Alteration of the Mitochondrial Apoptotic Pathway Is Key to Acquired Paclitaxel Resistance and Can Be Reversed by ABT-737

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Abstract

Paclitaxel is a microtubule-targeting antineoplastic drug widely used in human cancers. Even when tumors are initially responsive, progression of disease despite continued taxane therapy is all too common in the treatment of many of the most common epithelial cancers, including breast cancer. However, the mechanisms underlying paclitaxel resistance in cancer cells are not completely understood. Our hypothesis is that changes in the intrinsic (or mitochondrial) cell death pathway controlled by the BCL-2 family are key to the development of acquired paclitaxel resistance. Here we show that paclitaxel activates the mitochondrial apoptosis pathway, which can be blocked by BCL-2 overexpression. Treatment with ABT-737, a small-molecule BCL-2 antagonist, restores sensitivity to paclitaxel in BCL-2-overexpressing cells. To investigate the importance of changes in the intrinsic apoptotic pathway in the absence of enforced BCL-2 expression, we generated two independent breast cancer cell lines with acquired resistance to apoptosis induced by paclitaxel. In these lines, acquired resistance to paclitaxel is mediated either by increased antiapoptotic BCL-2 proteins or decreased proapoptotic BCL-2 proteins. In both cases, ABT-737 can engage the mitochondrial apoptosis pathway to restore sensitivity to paclitaxel to cell lines with acquired paclitaxel resistance. In summary, these findings suggest that alterations in the intrinsic apoptotic pathway controlled by BCL-2 protein family members may be crucial to causing paclitaxel resistance. Furthermore, our results suggest that combining small-molecule BCL-2 antagonists with paclitaxel may offer benefit to patients with paclitaxel-resistant tumors, an oncologic problem of great prevalence. [Cancer Res 2008;68(19):7985–94]

Introduction

Resistance to chemotherapy is a major obstacle for the success of cancer therapy. Resistance may be present at the onset of therapeutic intervention and patients initially fail to respond (intrinsic or innate chemoresistance), or it may emerge over time during chemotherapy (acquired chemoresistance), even after a dramatic initial response (1). In epithelial tumors including breast, ovarian, and lung cancers, resistance to chemotherapy is frequently observed and is associated with poor clinical outcome (2–5).

Paclitaxel is a diterpenoid compound initially isolated from *Taxus brevifolia* and is widely used against malignant epithelial tumors. Paclitaxel mainly targets β-tubulin at the molecular level and stabilizes microtubule dynamics to induce microtubule polymerization (6). There are a variety of mechanisms responsible for the development of chemoresistance in cancer cells: reduced drug uptake or increased drug efflux, increased repair of drug targets, alteration of drug targets that prevent drug binding or action, and blocks in biochemical pathways that mediate drug-induced cytotoxicity. Development of acquired paclitaxel-resistance has been attributed to such mechanisms, including overexpression of P-glycoprotein, alterations in tubulin composition, and mutations in β-tubulin (7–10). Nearly every patient with advanced or metastatic breast cancer in the United States receives a taxane as part of their chemotherapy. Therefore, those who die of their disease have generally ceased to respond to taxanes.

One can greatly simplify the pathway of chemotherapy response into three main steps. In the first step, drug must accumulate in the cell in sufficient concentration. In the second step, the drug must interact with target. In the third step, death signals generated from drug interaction with target must interact with a receptive apoptotic pathway. Acquired resistance due to blocking of the first two steps has been examined for paclitaxel. The third step, involving alteration of the intrinsic apoptotic pathway, has not been well explored as a mechanism of acquired paclitaxel resistance.

Apoptosis, a type of programmed cell death, is an essential mechanism for cell death following many types of chemotherapy. The mitochondrial (or intrinsic) apoptosis pathway is mainly regulated by the interplay between members of the BCL-2 protein family. Following apoptotic stimuli, including many kinds of chemotherapy, cytochrome c is released into the cytosol from mitochondria as a result of mitochondrial outer membrane permeabilization, which is followed by the formation of the apoptosome complex and caspase activation (11). The key, essentially irreversible step in the commitment to apoptotic death is the permeabilization of the mitochondrial outer membrane. It is this step that is controlled by the interaction of BCL-2 family proteins.

Proapoptotic BCL-2 proteins can be classified into two main groups: multidomain proapoptotic proteins (BAX and BAK) and BH3-only proteins (BID, BIM, BAD, NOXA, PUMA, BMF, BIK, and HRK). In response to death stimuli, BH3-only proteins are up-regulated by numerous means, including transcriptional up-regulation, posttranslational modification, and subcellular localization. Certain BH3-only proteins, the so-called activators, which include BID and BIM, induce the activation of BAX and BAK. Activated BAX and BAK undergo a conformational change, which can be recognized by conformation-specific antibodies, and oligomerize. Oligomerized BAX and BAK, perhaps in a complex with other proteins, induce permeabilization of the outer mitochondrial membrane, which allows diffusion of proapoptotic proteins, including cytochrome c from the intermembrane space. Antiapoptotic BCL-2 proteins BCL-2, BCL-X<sub>L</sub>, MCL-1, BCL-w, and BFL-1 control the mitochondrial release of cytochrome c by sequestering proapoptotic BCL-2 family proteins. BAD, NOXA, 

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PUMA, BMF, BIK, and HRK, known as sensitizers, act as inhibitors of the inhibitors of apoptosis, displacing activators from anti-apoptotic BCL-2 proteins, which are then freed to trigger activation and oligomerization of BAX and BAK (12, 13). Adding to this complexity, antiapoptotic BCL-2 proteins exhibit selective binding affinities to sensitizer BH3-only proteins, and the dynamic nature of these interactions frequently dictates the fate of the cell in response to apoptotic stimuli, either death or survival.

Here we systematically investigate, for the first time, whether alterations in the apoptotic pathway are responsible for resistance to paclitaxel. We find that breast cancer cell lines that acquire resistance increase antiapoptotic protein expression or decrease proapoptotic protein expression. ABT-737 is a small-molecule inhibitor of BCL-2, BCL-XL, and BCL-w that binds to the BH3-binding cleft with very high affinity (14). ABT-737 was reported to elicit apoptosis either as a single agent or in combination with chemotherapeutics against several cancer cell types, including non-Hodgkin's lymphoma, non–small cell lung cancer, and various leukemias (13, 15–27). An orally available derivative of ABT-737, ABT-263, is currently in study in clinical trials for non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and small-cell lung cancer. We find that we can reverse acquired resistance to paclitaxel using ABT-737. We suggest that combination therapy of taxanes with ABT-737 may be a useful strategy to attack the serious clinical problem of acquired taxane resistance in breast cancer.

Materials and Methods

Cell lines. MCF-7 and MDA-MB-468 cells were grown in DMEM/F12 (Invitrogen) supplemented with 2 mmol/L L-glutamine, 10% heat-inactivated fetal bovine serum (Sigma), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) in a humidified incubator at 37°C and 5% CO2. Insulin (5 μg/mL; Sigma) was added to DMEM/F12 used for the culture of MCF-7 and T47D cells.

The paclitaxel-resistant MCF-7 and MDA-MB-468 cells were derived as described before (28). Following selection of paclitaxel-resistant clonal lines, MCF-7 TaxR30 and MCF-7 TaxR50 were maintained in the presence of 30 and 50 mmol/L of paclitaxel, respectively; MDA-MB-468 TaxR was maintained in the presence of 15 mmol/L paclitaxel. All paclitaxel-resistant cell lines were grown in the presence of 5 μg/mL verapamil.

MCF-7 and T47D cells were stably transfected with either pCI-Neo.FlagBCL-2 (MCF-7 BCL-2 and T47D BCL-2) or pCI-Neo.FLAG plasmid (MCF-7 Mock and T47D Mock) using Fugene 6 (Roche), and clonal selection was carried out in the presence of G418 (1.2 mg/mL; Sigma). The resulting clones were assayed by detection of BCL-2 or FLAG-tag by immunoblot analysis. Selected clones were maintained in growth medium with 0.2 mg/mL G418.

Chemicals. Paclitaxel was purchased from Sigma and dissolved in DMSO. ABT-737 and ABT-737 enantiomer were provided by Abbott Laboratories. Caspase-9 inhibitor (z-LEHD-FMK), pancaspase inhibitor (z-VAD-FMK), and caspase-8 inhibitor (z-IETD-FMK) were obtained from BD Biosciences PharMingen.

Cell viability and apoptotic assays. Apoptosis was evaluated by Annexin V-FITC (BioVision) staining according to the manufacturer's protocols. Apoptosis was quantified by flow cytometry on a FACS Calibur (BD Biosciences), followed by analysis using WinMDI 2.9 software (Scripps Institute). For determination of EC50 values for paclitaxel in parental and resistant cell lines, cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit according to manufacturer's instructions following treatment with various concentrations of paclitaxel. The results were from three experiments in triplicate. The EC50 values for paclitaxel in parental and resistant cells were determined by nonlinear regression of sigmoidal dose-response curves using GraphPad Prism 3.0 software.

Colony-forming assay. Colony-forming assay was done as described previously (29). Briefly, paclitaxel-resistant MCF-7 and MDA-MB-468 cells were plated in 24-well plates and treated with drugs for 16 h. Cells were counted and replated into 60-mm tissue culture dishes (200 per plate). Following 10 d of incubation, tissue culture plates were stained with crystal violet [0.5% crystal violet in a 3:1 (v/v) mixture of distilled water/methanol] and colonies were counted. Results were expressed as the percentage of colony formation compared with untreated control cells.

Immunoprecipitation and immunoblotting. Total cell lysates were prepared in 1% Chaps buffer [5 mmol/L MgCl2, 137 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Chaps, 20 mmol/L Tris-HCl (pH 7.5), and protease inhibitors (Complete, Roche)] as described previously (30). To monitor the activation of BAX and BAK, proteins (800 μg) were immunoprecipitated with anti-BAX (6A7; BD Pharmingen) and anti-BAK (Ab-2, Oncogene Research) at 4°C for 2 h. Immunoprecipitates were captured by 50% slurry of protein G-Sepharose in lysis buffer (GE Healthcare) at 4°C for 2 h. Immunoprecipitates were then recovered by centrifugation and washed thrice in 1% Chaps buffer. Immunoprecipitates, total cell extracts (40 μg), and subfractionation lysates (30 μg) were separated on NuPage 10% Bis-Tris gels. Following SDS-PAGE, proteins were transferred onto polyvinylidene difluoride membranes (Immobilon, Millipore) and then blocked with 5% dried milk in PBS-Tween20. Membranes were incubated with primary and secondary antibodies (GE Healthcare) in a buffer containing 10% milk diluent blocking concentrate (KPL), detected with Western Lightning Chemiluminescence Reagent Plus (Perkin-Elmer), and exposed to Biomax MR film (Kodak). For detection of some immunoprecipitates, protein A-horseradish peroxidase (GE Healthcare) was used as a secondary detection agent. The following antibodies were used for immunoblotting: anti-FLAG (Sigma), anti–BCL-2, anti–BCL-XL, anti-BF, anti-BAD, anti-PUMA, anti–I-actin, anti–caspase-3, anti–caspase-9, anti–caspase-8, (Cell Signaling), anti-BAX (N20; Santa Cruz), anti-BIK (G-28; Santa Cruz), anti-BIM (22–40; Calbiochem), anti–cytochrome c (6H2.B4; BD Biosciences), and anti-CoxIV (Molecular Probes).

Caspase activation assays. The activity of caspase-3, caspase-9, and caspase-8 was determined using ApoAlert Caspase Profiling Plate (Clontech) according to the manufacturer's protocol. The release of fluorochrome 7-amino-4-methyl coumarin was analyzed at 380-nm excitation and 460-nm emission using a multiplate fluorescence spectrophotometer. Data shown are mean ± SE of three independent experiments in duplicate and expressed in arbitrary fluorescence units per milligram of protein.

Subcellular fractionation. Subcellular fractionation was done as described before (31). Briefly, cells were harvested and washed in ice-cold PBS and then resuspended in an isotonic buffer [250 mmol/L sucrose, 20 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L MgCl2, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitors (Complete, Roche)] on ice for 20 min. Following incubation, cells were homogenized with Dounce homogenizer and centrifuged at 800 × g for 10 min at 4°C. The resulting supernatant was centrifuged at 8,000 × g for 20 min at 4°C to obtain mitochondrial and cytosolic fractions. These fractions were used to monitor cytochrome c release from mitochondria. Mitochondrial fractions were lysed in 1% Chaps buffer for immunoblot analysis.

Statistical analysis. Statistical significance of the results was analyzed by Student's t test using GraphPad Prism 3.0 software. P < 0.05 and P < 0.01 were considered significant.

Results

ABT-737 treatment overcomes BCL-2-mediated resistance to paclitaxel-induced apoptosis. If a type of cell death operates via the mitochondrial apoptotic pathway, we would expect that BCL-2 overexpression would block the death. We first tested whether BCL-2 overexpression could block apoptosis due to paclitaxel treatment. MCF-7 and T47D cells were stably transfected with either pCI-Neo.FlagBCL-2 (MCF-7 BCL-2 and T47D BCL-2) or control vector (MCF-7 Mock and T47D Mock). The overexpression...
of BCL-2 was verified by immunoblotting of total cell extracts with anti–BCL-2 or FLAG antibody (Fig. 1A and C). Both parental and transfected cells were treated with 100 nmol/L paclitaxel for 48 hours and analyzed for apoptotic response by Annexin V staining. The results clearly showed the protection against paclitaxel-induced apoptosis by BCL-2 overexpression in MCF-7 and T47D cells (Fig. 1B and D). These results imply that the mitochondrial (or intrinsic) apoptotic pathway, which is controlled by the BCL-2 family of proteins, is necessary for apoptosis following paclitaxel treatment.

ABT-737 is a cell-permeable BCL-2, BCL-X<sub>L</sub>, and BCL-w inhibitor that was shown to induce apoptosis in various cancer cell types as a single agent and in combination with chemotherapeutics (18–20, 23–26). Therefore, we evaluated whether treatment with ABT-737 could reverse BCL-2–mediated protection against paclitaxel-induced apoptosis in MCF-7 BCL-2 and T47D BCL-2 cells. As shown in Fig. 1B and D, ABT-737 efficiently augmented paclitaxel-induced apoptosis in MCF-7 BCL-2 and T47D BCL-2 cells, although its negative control enantiomer did not exert such an effect. Notably, ABT-737 or negative control enantiomer did not elicit a significant effect when used alone. Moreover, combining ABT-737 or negative control enantiomer with paclitaxel did not enhance paclitaxel-induced apoptosis in parental or mock-transfected MCF-7 or T47D cells. Of interest, cotreatment of parental MCF-7 and T47D cells with ABT-737 and 0 to 100 nmol/L of paclitaxel did not elicit a major effect on apoptotic response, except a slight, but

Figure 1. BCL-2 protects against paclitaxel-induced apoptosis and ABT-737 might overcome BCL-2–mediated paclitaxel resistance. A, MCF-7 cells were stably transfected with control vector (MCF-7 Mock) or pCI.Neo.FlagBCL-2 (MCF-7 BCL-2). The expression of FLAG-BCL-2 in transfected cells was verified by immunoblotting with anti–BCL-2 or anti-FLAG antibody. β-Actin was probed as a loading control. B, cells were treated with paclitaxel (100 nmol/L), ABT-737 (100 nmol/L), the combination of paclitaxel (100 nmol/L) and ABT-737 (100 nmol/L), enantiomer of ABT-737 (100 nmol/L), or the combination of enantiomer of ABT-737 (100 nmol/L) and paclitaxel (100 nmol/L) for 48 h. The percent apoptotic response was evaluated by Annexin V staining. Columns, mean of three independent experiments; bars, SE. **, P < 0.01, paclitaxel plus ABT-737–treated with respect to paclitaxel only–treated. C, T47D cells were stably transfected with control vector (T47D Mock) or pCI.Neo.FlagBCL-2 (T47D BCL-2). The expression of FLAG-BCL-2 in transfected cells was verified by immunoblotting with anti–BCL-2 or anti-FLAG antibody. β-Actin was probed as a loading control. D, cells were treated with paclitaxel (100 nmol/L), ABT-737 (100 nmol/L), the combination of paclitaxel (100 nmol/L) and ABT-737 (100 nmol/L), enantiomer of ABT-737 (100 nmol/L), or the combination of enantiomer of ABT-737 (100 nmol/L) and paclitaxel (100 nmol/L) for 48 h. The percent apoptotic response was evaluated by Annexin V staining. Columns, mean of three independent experiments; bars, SE. **, P < 0.01, paclitaxel plus ABT-737–treated with respect to paclitaxel only–treated.
statistically insignificant, increase in Annexin V–positive cells when 100 nmol/L ABT-737 was combined with 50 nmol/L paclitaxel in MCF-7 cells (results not shown).

Because BCL-2 family proteins control the mitochondrial apoptotic pathway, our expectation was that the sensitization effect of ABT-737 on BCL-2–overexpressing breast cancer cells also operated through the mitochondrial apoptosis pathway. In support of this expectation, we found that cotreatment with paclitaxel and ABT-737 induced mitochondrial outer membrane permeabilization, as measured by cytochrome c release from the mitochondria (Fig. 2A). The conformational change in BAX and BAK that accompanies apoptosis via the mitochondrial pathway was also observed following cotreatment (Fig. 2B). Caspase-9 activation accompanies apoptosis via the mitochondrial apoptotic pathway, whereas activation of caspase-8 accompanies activation via the extrinsic apoptotic pathway. Caspase assays showed the activation of caspase-9, but not caspase-8, in MCF-7 BCL-2 cells treated with the combination of ABT-737 and paclitaxel (Fig. 2C). Demonstrating that caspase-9 activation was required for cell death and not simply a synchronous phenomenon, pretreatment with pancaspase inhibitor z-VAD-FMK and caspase-9 inhibitor z-LEHD-FMK, but not caspase-8 inhibitor z-IETD-FMK, attenuated

**Figure 2.** ABT-737–mediated sensitization of MCF-7 BCL-2 cells to paclitaxel-induced apoptosis via the mitochondrial apoptosis pathway. A, MCF-7 BCL-2 cells were treated with paclitaxel (100 nmol/L), ABT-737 (100 nmol/L), or the combination of paclitaxel (100 nmol/L) and ABT-737 (100 nmol/L) for 48 h. Cytosolic and mitochondrial fractions from MCF-7 BCL-2 cells were immunoblotted for cytochrome c. CoxIV was probed as a loading control for mitochondrial fractions. B, MCF-7 BCL-2 cells were treated with paclitaxel (100 nmol/L), ABT-737 (100 nmol/L), or the combination of paclitaxel (100 nmol/L) and ABT-737 (100 nmol/L) for 12 h. Activation of BAX and BAK was analyzed by immunoprecipitation with active conformation-specific anti-BAX (6A7) and anti-BAK (Ab-2) antibodies followed by immunoblot analysis of BAX and BAK. Five percent of the input for immunoprecipitation was also subjected to immunoblot analysis. j-Actin was probed as a loading control. C, MCF-7 BCL-2 cells were treated with paclitaxel (100 nmol/L), ABT-737 (100 nmol/L), or the combination of paclitaxel (100 nmol/L) and ABT-737 (100 nmol/L) for 48 h. Activation of caspase-9 and caspase-8 by the combination of paclitaxel and ABT-737 treatment was evaluated by fluorometric caspase activation assays as described in Materials and methods. Columns, mean relative fluorescence units (RFU) from three independent experiments; bars, SE. D, MCF-7 BCL-2 cells were pretreated with 20 μmol/L pancaspase inhibitor (Z-VAD-FMK), 20 μmol/L caspase-9 inhibitor (Z-LEHD-FMK), and 20 μmol/L caspase-8 inhibitor (Z-IETD-FMK) before treatment with paclitaxel plus ABT-737 for 48 h. The percent apoptotic response was evaluated by Annexin V staining. Columns, mean of three independent experiments; bars, SE.
ABT-737–mediated sensitization of MCF-7 BCL-2 cells to paclitaxel-induced apoptosis (Fig. 2D). These findings suggest that the apoptotic block against paclitaxel treatment imparted by BCL-2 overexpression could be overcome by cotreatment with the BCL-2 inhibitor ABT-737 in breast cancer cells, resulting in a restoration of death via the mitochondrial (or intrinsic) apoptotic pathway.

Acquired resistance to paclitaxel is mediated by alterations in BCL-2 protein family members. The above experiments established that enforced changes in the mitochondrial apoptotic pathway could mediate resistance to paclitaxel. We next asked whether changes in the mitochondrial apoptotic pathway were selected for in models of paclitaxel resistance in which resistance was passively derived by long-term exposure to the drug. To establish paclitaxel-resistant breast cancer cell lines, we exposed MCF-7 and MDA-MB-468 cells to increasing concentrations of paclitaxel for short times. MCF-7 is an estrogen receptor–positive, p53-wild-type breast cancer cell line whereas MDA-MB-468 is an estrogen receptor–negative, p53-mutated breast cancer cell line; they were chosen to represent the different genetic properties of primary breast cancer tumors. After resistant cell pools were developed, we selected individual resistant clones by continuous exposure to paclitaxel. Two different clones of MCF-7 with different levels of resistance against paclitaxel were generated and designated as MCF-7 TaxR30 and MCF-7 TaxR50. Additionally, a paclitaxel-resistant clone of MDA-MB-468 was established and designated as MDA-MB-468 TaxR. EC50 values for paclitaxel in parental and resistant cell lines were determined by nonlinear regression analysis of dose-response curves (Fig. 3A). MCF-7 TaxR30 and MCF-7 TaxR50 cells tolerated ~100- and ~150-fold greater doses of paclitaxel treatment in comparison with parental MCF-7 cells, respectively. Similarly, MDA-MB-468 TaxR cells tolerated ~15-fold greater doses of paclitaxel when compared with parental MDA-MB-468 cells.

The expressions of BCL-2 family proteins in parental and paclitaxel-resistant clones were compared. Because we were interested in permanent changes established by selection rather than changes due to presence of drug, we compared continuous paclitaxel to withdrawing paclitaxel from media for 24 hours before isolation of total proteins, although in practice this had little effect on our findings. We then analyzed proteins isolated from resistant clones grown in media in which paclitaxel was

![Figure 3](image)
either restored or absent. As shown in Fig. 3B, BCL-2 and BCL-X\textsubscript{L} levels were increased in MCF-7 TaxR30 and MCF-7 TaxR50 cells compared with parental cells. We could not detect any change in expression levels of other BCL-2 proteins. Correspondingly, there were decreased levels of both BCL-X\textsubscript{L} and BIM in paclitaxel-resistant MDA-MB-468 TaxR cells in comparison with parental MDA-MB-468 cells (Fig. 3C). Moreover, no significant alterations in other BCL-2 protein family members were found in MDA-MB-468 TaxR cells. These results indicate that the acquired resistance to paclitaxel may be promoted either by increased levels of anti-apoptotic BCL-2 proteins (BCL-2 and BCL-X\textsubscript{L}) or by decreased levels of proapoptotic BH3-only proteins (BIM) in breast cancer cells.

BCL-2 inhibitor ABT-737 restores the sensitivity to paclitaxel in paclitaxel-resistant breast cancer cells. ABT-737 antagonizes BCL-2 function by acting as a mimetic of the BH3 domain of proapoptotic BH3-only proteins. Thus, it seemed possible that treatment with ABT-737 might reverse resistance induced by either increased expression of antiapoptotic BCL-2 and BCL-X\textsubscript{L} or decreased expression of the proapoptotic BH3-only proteins BIM. As shown in Fig. 4A, treatment of paclitaxel-resistant MCF-7 TaxR30 and MCF-7 TaxR50 cells with ABT-737 resulted in restoration of paclitaxel sensitivity in these cells as determined by Annexin V staining. To verify that the observed effects were occurring via the mitochondrial pathway, we monitored cytochrome c release from mitochondria. Paclitaxel treatment induced mitochondrial cytochrome c release in parental MCF-7 cells, and as seen in Fig. 4B, increased sensitivity of MCF-7 TaxR50 to paclitaxel when combined with ABT-737 was also accompanied by increased translocation of cytochrome c into cytosol. To further delineate the molecular mechanisms of ABT-737–mediated sensitization to paclitaxel-induced apoptosis, MCF-7 TaxR50 cells were treated with paclitaxel, ABT-737, or

**Figure 4.** ABT-737 restores sensitivity to paclitaxel in paclitaxel-resistant MCF-7 cells. A, MCF-7 TaxR30 and MCF-7 TaxR50 cells were treated with paclitaxel (300 nmol/L), ABT-737 (300 nmol/L), or the combination of paclitaxel (300 nmol/L) and ABT-737 (300 nmol/L) for 48 h. The percent apoptotic response was evaluated by Annexin V staining. Columns, mean of three independent experiments; bars, SE. *, \( P < 0.05 \); **, \( P < 0.01 \). Paclitaxel plus ABT-737–treated with respect to paclitaxel only–treated. B, MCF-7 cells were treated with paclitaxel (100 nmol/L) for 0 to 48 h. MCF-7 TaxR50 cells were treated as in A. Cytosolic and mitochondrial fractions were blotted for cytochrome c. CoxIV was probed as a loading control for mitochondrial fractions. C, MCF-7 TaxR50 cells were treated with paclitaxel (300 nmol/L), ABT-737 (300 nmol/L), or the combination of paclitaxel (300 nmol/L) and ABT-737 (300 nmol/L) for 12 h. Activation of BAX and BAK was analyzed by immunoprecipitation with active conformation-specific anti-BAX (6A7) and anti-BAK (Ab-2) antibodies followed by immunoblot analysis of BAX and BAK. Five percent of the input for immunoprecipitation was also subjected to immunoblot analysis. \( \mu \text{g} \)–Actin was probed as a loading control. D, left, MCF-7 TaxR50 cells were treated as in A, and activation of caspase-9 and caspase-8 was evaluated by fluorometric caspase activation assays. Columns, mean relative fluorescence units from three independent experiments; bars, SE. Middle, total cell extracts were analyzed for the activation of caspase-9 and caspase-8 by immunoblotting. \( \mu \text{g} \)–Actin was probed as a loading control. Right, MCF-7 TaxR50 cells were pretreated with 20 \( \mu \text{mol/L} \) pancaspase inhibitor (Z-VAD-FMK), 20 \( \mu \text{mol/L} \) caspase-9 inhibitor (Z-LEHD-FMK), and 20 \( \mu \text{mol/L} \) caspase-8 inhibitor (Z-IETD-FMK) before treatment with paclitaxel plus ABT-737 for 48 h. The percent apoptotic response was evaluated by Annexin V staining. Columns, mean of three independent experiments; bars, SE.
both, and activation of BAX and BAK was evaluated by immunoprecipitation with conformation-specific antibodies. When paclitaxel-resistant cells were treated with the combination of paclitaxel and ABT-737, a significantly increased activation of BAX and BAK was detected in MCF-7 TaxR cells (Fig. 4C). No such effect was observed on exposure to either paclitaxel or ABT-737 alone. Thus, as expected, ABT-737 targets a pathway upstream of BAX and BAK activation in MCF-7 TaxR cells to promote sensitization to paclitaxel-induced apoptosis.

To confirm the engagement of caspases downstream of mitochondria, we assayed the activation of caspase-9 and caspase-8 in MCF-7 TaxR cells by immunoblot analysis and fluorometric caspase activation assays in response to treatment with paclitaxel, ABT-737, or the combination of both. As shown in Fig. 4D (middle), elevated levels of active caspase-9 were induced by paclitaxel and ABT-737 when compared with treatment with paclitaxel alone or CB-737 alone in MCF-7 TaxR50 cells. In contrast, caspase-8 was not activated in this cell line. Fluorometric caspase assays further confirmed the enhanced activation of caspase-9 by the combination of paclitaxel and ABT-737 in MCF-7 TaxR50 cells (Fig. 4D, left). Additionally, pretreatment with pancaspase inhibitor z-VAD-FMK and caspase-9 inhibitor z-LEHD-FMK markedly abolished ABT-737-mediated sensitization of MCF-7 TaxR50 cells to paclitaxel-induced apoptosis, although caspase-8 inhibitor z-IETD-FMK did not elicit such a similar effect (Fig. 4D, right). Thus, ABT-737 restores taxane sensitivity to resistant MCF-7 cells via the mitochondrial apoptotic pathway controlled by BCL-2 family proteins, consistent with ABT-737 acting at the expected target.

Similarly, the combination of paclitaxel and ABT-737 also led to a restored apoptotic response in MDA-MB-468 TaxR cells (Fig. 5A).

Figure 5. ABT-737 restores sensitivity to paclitaxel in paclitaxel-resistant MDA-MB-468 cells. A, MDA-MB-468 cells were treated with 100 nmol/L paclitaxel for 48 h. MDA-MB-468 TaxR cells were treated with paclitaxel (100 nmol/L), ABT-737 (200 nmol/L), or the combination of paclitaxel (100 nmol/L) and ABT-737 (200 nmol/L) for 48 h. The percent apoptotic response was evaluated by Annexin V staining. Columns, mean of three independent experiments; bars, SE. **, P < 0.01, paclitaxel plus ABT-737–treated with respect to paclitaxel only–treated. B, MDA-MB-468 cells were treated with paclitaxel (100 nmol/L) for 0 to 48 h; MDA-MB-468 TaxR cells were treated as in A. Cytosolic and mitochondrial fractions were blotted for cytochrome c. CoxIV was probed as a loading control for mitochondrial fractions. C, MDA-MB-468 TaxR cells were treated with–paclitaxel (100 nmol/L), ABT-737 (200 nmol/L), or the combination of paclitaxel (100 nmol/L) and ABT-737 (200 nmol/L) for 12 h. Activation of BAX and BAK was analyzed by immunoprecipitation with active conformation-specific anti-BAX (6A7) and anti-BAK (Ab-2) antibodies followed by immunoblot analysis of BAX and BAK. Five percent of the input for immunoprecipitation was also subjected to immunoblot analysis. h-Actin was probed as a loading control. D, left, MDA-MB-468 TaxR cells were treated as in A, and activation of caspase-3, caspase-9, and caspase-8 was evaluated by fluorometric caspase activation assays. Columns, mean relative fluorescence units from three independent experiments; bars, SE. Middle, total cell extracts were analyzed for activation of caspase-3, caspase-9, and caspase-8 by immunoblotting. h-Actin was probed as a loading control. Right, MDA-MB-468 TaxR cells were pretreated with 20 μmol/L pancaspase inhibitor (Z-VAD-FMK), 20 μmol/L caspase-9 inhibitor (Z-LEHD-FMK), and 20 μmol/L caspase-8 inhibitor (Z-IETD-FMK) before treatment with paclitaxel plus ABT-737 for 48 h. The percent apoptotic response was evaluated by Annexin V staining. Columns, mean of three independent experiments; bars, SE.
On the contrary, no such effect was observed in parental MDA-MB-468 cells when ABT-737 was used in combination with paclitaxel (0–100 nmol/L; results not shown). Treatment of MDA-MB-468 parental cells with paclitaxel (100 nmol/L) triggered apoptosis (Fig. 5A) and induced the release of cytochrome c into cytoplasm (Fig. 5B). In parallel to apoptotic response, paclitaxel plus ABT-737 treatment resulted in the release of cytochrome c into cytosol in MDA-MB-468 TaxR cells, although treatment with paclitaxel or ABT-737 alone did not show such an effect (Fig. 5B). Activation of BAX and BAK was also drastically enhanced in MDA-MB-468 TaxR cells treated paclitaxel plus ABT-737 in comparison with treatment with paclitaxel or ABT-737 alone (Fig. 5C). In addition, activation of caspase-3, caspase-9, and caspase-8 was detected when MDA-MB-468 TaxR cells treated with paclitaxel plus ABT-737 as shown by immunoblot analysis and fluorometric caspase activation assays (Fig. 5D, left, middle). Correspondingly, pretreatment with ABT-737 and paclitaxel on colony formation in paclitaxel-resistant MCF-7 and MDA-MB-468 cells. A, MCF-7 TaxR50 cells were treated with paclitaxel (300 nmol/L), ABT-737 (300 nmol/L), or the combination of paclitaxel (300 nmol/L) and ABT-737 (300 nmol/L) for 16 h. MDA-MB-468 TaxR cells were treated with paclitaxel (100 nmol/L), ABT-737 (200 nmol/L), or the combination of paclitaxel (100 nmol/L) and ABT-737 (200 nmol/L) for 16 h. Clonogenic survival was assessed by colony-forming assay. Data presented are percentage of colony formation normalized to untreated control cells. B, model of alterations in BCL-2 protein family members governing paclitaxel resistance. i, paclitaxel-sensitive cells; ii, paclitaxel-resistant cells, similar to MCF-7 TaxR50; iii, paclitaxel-resistant cells, similar to MDA-MB-468 TaxR.
z-VAD-FMK and z-LEHD-FMK attenuated ABT-737-mediated sensitization of MDA-MB-468 TaxR cells to paclitaxel-induced apoptosis; caspase-8 inhibitor z-IETD-FMK exerted only a partial protective effect (Fig. 5D, right).

To further show the cytotoxic effect of combining ABT-737 with paclitaxel in paclitaxel-resistant cell lines, MCF-7 TaxR50 and MDA-MB-468 TaxR cells were treated with paclitaxel, ABT-737, or the combination of both for 16 hours and the survival capability of cells was evaluated by colony formation assays. As shown in Fig. 6A, the combination of paclitaxel with ABT-737 led to the loss of colony formation capability of paclitaxel-resistant cells, although treatment with paclitaxel or ABT-737 did not exert a similar effect.

Thus, alterations of BCL-2 family proteins may contribute to the acquired resistance to paclitaxel in breast cancer cells, and ABT-737 could selectively restore paclitaxel-induced apoptosis via the mitochondrial pathway in these resistant cells. A summary of these alterations is presented in Fig. 6B. Briefly, in paclitaxel-sensitive cells before treatment, antiapoptotic BCL-2 proteins are present in sufficient quantity to sequester existing activator BH3-only proteins. Following paclitaxel treatment, additional BH3-only proteins are activated by changes in subcellular localization, increase in protein abundance, or posttranslational modifications. These new proteins compete for the BH3 binding site in antiapoptotic BCL-2 proteins, exceeding the total BH3 binding capacity. Unsequestered activator BH3-only proteins, including BIM, trigger apoptosis by activating BAX and BAK (Fig. 6A). We found that cancer cells could select to block this chain of events in two ways: First, in the MCF-7 cells, apoptosis could be blocked by increased expression of antiapoptotic BCL-2 proteins, preventing BAX and BAK activation through sequestration of more activators and sensitizers (Fig. 6B). Second, as in the MDA-MB-468 cells, decreased expression of BH3-only activators reduces the proapoptotic load so that death signaling induced by paclitaxel is insufficient to overwhelm the antiapoptotic BH3 domain binding capacity (Fig. 6C). ABT-737 can reverse these mechanisms of resistance by restoring increased competition for BH3 domain binding sites, thus yielding more unsequestered activator BH3-only proteins to activate BAX and BAK.

Discussion

Acquired resistance to treatment negatively affects the outcome of chemotherapy in solid tumors and hematologic malignancies (1). Many tumors show an initial sensitivity to chemotherapy, resulting in a good clinical response, even a complete remission with no remaining evidence of disease. All too often, however, the cancer recurs in a form that is newly resistant to the initially successful chemotherapy.

Many chemotherapeutics, including paclitaxel, have been shown to exert their effect through engagement and activation of the intrinsic apoptotic pathway (1, 32–34). However, most studies would reverse the resistance. The restoration of sensitivity to paclitaxel in breast cancer cell lines, that a key mediator of acquired resistance to paclitaxel in cancer cells.

In fact, our findings indicate, in two independently derived paclitaxel-resistant cell lines, that a key mediator of acquired resistance is indeed alteration in the control of the mitochondrial apoptotic pathway. As none of these changes was enforced, but rather selected by random mutation, it suggests that such alterations in apoptotic signaling may be quite important in cancers that acquire resistance to paclitaxel. BCL-XL is required for paclitaxel-induced apoptosis (37–39). It seemed, therefore, that alterations in the intrinsic apoptotic pathway might well play an important and heretofore unexamined role in the acquisition of resistance to paclitaxel in cancer cells.

We provide evidence here that paclitaxel uses the mitochondrial apoptotic pathway to induce apoptosis in breast cancer cell lines, consistent with previous reports (35, 36) When drugs kill using this pathway, they do so by increasing the balance of proapoptotic proteins compared with antiapoptotic proteins at the mitochondria. BH3-only proteins are often the most dynamic participants in this process, and we and others have found that, indeed, signaling by BH3-only proteins is required for paclitaxel-induced apoptosis (37–39).1 It seemed, therefore, that alterations in the intrinsic apoptotic pathway might well play an important and heretofore unexamined role in the acquisition of resistance to paclitaxel in cancer cells.

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References


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