Influence of Bcl-2 Family Members on the Cellular Response of Small-Cell Lung Cancer Cell Lines to ABT-737


Abstract

ABT-737 is a novel and potent Bcl-2 antagonist with single-agent activity against small-cell lung cancer (SCLC) cell lines. Here, we evaluated the contribution of Bcl-2 family members to the in vitro cellular response of several SCLC cell lines to ABT-737. Relatively higher levels of Bcl-2, Bcl-XL, Bim and Noxa, and lower levels of Mcl-1 characterized naïve SCLC cell lines that were sensitive to ABT-737. Conversely, a progressive decrease in the relative levels of Bcl-2 and Noxa and a progressive increase in Mcl-1 levels characterized the increased resistance of H146 cells following chronic exposure to ABT-737. Knockdown of Mcl-1 with small interfering RNA sensitized two resistant SCLC cell lines H196 and DMS114 to ABT-737 by enhancing the induction of apoptosis. Likewise, up-regulation of Noxa sensitized H196 cells to ABT-737. Combination treatment with DNA-damaging agents was extremely synergistic with ABT-737 and was associated with the down-regulation of Mcl-1 and the up-regulation of Noxa, Puma, and Bim in H196 cells. Thus, SCLC cells sensitive to ABT-737 expressed the target proteins Bcl-2 and Bcl-XL, whereas Mcl-1 and factors regulating Mcl-1 function seem to contribute to the overall resistance of SCLC cells to ABT-737. Overall, these observations provide further insight as to the mechanistic bases for ABT-737 efficacy in SCLC and will be helpful for profiling patients and aiding in the rational design of combination therapies. [Cancer Res 2007;67(3):1176–83]

Introduction

Lung cancer is the leading cause of cancer-related death in the industrialized Western nations, and there are more than 1 million new cases of lung cancer reported worldwide each year (1). Small-cell lung cancer (SCLC) makes up ~15% to 20% of all lung cancers, and it is strongly associated with smoking and tends to grow and spread quickly. If left untreated, the median survival time of SCLC patients is 2 to 4 months (2). Initially, most SCLC tumors respond to chemotherapy and radiotherapy. Typical chemotherapies involve platinum-based regimens combined with etoposide (3–5). Unfortunately, relapse is common because the tumors become resistant to further treatment, with a 5-year survival rate of ~5% to 10%. One of the factors implicated in the resistance of SCLC to chemotherapy is the overexpression of the antiapoptotic protein Bcl-2 (6–9). Unlike other oncogenes, Bcl-2 promotes tumorigenesis by attenuating cell death as opposed to promoting cell proliferation (10, 11). Thus, Bcl-2 allows tumor cells to ignore environmental cues that signal cells to undergo apoptosis.

The bcl-2 oncogene was the first member of about 20 evolutionarily conserved genes identified that encode closely related proteins that possess either proapoptotic or antiapoptotic activity (11, 12). The interaction of the Bcl-2 family members with each other is largely responsible for the commitment of cells to undergo apoptosis (13). One distinguishing feature is that they share up to four Bcl-2 homology (BH) domains (14). The multi-domain antiapoptotic Bcl-2 members, such as Bcl-XL, Bcl-2, Bcl-w, A1, and Mcl-1, inhibit cytochrome c release by blocking the activation of the multidomain proapoptotic proteins Bax and Bak (15). Bax and Bak are direct mediators of apoptosis and are absolutely required for the initiation of the mitochondrial apoptosis pathway (16, 17). The other proapoptotic members, such as Bad, Bik, Bid, Bim, Hrk, Bmf, Noxa, and Puma, act as molecular sensors of cellular stress or damage (18). They are distinguished from Bax and Bak in that they possess only the BH3 domain. They are mobilized and activated to initiate apoptosis by modulating the activity of the other Bcl-2 family members (17, 19–22).

The overexpression of Bcl-2 has been observed in a variety of cancers and contributes to chemotherapeutic resistance (23). In SCLC patients, the overexpression of Bcl-2 has been reported to occur in 55% to 90% of all SCLC cases, and it has been suggested to be a key factor involved in both the genesis and maintenance of SCLC (24–28). Studies have shown that Bcl-2 also plays a direct role in SCLC cell resistance to external apoptotic stimuli (8, 9, 29, 30). For example, the apoptotic response of SCLC cells to mitomycin C, irinotecan, and Adriamycin could be inhibited by ectopically overexpressing Bcl-2 (8). Conversely, treatment with Bcl-2 antisense oligodeoxynucleotides acted synergistically with etoposide, doxorubicin, and cisplatin to enhance the cell killing of SCLC cell lines in vitro (31).

There have been several efforts to target Bcl-2 family members for the treatment of neoplastic disease given their association with cancer and resistance to numerous cytotoxic drugs (32–34). Recently, we reported the discovery and biological properties of ABT-737, a novel and potent Bcl-2 antagonist developed at Abbott Laboratories (35). ABT-737 binds with high affinity (Ki ≤ 1 nmol/L) to Bcl-2, Bcl-XL, and Bcl-w and is two to three orders of magnitude more potent than previously reported compounds (36). ABT-737 does not, however, bind strongly to Bcl-B, Mcl-1, or A1 (Ki ≥ 0.46 ± 0.11, >1, and >1 μmol/L, respectively). ABT-737 exhibited single-agent activity against several cell lines in vitro and in vivo. However,
the response varied depending on the cell line, indicating that one or more cellular-based mechanisms were contributing to the sensitivity to ABT-737.

Here, we evaluated the contribution of Bcl-2 and other Bcl-2 family members to the cellular response of SCLC cell lines to ABT-737 by first establishing the co-relationship of Bcl-2 family members with sensitivity to ABT-737 in a panel of 11 naïve SCLC cell lines and in the progressive adaptation of the sensitive cell line H146 to ABT-737. Because correlative expression may only be an epiphenomenon, we evaluated the mechanistic contribution of two family members, Mcl-1 and Noxa, to SCLC cellular resistance to ABT-737 by down-regulating Mcl-1 or ectopically overexpressing Noxa in resistant SCLC cell lines. Finally, we show that the cellular sensitivity of H196 cells treated in combination with DNA-damaging agents and ABT-737 was synergistic, and that the increase in sensitivity was associated with decreased Mcl-1 levels and increased levels of the BH3-only proteins Noxa, Puma, and Bim.

Materials and Methods

Chemicals. ABT-737 (4R)-4-(3-dimethylamino-1-phenylsulfanyl methylpropylamino)-N-[4-[4-(4-chloro-biphenyl-2-ylmethyl)-piperazin-1-yl]-benzoyl]-3-nitro-benzenesulfonamide) was synthesized at Abbott Laboratories (Abbott Park, IL). Carboplatin and etopside were purchased from Sigma (St. Louis, MO).

Cell culture. The SCLC cell lines NCI-H889, NCI-H1963, NCI-H1417, NCI-H146, NCI-H187, DMS79, NCI-1048, NCI-H196, H69AR, and DMS114 were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 (Invitrogen Corp, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen), 1% sodium pyruvate, 25 mmol/L HEPES, 4.5 g/L glucose, and 1% penicillin/streptomycin (Sigma). All cell lines were maintained in a humidified chamber at 37°C containing 5% CO2.

The ABT-737-resistant H146 cells were derived from H146 cells by initially adding 40 nmol/L ABT-737 to their culture medium and thereafter doubling the concentration over a period of several months. Several drug-resistant variants that adapted to and maintained in the presence of 40, 80, 160, 320, 640, and 1,280 nmol/L ABT-737 were generated in this manner and were designated as H146-40, H146-80, etc.

Cell treatment and viability assays. Cells were treated for 48 h in 96-well tissue culture plates in a total volume of 100 μL of tissue culture medium supplemented with 10% human serum (Invitrogen). Each concentration was tested in duplicate at least thrice separately. Viable cells were determined using the CellTiter 96 AQueous nonradioactive cell proliferation MTS assay (Promega Corp., Madison, WI). The activation of caspase 3 was measured by the cleavage of the fluorometric substrate Ac-DEVD-AMC (Biomol Research Laboratories, Plymouth Meeting, PA) or Z-DEVD-R110 (Promega) as described previously (37). For combination treatment, H196 cells were plated at 1 x 10^4 cells/100 μL and treated concurrently with increasing concentrations of carboplatin/etoposide (at a fixed ratio of 2.5:1) and ABT-737 in the presence of 10% human serum for 72 h. Cell viability was determined using Promega’s CellTiter Glo assay. The combination index (CI) as described by Chou and Talalay (38) was assessed using the software program CalcuSyn (Biosoft, Ferguson, MO) to quantitatively assess the effect of drug-combination treatment. A CI value of 1.0 indicated an additive effect, CI values of <1.0 reflected a synergistic effect, and a CI value of >1.0 reflected an antagonistic effect.

Western blot analysis. Total protein from cell lysates were separated on 4% to 20% gels by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). The membranes were blocked with TBS containing 5% nonfat dry milk and 0.1% Tween 20 (blocking buffer) for 1 h at room temperature and then incubated overnight at 4°C with antibodies diluted in blocking buffer. We used anti-Bcl-XL (1 μg/mL; 2300-Mc), anti-Bax (1 μg/mL; 807014), and anti-Bid (1 μg/mL; AF-846) from R&D Systems (Minneapolis, MN); anti-Bcl-2 (1:500; 610539) and anti-Mcl-1 (1:500; 554103) from BD PharMingen (San Diego, CA); anti-Bak (1:1000; 3792) and anti-αPuma (1:250; 4976) from Cell Signaling (Beverly, MA); anti-Bim (1 μg/mL; AAP-330) from Stressgen (Victoria, BC); anti-Noxa (2 μg/mL; IMG-349) from Imgenex (San Diego, CA); and anti-actin (1:10,000; ab6276) from Abcam (Cambridge, MA). Primary antibodies were detected using either a goat anti-mouse or goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce Biotechnology, Rockford, IL) diluted 1:500 in blocking buffer for 0.5 h at room temperature. The membranes were washed after each antibody step thrice for 5 min with TBS containing 0.2% Tween 20. Bands were detected using Enhanced Chemiluminescence Plus (GE Healthcare, Piscataway, NJ) and a STORM 860 molecular imager (GE Healthcare). Quantitation of the protein level changes was done by densitometry using ImageQuant (GE Healthcare). The percent increase in protein levels is defined as ((band intensity at time x - band intensity at time 0 h)/band intensity at time 0 h) x 100%.

Transfection protocols. H196 cells were plated at 1 x 10^6 cells/100 μL in 96-well tissue culture plates and allowed to attach overnight. Small interfering (si) RNA transfections were done using four siRNA duplexes directed at different regions of the Mcl-1 gene (M-004501-02, M-004501-07, M-004501-08, and M-004501-09, designated as siRNA-1, siRNA-2, siRNA-3,
and siRNA-4, respectively) or scrambled siRNA pool (four negative control siRNAs, D-001206-13) from Dharmacon (Lafayette, CO). For Mc-1 siRNA transfections, 1.5 μL of a 20-μmol/L stock of Mc-1 siRNAs and 3 μL of LipofectAMINE 2000 (Invitrogen) were each incubated separately with 100 μL Opti-MEM (Invitrogen) for 10 min, mixed together for 20 min at room temperature, and then 20 μL was applied to the cells (final siRNA concentration was 25 nmol/L). After incubating the cells for 3 to 4 h, the medium was changed to RPMI supplemented with 10% human serum. The following day, siRNA-transfected cells were either treated with ABT-737 for 48 h, and cell viability was assessed using Perkin-Elmer's (Boston, MA) ATPlite assay and used to measure the cleavage of Z-DEVD-R110 before and following day, siRNA-transfected cells were either treated with ABT-737 for 48 h after the addition of ABT-737 to assess the induction of apoptosis. DMS114 siRNA transfections were done using the siRNA duplex for Mc-1 (M-005301-02; siRNA-1), or scrambled siRNA (D-001210-01) from Dharmacon as described above, and their viability was determined using the MTS assay.

For Noxa transfections, H196 were plated at 6.0 to 7.5 × 10⁵ cells in six-well tissue culture plates and allowed to attach overnight. The Flag-tagged Noxa expression vector (1 or 2 μg, GNO12925, ReSeq no. NM_021127, Genscript Corp., Piscataway, NJ) or control pDream2.1-cGFP vector (SD0220, Genscript), and 10 μL LipofectAMINE 2000 (Invitrogen) was added to 250 μL Opti-MEM. Both were incubated for 10 min separately and then together for 20 min at room temperature. The mixture (500 μL) was added to the cells in 2 mL of culture medium. After incubating the cells for 3 h, the medium was changed with fresh medium containing 10% human serum. The following day, plasmid-transfected cells were transferred to 96-well plates at 10 × 10⁴ cells per well and treated with ABT-737 for 48 h in medium containing 10% human serum. Cell viability was assessed using Promega’s MTS assay.

**Microarray analysis.** Total RNA was isolated from cell lines, and 5 μg was used for microarray analysis using the standard protocol provided by Affymetrix Inc. (Santa Clara, CA). Fragmented, labeled cRNA synthesized using an IVT labeling kit, (Enzo, New York, NY) was hybridized to an Affymetrix human genome U133A version 2.0 Affymetrix microarrays at 45°C overnight. The scanned image and intensity files were imported in the Rosetta Resolver gene expression analysis software version 5.1 (Rosetta Inpharmatics, Kirkland, WA). Resolver’s Affymetrix error model was applied, and replicates were combined. Combined intensity values were exported into Spotfire (Spotfire, Inc., Somerville, MA) to generate heat maps, with red indicating a high expression and green indicating a low expression using intensity z score values normalized for each gene individually, across all experiments being analyzed. Expression profiles were derived from mRNA from two or more independent samples for each cell line.

**Results**

**ABT-737 inhibits proliferation and induces apoptosis in a subset of SCLC cells.** The ability of ABT-737 to inhibit cell proliferation with single-agent activity was evaluated against a panel of 11 naïve SCLC cell lines (Fig. 1). The cellular response based on the cell proliferation EC₅₀ values ranged from as low as 20 nmol/L for NCI-H889 cells to >100 μmol/L for DMS114 cells following treatment for 48 h (Fig. 1A). To assess the ability of ABT-737 to directly induce apoptosis, we measured the cleavage of Ac-DEVD-AMC, a substrate for activated caspase 3, following treatment of H146 cells for 24 h. A dose-dependent increase in apoptosis coincided with a dose-dependent decrease in cell viability following ABT-737 treatment (Fig. 1B) suggesting that ABT-737 inhibits cell proliferation through the induction of apoptosis.

**Expression levels of Bcl-2 family proteins that correlate with cellular response to ABT-737.** The relative mRNA and protein expression profiles of several Bcl-2 family members were evaluated in 11 SCLC cell lines to determine if their expression patterns correlated with cellular response to ABT-737 using microarray and Western blot techniques. From the microarray analysis, Bcl-2 and Noxa mRNA levels correlated directly with the cellular response to ABT-737 (Fig. 2A; Supplemental Table 1) such that Bcl-2 and Noxa expression were higher in cells that were more sensitive and lower in cells that were more resistant to ABT-737. The same expression pattern of Bcl-2 and Noxa were evident also at the protein level (Fig. 2B). The protein levels of Bcl-2 also paralleled that of Bcl-2; however, it was not reflected at the RNA level, suggesting that
Bcl-XL expression may be differentially regulated at the translational or protein stability level. The protein levels of the proapoptotic Bcl-2 family member Bim also trended similarly to Bcl-2 such that it was lower in cells lines with increased resistance to ABT-737.

Unlike Bcl-2 and Bcl-XL, an inverse correlation with cell sensitivity was observed with the antiapoptotic Bcl-2 family member, Mcl-1. Mcl-1 mRNA and protein levels were higher in cells that were resistant to ABT-737 and lower in cells that were sensitive to ABT-737 (Fig. 2B). As for the other proapoptotic family proteins Bax, Bak, and Bid, there was no clear correlation between their expression and cellular response to ABT-737.

Bcl-2 and Noxa levels decrease and Mcl-1 levels increase in H146 cells following chronic exposure to ABT-737. We next evaluated the relative changes to the expression levels of Bcl-2 family members in H146 cells that became progressively resistant to ABT-737 following continuous exposure for several months. Several drug-resistant variants that were adapted to and maintained in the presence of 40, 80, 160, 320, 640, and 1,280 nmol/L ABT-737 were generated. The cellular sensitivity for the resistant variants ranged from an EC_{50} of 100 nmol/L for the parental cells up to 1.5 μmol/L for the H146-1280–resistant variants (Fig. 3A).

A reduction in the expression of both Bcl-2 and Noxa was observed by microarray and Western blot analysis upon progressive adaptation to ABT-737 in H146 cells (Fig. 3B and C; Supplemental Table 2). On the other hand, there was a progressive increase in Mcl-1 expression levels as H146 cells adapted to ABT-737. Because similar trends were observed in the naïve SCLC panel of cells, it is suggestive that these proteins may contribute mechanistically to the cellular response to ABT-737.

Decrease in Mcl-1 expression with siRNA sensitizes H196 cells to ABT-737. Given that expression correlations alone are not sufficient to show a mechanistic role in resistance, functional validation studies were conducted using RNAi technology. We initially transfected one of the highly resistant SCLC cell lines, H196, with Mcl-1 siRNA to show a causal relationship between Mcl-1 expression and cellular resistance to ABT-737. H196 cells (EC_{50} ~ 55 μmol/L) were transfected with four different Mcl-1–specific siRNAs at a final concentration of 25 nmol/L and then treated with or without 20 μmol/L ABT-737 (EC_{50}). Transfection with each of the four Mcl-1 siRNAs resulted in knockdown in Mcl-1 protein levels compared with the scrambled siRNA control, but had minimal effects on cell growth alone (Fig. 4A and B). Importantly, Mcl-1 knockdown in the presence of ABT-737 corresponded to enhanced H196 cell death (Fig. 4B). Notably, siRNA-1 and siRNA-4 that decreased Mcl-1 levels to the greatest extent also sensitized H196 cells to ABT-737 to the greatest degree. There was a clear dose response to ABT-737 in the presence of 25 nmol/L Mcl-1 siRNA-1 with an apparent EC_{50} of 0.14 μmol/L, which represented approximately a 400-fold increase in the sensitivity of H196 cells to ABT-737 treatment (Fig. 4C). Increased sensitivity to ABT-737 was also observed following Mcl-1, but not scrambled siRNA transfection of the most resistant SCLC cell line, DMS114 (Fig. 4C). Unlike H196, however, the viability of DMS114 cells was reduced by >50% following Mcl-1 siRNA transfection alone and was further reduced following treatment with ABT-737. The apparent EC_{50} of ABT-737 in DMS114 cells following Mcl-1 transfection was <0.2 μmol/L. In both cell lines, an analogous dose response in the presence of 25 nmol/L scrambled siRNA did not enhance the cellular sensitivity of ABT-737, suggesting that the increased sensitivity was specific to the knockdown of Mcl-1 and not due to the transfection conditions alone.

To determine the mechanism by which Mcl-1 knockdown mediated its effect on ABT-737 sensitivity, the induction of apoptosis was accessed in cells treated 24 h posttransfection...
with ABT-737 for 6 h. Increases in ABT-737 mediated apoptosis was observed in both H196 and DMS114 cells transfected with Mcl-1, but not scramble, siRNA relative to each cell lines scramble controls (Fig. 4D). Consistent with the cell viability dose-response data (Fig. 4C), the induction of apoptosis was greater in DMS114 cells as compared with H196 cells, following Mcl-1 siRNA transfection alone and in combination with ABT-737.

**Noxa overexpression sensitizes H196 cells to ABT-737.** To investigate the relationship between Noxa and cellular response to ABT-737, we transfected H196 cells with a Noxa or control expression vector, and cell viability was accessed following treatment with or without 20 μmol/L ABT-737. Transfection with the Noxa expression vector resulted in enhanced expression of Noxa (Fig. 5A). The increased expression of either Noxa or ABT-737 treatment alone slightly reduced the viability of H196 cells. However, the combination of both Noxa overexpression and ABT-737 treatment decreased H196 viability by ~50% to 60% as compared with the nontransfected controls (Fig. 5B). The viability of the control vector–transfected cells was similar to the nontransfected controls, suggesting that the increased sensitivity to ABT-737 was due to the expression of Noxa and not the transfection conditions alone.

**Synergy between carboplatin/etoposide and ABT-737 involves the down-regulation of Mcl-1 and concurrent up-regulation of Puma, Noxa, and Bim in H196 cells.** We evaluated the effect of concurrent treatment of ABT-737 and carboplatin/etoposide (kept at a fixed ratio of 2.5:1) in H196 cells. H196 viability was ~90% of untreated controls following treatment with 1.56 μmol/L ABT-737 and 70%, 60%, and 40% following treatment with 4.7:1.9, 9.4:3.8, or 75:30 μmol/L carboplatin/etoposide, respectively, for 72 h (Fig. 6A). In contrast, cell viability following concurrent treatment with 4.7:1.9, 9.4:3.8, or 75:30 μmol/L carboplatin/etoposide with 1.56 μmol/L ABT-737 was 40%, 30%, and 20%, respectively. The CI as described by Chou and Talalay (38) was used to quantitatively assess the effect of the drug-combination treatment where the ratio of carboplatin, etoposide, and ABT-737 was kept at a fixed ratio of 48:19:1, respectively. The CI values between the dose-effect levels 0.50 to 0.90 were <0.3, indicating that the combination of carboplatin/etoposide and ABT-737 was strongly synergistic (Fig. 6B).
To determine if Mcl-1 played a role in the synergism observed with ABT-737/carboplatin/etoposide treatment, H196 cells were treated with carboplatin/etoposide at 100:40 μmol/L over a 24-h period. Carboplatin/etoposide treatment alone resulted in a decrease in Mcl-1 levels by 60% after 24 h (Fig. 6C). In contrast, Noxa, Puma, and Bim increased following carboplatin/etoposide treatment. Both Bim and Puma levels increased by 150% and 230%, respectively, 24 h after treatment. On the other hand, Noxa levels increased transiently by 74% at 6 h, as compared with untreated controls, and then decreased by 24 h (Fig. 6C). Over the course of 24 h, there was also a slight stabilization of p53, which is known to directly regulate Noxa and Puma (39). Quantitative PCR analysis of H196 cells treated with carboplatin/etoposide confirmed that both Noxa and Puma mRNA expression levels increased significantly within 4 h of treatment, indicating that the increase in protein levels was due to regulation at the transcript level (data not shown).

Discussion

Based on the expression studies in naïve SCLC cell lines, we show that higher levels of Bcl-2, Bcl-X<sub>L</sub>, Bim, and Noxa and lower levels of Mcl-1 characterize SCLC cell lines sensitive to ABT-737. The converse was true in the ABT-737-resistant H146 cells following chronic exposure to ABT-737. Here we observed a progressive decrease in the relative levels of Bcl-2 and Noxa and an increase in the relative levels of Mcl-1 with increasing resistance to ABT-737 in H146 cells. The fact that we observed similar changes in the relative levels of Bcl-2 family members in naïve and ABT-737-resistant H146 cells suggests that these changes play an important role in SCLC survival and response to ABT-737. The apparent association of Mcl-1 protein levels with increased resistance to ABT-737 and higher levels of Bcl-2 and Bcl-X<sub>L</sub> in sensitive cell lines is consistent with the specificity profile of ABT-737 because it binds tightly to the antiapoptotic members, Bcl-2 and Bcl-X<sub>L</sub>, but not Mcl-1 (35).

To evaluate the mechanistic contribution of Bcl-2 family members to the cellular response to ABT-737, we used both molecular and chemical approaches. We found that Mcl-1 knockdown sensitized SCLC cells to ABT-737 treatment by enhancing ABT-737–induced apoptosis. Mcl-1 knockdown seems to contribute to ABT-737 sensitivity by lowering the apoptotic threshold which is consistent with previous reports that show Mcl-1 and Bcl-X<sub>L</sub> both are potent inhibitors of apoptosis by inhibiting Bak activation (13, 20). The enhanced apoptosis when ABT-737 was used in combination with Mcl-1 knockdown indicates further that Mcl-1 is an important regulator of apoptosis, and that the combination of ABT-737 with other treatment therapies that modulate Mcl-1 has potential utility in SCLC malignancies.

Of interest, we observed that there was an increase in apoptosis in DMS114 cells following Mcl-1 knockdown alone. This is consistent with the idea that some cells are “primed for death,” and that they are dependent or “addicted” to certain Bcl-2 family members for survival (40). In this case, Mcl-1 would seem to play a crucial role in DMS114 cell survival, and inhibition of other antiapoptotic proteins such as Bcl-X<sub>L</sub> can further enhance cell death. Thus, Mcl-1 may play a critical role in SCLC drug resistance to ABT-737 by functionally compensating for other Bcl-2 antiapoptotic family members.

Here, we show that up-regulation of the BH3-only protein Noxa increased the sensitivity of H196 cells to ABT-737. The BH3-only proteins act as molecular sensors of cellular stress or damage, and they are activated to initiate apoptosis by modulating the activity of the other Bcl-2 family members (17–22). Recently, Noxa has been shown to specifically bind to Mcl-1 and A-1 but not Bcl-2, Bcl-X<sub>L</sub>, or Bcl-w (19). This highly specific interaction of Noxa for Mcl-1 suggests that it is a key regulator of Mcl-1. This was shown previously in transformed mouse embryo fibroblast cells where overexpression of Noxa was shown to promote Mcl-1 but not Bcl-X<sub>L</sub> degradation and promote the release of proapoptotic Bak from Mcl-1 (20). Furthermore, it was shown that Bak binds to both Bcl-X<sub>L</sub> and Mcl-1, and that a loss of Bcl-X<sub>L</sub> sensitized mouse embryo fibroblasts to Noxa-induced killing (20). Coincidentally, we observed higher Noxa levels in cell lines sensitive to ABT-737, and ectopic expression of Noxa in a resistant cell line increased their sensitivity to ABT-737. This suggests that Noxa may play an important role in governing the cellular response of SCLC cells to ABT-737, in part by neutralizing Mcl-1 in SCLC cells.

Carboplatin, cisplatin, and etoposide are cytotoxic agents that interfere with the DNA replication process during S phase and elicit a DNA damage response that causes cells to arrest and undergo apoptosis (41, 42). In cells containing wild-type p53, p53 induces the transcriptional up-regulation of several proapoptotic genes including BAX, PUMA, and NOXA (39). Here, we show that
combination treatment with carboplatin/etoposide and ABT-737 was extremely synergistic (CI < 0.3). This was associated with the down-regulation of Mcl-1 and the concerted up-regulation of multiple BH3-only proteins Noxa, Puma, and Bim. Noxa, Puma, and Bim have been shown to be potent inducers of apoptosis (13, 17, 19). Unlike Noxa, however, Puma and Bim bind with high affinity to all of the multidomain antiapoptotic Bcl-2 family members (19). Thus, ABT-737 synergism with carboplatin/etoposide may involve in part the combined activities of BH3-only proteins to neutralize the prosurvival activity of Mcl-1, together with the inhibition of Bcl-2/Bcl-X₅ by ABT-737. This suggests that the chemotherapeutics that neutralize Mcl-1 either directly or indirectly by modulating key factors that regulate it will enhance the activity of ABT-737.

Thus, a balance between antiapoptotic and proapoptotic proteins seems to govern the cellular response of SCLC cells to ABT-737. Presumably, the presence of intrinsic and extrinsic death signals necessitates the expression of one or more antiapoptotic Bcl-2 family members for SCLC survival. In SCLC cell lines sensitive to ABT-737, the expression of Bcl-2, Bcl-X₅, and, to a lesser extent, Mcl-1 governs cell survival. In SCLC cell lines resistant to ABT-737, elevated Mcl-1 expression seems to compensate for the decreased expression of Bcl-2 and Bcl-X₅. Lower expression levels of Noxa in resistant cell lines would further increase the antiapoptotic potency of Mcl-1. Similarly, lower levels of Puma and Bim would also increase the antiapoptotic potency of all prosurvival members. Overall, these data suggest that cell dependence on Bcl-2 and Bcl-X₅ for survival is requisite for cellular sensitivity to ABT-737, and their absence is indicative of resistance in SCLC cell lines. Conversely, the presence of Mcl-1 alone does not necessarily signify resistance; however, higher Mcl-1 expression may be one mechanism by which resistance can occur.

In summary, these findings provide compelling evidence that certain Bcl-2 family members play a role in the cellular response of SCLC cell lines to ABT-737. In a broader context, our results provide information that may be useful in the development of biomarkers for the stratification of SCLC patients and the design of better rational-based combination therapies for clinical trials. In addition, they provide further insight into the relative contribution and interaction of Bcl-2 family members in regulating apoptosis in SCLC that may lead to the design of more effective targeted therapies in the future.

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Figure 6. Synergistic interaction of ABT-737 and carboplatin/etoposide (C/E) in H196 cells. H196 cells were treated for 72 h either individually or concurrently with ABT-737 and carboplatin/etoposide. A, H196 cell viability following treatment with varying doses of ABT-737, carboplatin/etoposide, or concurrent treatment with ABT-737 and carboplatin/etoposide. Columns, mean of three independent experiments, n = 2. B, experimentally derived CI values at various dose-effect levels for the combination of carboplatin/etoposide and ABT-737 in H196 cells based on Chou-Talalay’s analysis method as described in Materials and Methods. Data were representative of three experiments. C, Western blot analysis of H196 cells following treatment with carboplatin/etoposide (100/40 μmol/L) for 0, 6, and 24 h.


