor clearance. It has been argued that because apoptosis proceeds with an almost intact plasma membrane, it prevents the onset of an inflammatory response and favors phagocytic elimination of cells by macrophages. However, these ideas have been challenged by novel findings demonstrating that exposure of macrophages to necrotic dead cells resulted in pronounced stimulation of macrophage antitumor activity. This response might in fact positively contribute to tumor eradication (44). Furthermore, it has been proposed that the antitumor effect brought about by the nonspecific activation of the immune system by necrotic cells might lead to a longer lasting effect than that achieved by apoptotic cells (43–45). Compounds like the mitochondriotoxic molecule F16,
that possess dual ability to induce apoptosis as well as necrosis might present an added advantage for inducing cell death in a diverse array of malignant cells.

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F16, a Mitochondriotoxic Compound, Triggers Apoptosis or Necrosis Depending on the Genetic Background of the Target Carcinoma Cell

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