

Alterations in the Noxa/Mcl-1 axis determine sensitivity of small cell lung cancer to the BH3 mimetic ABT-737

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Abstract

To understand the molecular basis for variable sensitivity to the BH3 mimetic drug ABT-737, the abundance of Bcl-2 family members was assayed in a panel of small cell lung cancer cell lines whose sensitivity varied over a 2-log range. Elevated Noxa and Bcl-2 levels directly correlated with sensitivity to ABT-737, whereas Mcl-1 levels were similar in all cell lines tested regardless of sensitivity. Transgenically enforced expression of Noxa but not Bcl-2 resulted in increased sensitivity to ABT-737 in multiple cell lines. This increase was especially pronounced in the H209 cell line in which expression of Noxa resulted in a proportionate decline in Mcl-1 expression. Although overexpression of Noxa enhanced sensitivity of the H526 and H82 cell lines to ABT-737, it did not result in altered Mcl-1 levels. Similarly, small interfering RNA-mediated knockdown of Noxa expression in the H146 cell line, which increased resistance to ABT-737, did not result in altered Mcl-1 levels. Therefore, three of four cell lines studied failed to show Noxa-mediated regulation of Mcl-1 expression. However, despite failure to regulate Mcl-1 levels, Noxa blocked binding of Bim to Mcl-1 following its release from Bcl-2 by ABT-737. Finally, we observed that a 24-hour incubation of the H526 and WBA cell lines with ABT-737 resulted in increased Noxa expression, suggesting that Noxa may play a direct role in ABT-737-mediated apoptosis. These results indicate that Noxa expression is the critical determinant of ABT-737 sensitivity and loss of Noxa-mediated regulation of Mcl-1 expression may be an important feature of small cell lung cancer biology. [Mol Cancer Ther 2009;8(4):883–92]

Introduction

Small cell lung cancer (SCLC) accounts for ~15% of all lung cancers, with 90% to 95% of affected individuals dying of

the disease within 5 years (1). Although SCLC is initially a highly chemotherapy-responsive disease, relapse and progressive development of chemotherapy resistance is the rule. Trials of chemotherapy dose escalation or addition of multiple agents to standard two-drug regimens, such as cisplatin and etoposide, have resulted in minimal increases in response rates and increased toxicity without a survival advantage (1). Improvement in long-term survival will depend on the development of novel therapeutic approaches that can prevent or overcome the development of clinical resistance to cytotoxic agents. One promising approach would be to use agents that target molecular abnormalities that regulate resistance to apoptosis in SCLC.

Key molecular abnormalities in the pathogenesis of SCLC seem to be the inactivation of the *p53* and *Rb* tumor suppressor genes. It has been estimated that >90% of all SCLC tumors carry genetic alterations in *p53* and *Rb* (2). Two recently developed animal models of SCLC, each of which dually inactivate *p53* and *Rb* in bronchial epithelial cells using different methodologies (3, 4), confirm the seminal role of inactivation of both tumor suppressors in the initiation of SCLC. However, in both models, there is significant tumor latency, suggesting that additional genetic changes are necessary for the development of SCLC. One alteration seems to be overexpression of *Myc* family genes (5), which is a feature of >95% of SCLC tumors and cell lines (6, 7). Although expression of *Myc* stimulates proliferation, it also provides a potent apoptotic stimulus (8). Perhaps as a result, SCLC is highly dependent on signaling from the antiapoptotic phosphatidylinositol 3-kinase–Akt pathway, which is activated by growth factors, such as stem cell factor, insulin-like growth factor-I, or hepatocyte growth factor (9, 10), or by genetic alterations in *PTEN* (11) or *PIK3CA* (12). Another prominent feature of SCLC is overexpression of antiapoptotic members of the Bcl-2 family (13), which regulate apoptosis mediated by the intrinsic or mitochondrial pathway (14).

Mitochondrial integrity and cellular survival is promoted by antiapoptotic family members, such as Bcl-2 itself, Bcl-X_L, and Mcl-1 (14–16). They possess a hydrophobic binding groove, which can bind proapoptotic family members containing the conserved BH3 domain. Apoptosis is initiated when the free proapoptotic Bax and Bak proteins oligomerize and permeabilize the outer mitochondrial membrane, leading to activation of the caspase cascade. Under prosurvival conditions, the antiapoptotic family members bind to Bax and Bak and prevent mitochondrial membrane permeabilization. Under proapoptotic conditions, BH3-only proteins, such as Bim, Bid, Bad, Puma, and Noxa, compete for binding to the prosurvival members and displace Bax and Bak. Bim and Bid may also enhance the ability of free Bax and Bak to activate the apoptotic cascade (17). Bim, Bid, and Puma can bind to all of the prosurvival members, whereas Bad binds preferentially to Bcl-2

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and Bcl-X_L. Noxa is unique because its predominant binding partner is Mcl-1.

BH3 mimetics represent a novel class of drugs designed to promote tumor cell apoptosis by blocking interaction between the prosurvival and proapoptotic members of the Bcl-2 family. ABT-737, the prototypic drug in this class, efficiently blocks Bcl-2 and Bcl-X_L function but does not effectively bind Mcl-1 (18). It is very effective in augmenting apoptosis in many cases, but SCLC is one of the few tumor types in which it induces apoptosis without an exogenous apoptotic stimulus. However, there is considerable variation in the sensitivities of different SCLC cell lines to this drug (18, 19). Studies in a variety of cell types have suggested that indicators of sensitivity include low levels of Mcl-1 and high levels of Bcl-2, Bcl-X_L, Bim, and Noxa expression (19–25). In particular, studies in SCLC have documented that decreased Mcl-1 expression (19, 20) and amplification of a region of chromosome 18 containing both the *BCL-2* and *NOXA* genes correlates with increased sensitivity to ABT-737 (24).

To further explore the underlying basis for the diverse sensitivities of SCLC cell lines to ABT-737 with the hope of identifying important predictors of clinical sensitivity, the levels of the Bcl-2 family members were analyzed in a representative selection of cell lines. Critical candidates correlating with sensitivity were identified and their functional role in modulating ABT-737–induced apoptosis was prospectively studied through molecular manipulation of their expression in selected cell lines.

Materials and Methods

Compounds

ABT-737 {(R)-4-(3-dimethylamino-1-phenylsulfanyl-methyl-propylamino)-N-[4-[4-(4'-chloro-biphenyl-2-ylmethyl)-piperazin-1-yl]-benzoyl]-3-nitro-benzenesulfonamide)} was synthesized and kindly provided by Abbott. Etoposide (Calbiochem) and ABT-737 stock solutions were made in 100% DMSO (Sigma Chemical Co.) and diluted with culture medium before use. The final DMSO concentration in all cultures, including vehicle controls, was 0.1%.

Cell Culture

SCLC cell lines (NCI-H69, NCI-H69AR, NCI-H82, NCI-H146, NCI-H209, NCI-H526, and WBA) were maintained in RPMI 1640 (Life Technologies, Invitrogen Corp.) containing 10% (v/v) fetal bovine serum (Life Technologies), 2 mmol/L L-glutamine (Life Technologies), and 50 units/mL penicillin/streptomycin (Life Technologies) in a humidified chamber at 37°C containing 5% CO₂. H526, H209, and H82 transfected with the pCEP4 expression vector (Invitrogen) were maintained in the above medium containing hygromycin (Calbiochem) and then cultured without hygromycin for 48 h before and during experimentation.

Transfection of *NOXA* and *BCL-2* Expression Plasmids

The construction of the pCEP4-*BCL-2* expression plasmid has been previously described (26). A full-length cDNA encoding *NOXA* (*PMAIP1*) in the pCMV-SPORT6 expression vector (Invitrogen) was excised using a *KpnI/XhoI* restric-

tion digest and cloned into the *KpnI/XhoI* restriction sites of the pCEP4 expression vector (Invitrogen), which reproducibly produces high levels of constitutive expression in SCLC cell lines. Transfections of the above expression plasmids and the empty vector controls were conducted using the Amaxa nucleofector device (Amaxa) and proprietary solutions according to the manufacturer's instructions. Specifically, 1 × 10⁶ H526 and H82 cells were transfected with 2 μg DNA diluted in Solution L with program D-023 and H209 cells were transfected in Solution L using the X-01 program. After 72 h, cells were centrifuged and resuspended in medium containing hygromycin and plated in 96-well plates, and single-cell clones were selected over the ensuing 4 to 6 wk.

Silencing of Noxa Expression

Silencing of Noxa was accomplished by transfection of 2 × 10⁶ H146 cells in Solution L using Amaxa nucleofector program D-023, with 4 μmol/L Dharmacon (ThermoFisher) ON-TARGET plus SMARTpool small interfering RNA (siRNA) duplexes targeting *NOXA* (*PMAIP1*). Cells were maintained in growth medium for 24 h before use in cell viability, drug sensitivity, and expression studies as described. Low GC content nontargeting siRNA (Invitrogen) controls were transfected simultaneously under identical conditions.

Cell Growth and Viability Assay

Relative viable cell number was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical) colorimetric dye reduction method (27). Duplicate plates containing eight replicate wells per assay condition were seeded at a density of 1 × 10⁴ cells in 0.1 mL of medium and the relative change in viable cell number under the experimental conditions was determined by the change in absorbance at 540 nm over 72 h compared with initial values obtained 3 h after plating. For each experiment, a dose-response curve was generated by plotting the change in absorbance compared with the vehicle control or the fraction affected [FA = 1 – (experimental/control absorbance at 72 h)]. All experiments were repeated in triplicate.

Western Blotting

Whole-cell lysates were prepared by resuspending the cells in cold SDS sample buffer [1% SDS, 0.04 mol/L Tris-HCl (pH 6.8), 5% glycerol]. The lysates were boiled and sheared through a 25-gauge needle and protein concentrations were determined using a commercial assay kit (bicinchoninic acid, Pierce); 100 μg of protein were resolved on either a 10% SDS-polyacrylamide gel or a 10% to 20% gradient gel specifically for detection of Noxa. Bromophenol blue and DTT were added to samples before loading. Western blotting was done using standard procedures with the following primary antibodies: Noxa monoclonal (Kamiya), Mcl-1 monoclonal (BD Pharmingen), Bcl-X_L polyclonal (Santa Cruz Biotechnology), Bcl-2 monoclonal (Dako), β-actin monoclonal (Sigma Chemical), and poly(ADP-ribose) polymerase (PARP) polyclonal (Cell Signaling Technology). Detection was achieved using the West Pico Chemiluminescent System (Pierce/ThermoFisher) and a

Raytest cooled charge-coupled device camera imaging system equipped with the AIDA 4.15 software package for quantitation. All experiments were repeated in triplicate.

Immunoprecipitation

Cells were lysed in ice-cold CHAPS buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L MgCl₂, 1 mmol/L EGTA, 10% glycerol, 1 mmol/L NaVO₄, 1% (v/v) protease inhibitor cocktail (Pierce/ThermoFisher), 1% CHAPS] using 25 strokes of a Dounce homogenizer with a tight pestle. After clearing the lysate, 2.5 µg of rat monoclonal anti-Bim (14A8; Calbiochem) per mg protein was added to the lysate and then the mix was incubated overnight at 4°C. Protein A+G beads (Santa Cruz Biotechnology) were then added and incubated with end-over-end mixing for 2 h. The beads were then washed four times in lysis buffer and analyzed by Western blotting as described.

Quantitation of NOXA mRNA

Total RNA was isolated using Trizol reagent (Invitrogen) and cDNA was synthesized using the High Capacity cDNA Archive kit (Applied Biosystems) according to the manufacturers' protocols. Real-time PCR was done using Taqman Fast Universal Master Mix, predeveloped primers for NOXA and GADPH (endogenous control), and the 7500 Real-Time PCR System (Applied Biosystems). The denaturation cycle was set at 95°C for 20 s followed by 40 cycles of 95°C for 3 s/60°C for 20 s. Quantitation relative to the endogenous control was done using the Applied Biosystems 7500 Fast System SDS software.

Results

Expression Levels of Bcl-2 and Noxa, but not Mcl-1, Are Correlated with ABT-737 Sensitivity

To help identify factors that may be responsible for the variable sensitivity of SCLC to ABT-737, we identified a representative panel of SCLC cell lines (H69, H69AR, H146, H209, H526, and WBA) with levels of sensitivity that ranged over >2 logs (0.1 to >10 µmol/L) as assessed by a MTT viability assay (Fig. 1A). The sensitivities of these cell lines are in good agreement with those previously published (18). Expression of Bcl-2 family members previously determined to influence sensitivity to ABT-737 was assessed in this panel of cell lines. Surprisingly, Mcl-1 levels, strongly implicated in sensitivity of SCLC to the drug (19, 20, 25), were remarkably constant in all cell lines tested, except for an ~50% lower amount in the H146 cell line. This finding was even more surprising given the fact that Noxa levels varied by ~10-fold in these cell lines and Noxa has been shown to regulate proteasomal degradation of Mcl-1 (23, 28, 29). Higher levels of both Noxa and Bcl-2 correlated with increased sensitivity to ABT-737, in good agreement with a previous study showing that coamplification of these linked genes predicted sensitivity to the drug (24). There was also a trend toward increased Bcl-X_L expression in the more resistant cell lines. The exception to both of these trends was the H209 cell line, which had intermediate sensitivity with undetectable levels of Noxa and relatively low levels of both Bcl-2 and Bcl-X_L.

Overexpression of Bcl-2 in H526 Does Not Result in Enhanced Sensitivity to ABT-737

To determine the relative contribution of high levels of Noxa and Bcl-2 expression to ABT-737 sensitivity, we chose to overexpress these genes in H526, a resistant cell line that has low levels of Noxa and Bcl-2 expression. Using the pCEP4-BCL-2 vector, numerous clones constitutively expressing high levels of Bcl-2, comparable with or greater than that in the very sensitive H146 cell line, were obtained (Fig. 1B). Overexpression of Bcl-2 did not result in changes in expression of other Bcl-2 family members. Figure 1C illustrates MTT viability assays conducted to assess the dose response of the transfected clones to ABT-737. There was no indication that enhanced Bcl-2 expression in turn enhanced sensitivity to ABT-737. In fact, most BCL-2 transfectants seemed modestly more resistant than the empty vector control. Therefore, it seems that high levels of Bcl-2 expression alone do not augment sensitivity to ABT-737 and could result in increased resistance due to the overabundance of one of the primary targets of the drug.

Increased Noxa Expression Results in Increased Sensitivity to ABT-737

Transfection of the pCEP4-NOXA construct into H526 cells resulted in up to an ~8-fold increase in constitutive Noxa expression compared with the empty vector control, with considerable variation among individual clones (Fig. 2A). The NOXA transfectants grew very slowly, taking at least 6 weeks from the time of transfection to generate sufficient numbers of cells for experimentation, with large numbers of apoptotic-appearing cells in the cultures despite maintenance of optimum growing conditions. Expression of Mcl-1 and Bcl-X_L was relatively invariant among the clones, whereas expression of Bcl-2 varied considerably (Fig. 2B). Similar to the comparison across cell lines (Fig. 1A), there was absolutely no indication of an inverse relationship between Noxa and Mcl-1 expression that has been observed in other cell types (29). The two clones with the highest levels of Noxa expression (1C4 and 2A1) had relatively high levels of Bcl-2 expression, but the other clones showed no discernible pattern between Noxa and Bcl-2 expression (Fig. 2B). All NOXA transfectants showed a marked increase in sensitivity to ABT-737 relative to the empty vector control (Fig. 2C). Whereas the NOXA transfectants responded to 0.12 µmol/L ABT-737, the vector control remained relatively resistant until concentrations >3.3 µmol/L ABT-737 were reached. There was not a very good correlation between the degree of Noxa overexpression and increased sensitivity, presumably due to clonal variation (exemplified by varying Bcl-2 expression) in other factors influencing drug sensitivity. The two highest expressing clones (1C4 and 2A1), which expressed Noxa on a comparable level to H146 and H69, showed sensitivity comparable with these cell lines, suggesting that Noxa is the primary determinant of ABT-737 sensitivity in SCLC.

To clearly show that Noxa expression resulted in an increased apoptotic rate, PARP cleavage, one of the later steps in the apoptotic cascade, was assessed under optimal

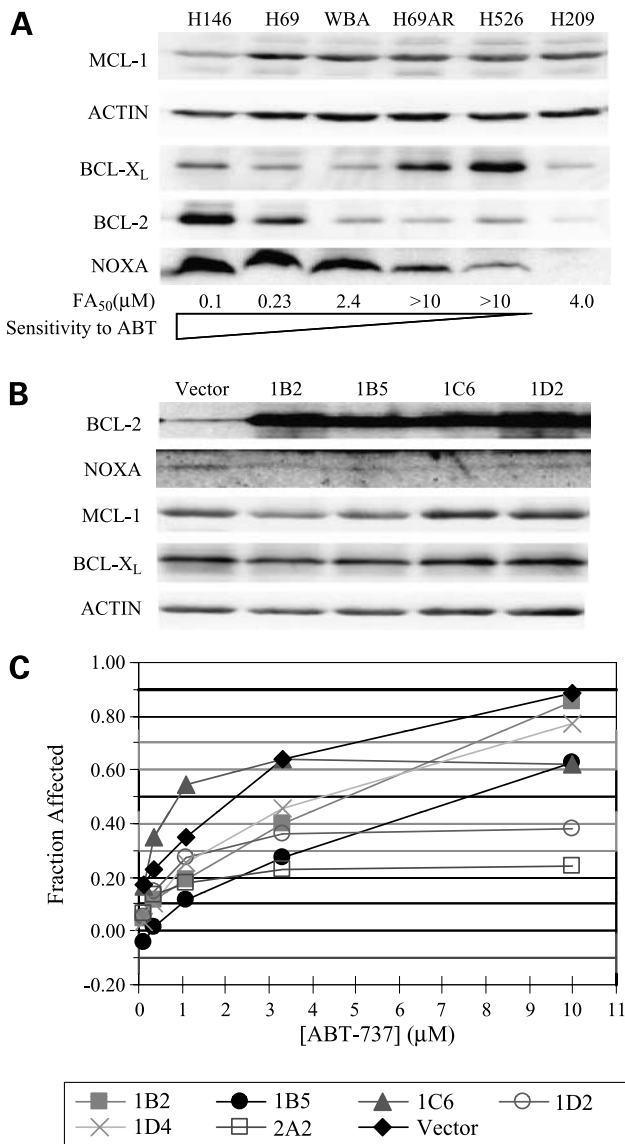


Figure 1. Expression levels of Bcl-2 and Noxa correlate with ABT-737 sensitivity but overexpression of Bcl-2 did not result in enhanced sensitivity to ABT-737. **A**, expression of Bcl-2 family members implicated in determining sensitivity to ABT-737 was studied by Western blot in six SCLC cell lines representing more than a 2-log variation in sensitivity to ABT-737. The FA₅₀, determined by MTT assay done in triplicate, is listed below the blot. Cell lines are ordered in decreasing sensitivity with the exception of H209, which stands out because of low Bcl-X_L, Bcl-2, and Noxa expression. **B**, Western blot of representative stable clones derived from the H526 cell line transfected with a BCL-2 expression vector or the empty vector. All BCL-2 transfectants displayed uniformly high levels of Bcl-2 expression (clones 1D4 and 2A2 not shown) with little variation in the expression of other family members compared with the vector control. **C**, MTT growth assays of BCL-2 transfectants were conducted to assess their dose response to ABT-737, with the data expressed as FA at 72 h. Data are representative of three independent experiments. For ease in distinguishing the individual curves, error bars were not included. SDs of data points for all clones were <7% of the indicated values, except for clone 1C6, which was <10%.

growth conditions and in the presence of 3.3 μmol/L ABT-737 or DMSO vehicle for 24 hours. Under optimal conditions, most of the transfectants showed increased PARP

cleavage compared with the vector control (data not shown). As expected, compared with the DMSO control, there was more cleaved PARP in the presence of ABT-737 (Fig. 2D). This increase was even more pronounced in the NOXA transfectants, in which the majority of immunodetectable PARP is in the cleaved form following ABT-737 treatment, whereas the reverse is true in the vector and vehicle controls.

To determine whether increased expression of Noxa increases sensitivity to all apoptotic stimuli, which might be expected if Noxa expression only lowered the apoptotic threshold, we did a MTT viability assay in the presence of increasing concentrations of etoposide, a potent inducer of the intrinsic apoptotic cascade. For comparison, we also studied the clones expressing Bcl-2, which has been shown to increase resistance to etoposide (30, 31). As expected, H526 clones overexpressing Bcl-2 show decreased sensitivity to etoposide compared with the empty vector control (Fig. 3A), whereas those overexpressing Noxa, on average, showed a similar sensitivity to the control (Fig. 3B). Therefore, Noxa overexpression has a selective effect on sensitivity to ABT-737.

To determine whether the above findings in the H526 cell line could be duplicated in a second SCLC cell line, we introduced the pCEP4-NOXA construct into H209, an atypical cell line with low Bcl-2 and Bcl-X_L and absent Noxa expression. Even more so than with H526, Noxa-expressing clones were difficult to derive and only three clones, expressing variable amounts of Noxa, were obtained from multiple transfections (Fig. 4A). As opposed to results in the H526 cell line, whereas Bcl-X_L or Bcl-2 expression remained relatively constant compared with the parental cell line and vector control, Mcl-1 expression was markedly decreased in proportion to the degree of Noxa expression. Viability assays illustrated that, similar to the H526 transfectants, the H209 NOXA transfectants were more sensitive to ABT-737 compared with the vector control (Fig. 4B). However, with the H209 transfectants, there was a much clearer proportionality between the amount of Noxa expressed and the loss of viability, as well as the induction of PARP cleavage (Fig. 4C) in response to ABT-737. The distinction between the high and low Noxa-expressing clones may be magnified by the proportional decrease in Mcl-1 expression with increased Noxa expression in this cell line.

The results obtained using the H526 and H209 cell lines were consistent with the exception that in H526 increased Noxa expression did not result in a concomitant decrease in Mcl-1 expression, whereas in H209 there was such a decrease. To better define how common the loss of Noxa-mediated regulation of Mcl-1 expression was in SCLC, four additional cell lines were transfected with the NOXA expression construct. Only one of those cell lines, H82, yielded clones stably expressing Noxa. As illustrated in Fig. 4D, increased Noxa expression did not have a consistent effect on Mcl-1 levels in this cell line. As with H526 and H209, Noxa-expressing clones were more sensitive to ABT-737 than the vector control (data not shown).

Decreased Noxa Expression Is Associated with Increased Resistance to ABT-737

Because stable transfection may result in selection for secondary genetic changes that could influence phenotype, we sought to confirm the importance of Noxa in ABT-737 sensitivity by silencing Noxa expression in H146, a sensitive cell line with endogenously high levels of Noxa (Fig. 1A). Transfection of siRNA duplexes targeting *NOXA* yielded a 30% to 40% decline in Noxa levels relative to the nontargeting controls (Fig. 5A). At effective concentrations of ABT-737, knockdown of Noxa increased resistance to the drug by 2- to 3-fold in three replicate experiments (Fig. 5B). Importantly, decreased Noxa expression did not result in increased Mcl-1 levels, showing that Noxa-mediated regulation of Mcl-1 expression does not occur in the H146 cell line.

Noxa Blocks Mcl-1 from Interacting with Bim after ABT-737 Exposure

ABT-737 has been shown to release proapoptotic BH3-containing proteins from Bcl-2 and Bcl-X_L. Sequestration of these proteins by Mcl-1 (which then acts as the primary sink because it binds poorly to ABT-737) inhibits the apoptotic cascade (18, 20, 22, 23, 25). Noxa, whose primary high-affinity binding partner is Mcl-1, should therefore limit transfer of other BH3 proteins from Bcl-2 and Bcl-X_L to Mcl-1 in the presence of ABT-737, thus promoting apoptosis. To determine if this hypothesis explained the increase in ABT-737 sensitivity in our *NOXA* transfectants, we studied Bcl-2 family members associated with the abundant BH3 protein Bim in the H526-*NOXA* 1A3 clone, along with the empty vector

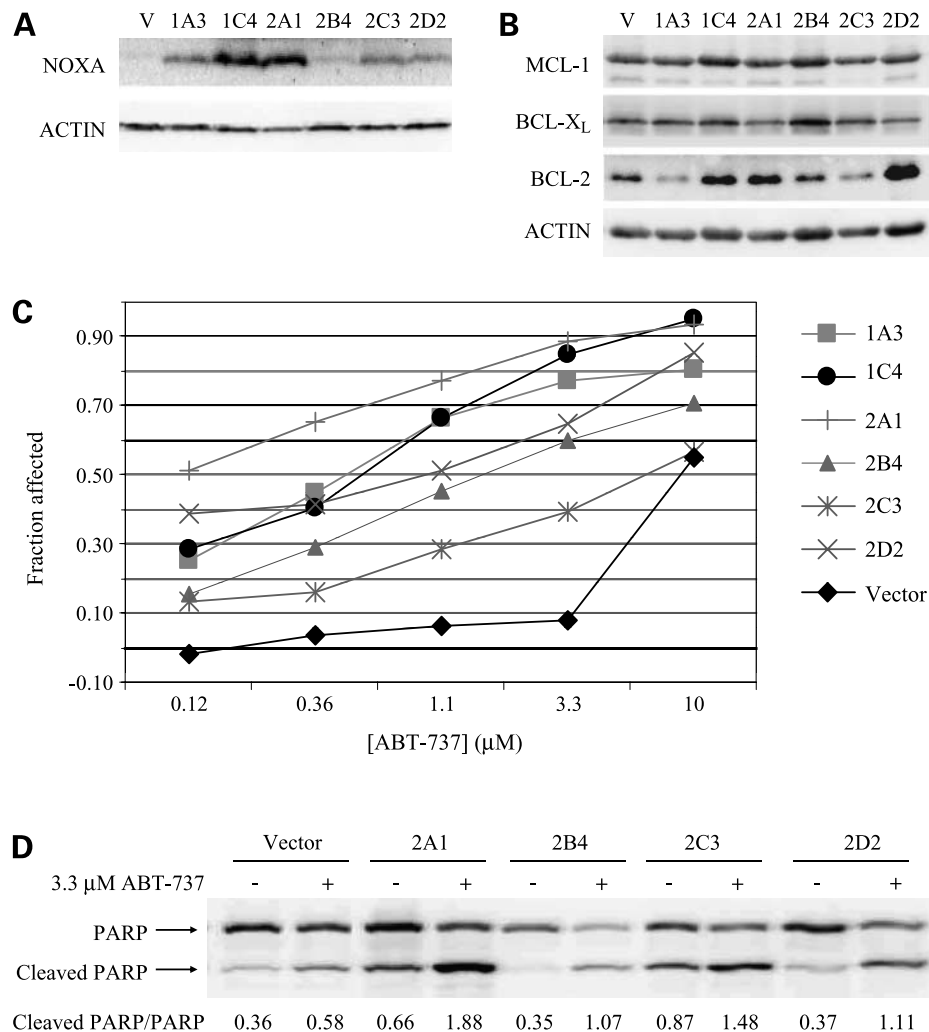


Figure 2. Overexpression of Noxa in H526 enhanced sensitivity to ABT-737. **A**, Western blot of Noxa expression in stable clones derived from the H526 cell line transfected with a *NOXA* or empty expression vector. **B**, the same protein samples in **A** analyzed for other Bcl-2 family members. Note that Mcl-1 levels are essentially invariant. **C**, MTT growth assays were conducted to assess the dose response of these transfectants to ABT-737, with the data expressed as FA at 72 h. Data are representative of three independent experiments. SDs for all data points were <7% of the indicated values. **D**, PARP cleavage was also assessed in cells that were incubated with an intermediate concentration of ABT-737 for 24 h and compared with the DMSO control (-). The ratio of cleaved PARP to intact PARP is indicated below each lane. Data are representative of experiments done in triplicate.

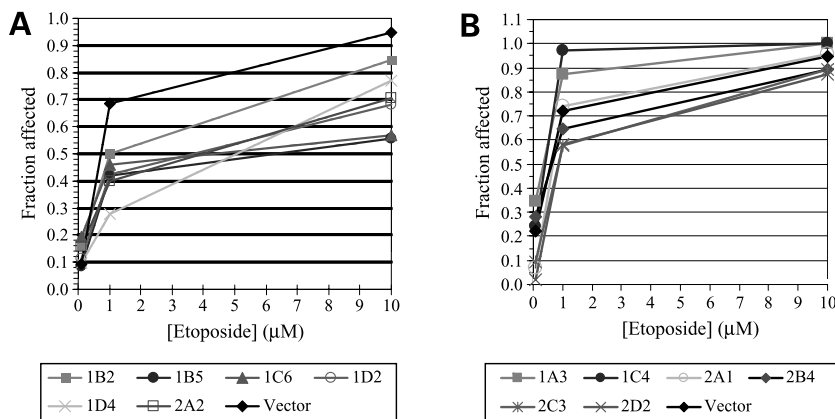


Figure 3. Sensitivity to etoposide is influenced by Bcl-2 levels but not by Noxa levels. **A**, MTT growth assays showing that H526 *BCL-2* transfectants are uniformly more resistant to etoposide compared with the vector control. SDs of all data points were <7% of the indicated values. **B**, MTT growth assays showing that sensitivity of H526 *NOXA* transfectants to etoposide is not distinguishably different than the vector control. SDs for all data points were <8% of the indicated values. Data are representative of experiments done in triplicate.

control, in the presence and absence of ABT-737. Following overnight exposure to 3.3 μmol/L ABT-737 or DMSO vehicle, Bim was immunoprecipitated and associated proteins were detected by Western blot. As evident in Fig. 6A, drug treatment efficiently disrupted Bim/Bcl-2 complexes in both the control and *NOXA* transfectant. Bim/Mcl-1 complexes markedly increased in control cells after ABT-737 exposure, but not in the *NOXA* transfectant, as would be expected if Noxa was already bound to the available Mcl-1.

Treatment with ABT-737 Results in Increased Noxa Expression

During the process of studying Bcl-2 family expression following exposure to ABT-737, we found that a relatively short-term (24 hours) exposure to 5 μmol/L ABT-737 resulted in a 2.4- and 1.4-fold increase in Noxa expression in H526 and WBA cells, respectively, whereas expression levels of Bcl-2, Mcl-1, and Bcl-X_L remained the same (Fig. 6B). This raises the possibility that the increase in Noxa expression may act as an amplification mechanism in

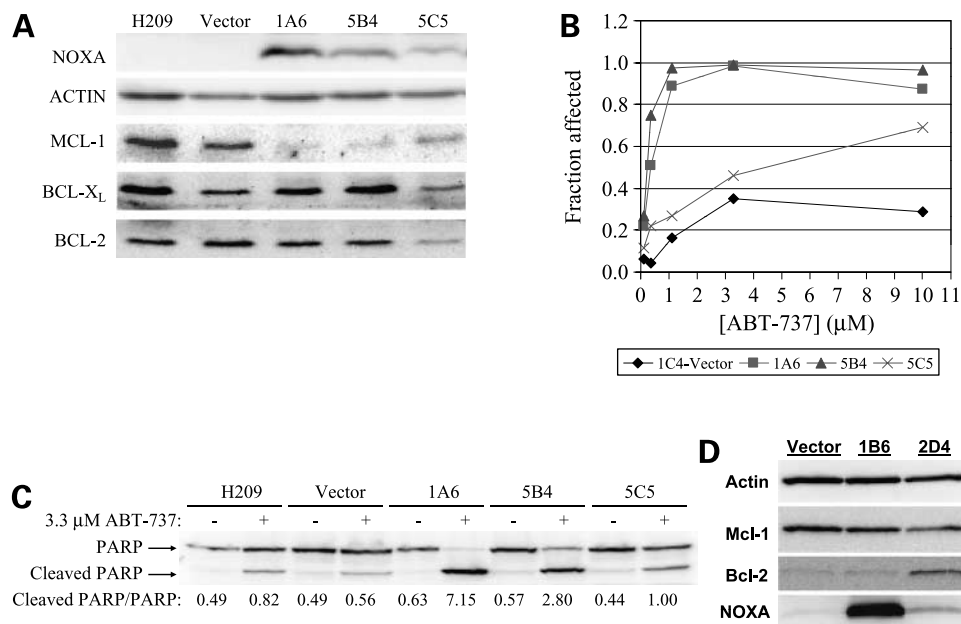


Figure 4. Overexpression of Noxa in H209, but not H82, leads to decreased expression of Mcl-1. **A**, Western blot showing the levels of Noxa expression achieved in H209 transfectants compared with empty vector transfectants and the parental cell line. As opposed to results in the H526 cell line, Noxa overexpression resulted in a proportionate decrease in expression of Mcl-1, but not Bcl-X_L or Bcl-2. **B**, MTT growth assays were conducted to assess the dose response of H209 transfectants to ABT-737, with the data expressed as FA at 72 h. SDs for all data points were <5% of the indicated values. **C**, PARP cleavage was assayed in H209 cells incubated in an intermediate concentration of ABT-737 for 24 h. Compared with the DMSO control (-), there is more cleaved PARP in the presence of ABT-737. This increase is even more pronounced in the transfectants. **D**, Western blot showing little change in Mcl-1 levels in H82 clones transfected with a *NOXA* expression vector. Data are representative of three independent experiments.

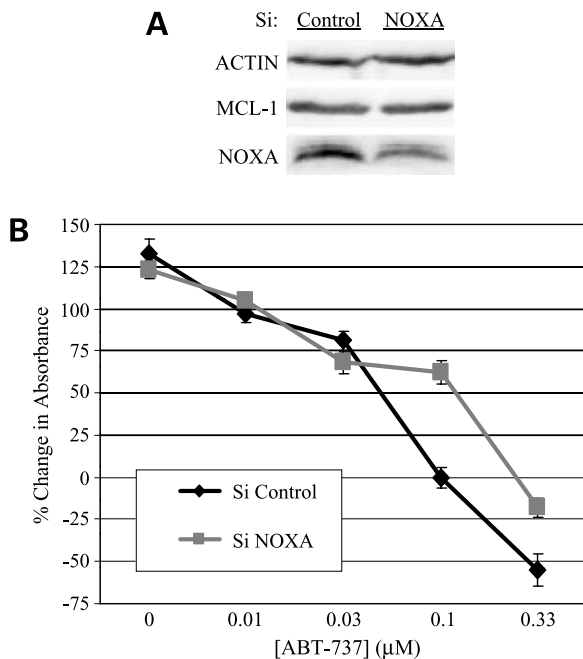


Figure 5. Silencing of Noxa expression is associated with increased resistance to ABT-737. **A**, Noxa was silenced by transient transfection of siRNA in the H146 cell line, which expresses high levels of Noxa and is very sensitive to ABT-737. **B**, MTT assay showing increased resistance of cells transfected with a siRNA targeting *NOXA* compared with the control duplex. On the indicated scale, a change of 100% represents a doubling of the cell population, whereas a change of -100% represents complete cell death of the starting cell population. The shift of the curve to the right at concentrations of ABT-737 of 0.1 μmol/L and higher was replicated in three independent experiments, despite achieving only a 30% to 40% decrease in Noxa expression. Bars, SD.

enhancing ABT-737-mediated cytotoxicity in a subset of SCLC cell lines.

To confirm the effect of ABT-737 on Noxa expression and identify potential mechanisms for the effect, H526 cells were

treated for 6 hours with 5 μmol/L ABT-737 or DMSO (control) and analyzed by quantitative real-time PCR. On exposure to ABT-737, *NOXA* mRNA abundance was ~1.5-fold higher than the control relative to *GAPDH* (Fig. 6C). Therefore, the increase in Noxa protein levels on ABT-737 treatment is at least in part due to increased RNA levels.

Discussion

Unique among solid tumors, SCLC cell lines have been shown to undergo extensive, albeit variable, apoptosis in the presence of ABT-737 without other exogenous apoptotic stimuli (18). It is vital to understand the underlying causes of this variability to effectively incorporate BH3 mimetic drugs into SCLC treatment regimens. Whereas ABT-737 is not appropriate for clinical trials because of unfavorable pharmacologic properties, its highly related derivative ABT-263 (32) has entered clinical trials and has a very similar activity profile against SCLC cell lines (33).

Initial attention has focused heavily on the level of Mcl-1 expression as a rational explanation for variable sensitivity to ABT-737 based in part on the observation that it is the one prosurvival Bcl-2 family member present in SCLC that binds the drug poorly (19, 20). As would be expected, cell lines with low Mcl-1 tend to be more sensitive to the drug and knockdown of Mcl-1 enhances sensitivity. However, it is also clear that even in the initial study correlating SCLC sensitivity with Bcl-2 family expression (19), there was a wider range of variation in expression of other family members that correlated with sensitivity, such as Bcl-2 and Noxa, than there was in Mcl-1. When we assessed Bcl-2 family expression in a panel of SCLC cell lines with more than a 2-log difference in sensitivity to ABT-737, we were quite surprised to observe similar levels of Mcl-1 expression across the range (Fig. 1A). This lack of correlation of sensitivity with Mcl-1 expression has also been noted in another recent study using a set of cell lines overlapping with ours (21).

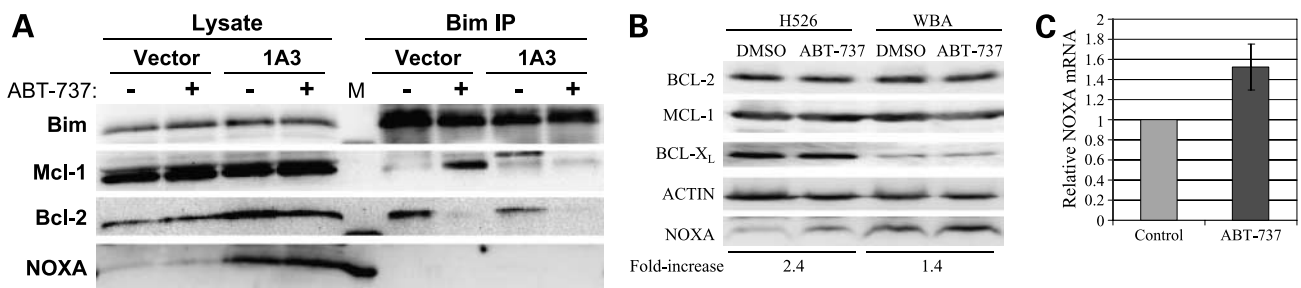


Figure 6. Enforced Noxa expression results in decreased Bim association with Mcl-1 following exposure to ABT-737, which also induces Noxa expression. **A**, the H526-*NOXA* 1A3 clone, along with the vector control, was incubated in 5 μmol/L ABT-737 or DMSO overnight and lysates were made in CHAPS buffer. Bim was immunoprecipitated (IP) and the amount of Bim, Bcl-2, and Mcl-1 was assessed by Western blot in both the starting lysate and following immunoprecipitation. ABT-737 causes a marked shift of Bim from Bcl-2 to Mcl-1 in the vector control but not in the *NOXA* transfectant. *M*, molecular weight marker lane separating the lysate from the immunoprecipitated lanes. The 10-kDa marker is adjacent to the Noxa band. Data are representative of triplicate experiments. **B**, H526 and WBA cell lines were exposed to 5 μmol/L ABT-737 for 24 h and levels of Bcl-2, Mcl-1, and Bcl-X_L were assessed by Western blot. Densitometry showed a 2.4- and 1.4-fold increase in Noxa expression in H526 and WBA cells, respectively. **C**, H526 cells were treated for 6 h with 5 μmol/L ABT-737 or DMSO (control) and analyzed by quantitative reverse transcription-PCR. On exposure to ABT-737, *NOXA* mRNA abundance was ~1.5-fold higher than the control relative to *GAPDH*. Columns, mean of three independent experiments; bars, SD.

Given the prior expression and genetic data suggesting that high levels of Bcl-2 and Noxa expression and coamplification of these two linked genes correlate with ABT-737 sensitivity (19, 24), we chose to prospectively manipulate their expression in a common genetic background to determine effects on drug sensitivity. Overexpression of Bcl-2 by itself did not sensitize H526 (Fig. 1B and C) or H209 cells (data not shown) to ABT-737, a phenomenon that has also been noted in lymphoma cell lines (25). On the other hand, enforced expression of Noxa markedly enhanced sensitivity of H526 (Fig. 2), H209 (Fig. 4), and H82 (data not shown) to the drug. In fact, H526 clones with similar levels of Noxa expression to the very sensitive H146 and H69 cell lines had a similar dose response to ABT-737, suggesting that Noxa expression is the major determinant of ABT-737 sensitivity in the panel of cell lines tested. Silencing of Noxa in the H146 cell line, which correlated with decreased sensitivity (Fig. 5), confirmed the above observation and ruled out potential artifacts related to selection of stable cell lines as an explanation of our results.

Manipulation of Noxa expression also resulted in the unexpected and novel observation that in the H526, H82, and H146 cell lines, changes in Noxa expression did not result in changes in Mcl-1 expression. This is contrary to the observation that Noxa mediates Mcl-1 degradation in mouse embryo fibroblasts and HeLa cells based in part on the unique properties of the COOH-terminal region of the Noxa BH3 domain (28, 29). One might attribute the differences to cell type-specific variation, but an inverse correlation between Noxa and Mcl-1 expression has been seen in a variety of cell types (34, 35) and expression of Noxa in the H209 cell line clearly leads to a proportionate decline in Mcl-1 expression (Fig. 4). It is very interesting that the one cell line in which we were able to show Noxa-mediated regulation of Mcl-1 expression is a cell line in which basal levels of Noxa expression are undetectable (Figs. 1A and 4A). This raises the possibility that loss of Noxa-mediated regulation of Mcl-1 expression, which could either be accomplished by abrogating Noxa-mediated Mcl-1 degradation or by eliminating expression of Noxa, is an important feature of SCLC biology. Because the mechanism of Noxa-mediated degradation of Mcl-1 is poorly understood at present (28), further exploration of this hypothesis will have to be the subject of future work.

Despite the apparent inability of Noxa to efficiently mediate decreased Mcl-1 expression in SCLC cell lines, it is clear that Noxa still impedes Mcl-1 function, as shown in our coimmunoprecipitation studies (Fig. 6A). After ABT-737 exposure, Bim was effectively transferred from Bcl-2 to Mcl-1 in control cells but not in cells overexpressing Noxa. This result indicates that excess Noxa increases the amount of free BH3-containing proapoptotic Bcl-2 family members in the presence of ABT-737 and provides a mechanistic basis for the increase in sensitivity to the drug in all our *NOXA* transfectants. Recently, it has been shown that an artificial BH3 ligand capable of specifically interacting with Mcl-1 without inducing its degradation can block its prosurvival function, showing that the ability to compete for BH3 binding and the ability to induce Mcl-1 degradation are separa-

ble functions of Mcl-1 antagonists (36). However, when Noxa can do both functions, as in the H209 cell line, even a modest increase in expression can lead to dramatic sensitization to ABT-737 (Fig. 4B).

Our results confirm and extended findings of the initial study of SCLC sensitivity to ABT-737 (19), which showed that interference with Mcl-1 function or expression resulted in increased sensitivity to the drug. In addition, the investigators showed that in H146 cells selected for ABT-737 resistance by stepwise culture in increasing drug concentrations, both Bcl-2 and Noxa levels dropped and Mcl-1 increased. Our results would indicate that the drop in Noxa, along with the increase in Mcl-1, would be the most important drivers of resistance.

Hann et al. (21) also recently published a study of ABT-737 sensitivity in SCLC cell lines and xenografts. Using a collection of continuous cell lines overlapping with our own, in addition to primary xenografts, they noted no correlation between sensitivity and Mcl-1 expression, in agreement with our data. They also noted decreased Bim expression in a subclone derived from a H187 xenograft resistant to ABT-737, consistent with the observation in non-SCLC that an increase in Bim after epidermal growth factor receptor kinase inhibitor treatment is critical for synergistic activity with ABT-737 (37–39). Contrary to our findings, however, they noted no correlation between sensitivity and Noxa protein expression even in cell lines common to our study, such as H146 and H209. Although this difference may represent divergent clonal evolution of cell lines maintained for years in individual laboratories, caution must be used in interpreting Western blot detection of Noxa because, in our experience, many of the commercial antibodies detect numerous background bands in the 10- to 15-kDa region, which are difficult to distinguish from authentic Noxa. We used 10% to 20% gradient gels to maximally separate these bands as well as clones transfected with a sequenced *NOXA* cDNA to verify the identity of Noxa and define the most specific antibody for routine blotting purposes.

Several preclinical studies have now suggested that modulation of Noxa and/or Mcl-1 expression with therapeutic intent can be used to enhance sensitivity to ABT-737 alone or synergistic activity with other anticancer agents. Mcl-1 down-regulation using cyclin-dependent kinase inhibitors or a siRNA approach has been shown to increase sensitivity of leukemia and epithelial cancer cells to ABT-737 (22, 23, 40). Likewise, arsenic trioxide-mediated increases in Noxa expression enhance sensitivity of multiple myeloma cell lines to ABT-737 (41). There were early indications that down-regulation of Mcl-1 could be useful in sensitizing SCLC to ABT-737 (17), and in the present study, we have clearly shown a similar finding for enhanced expression of Noxa, especially given the observation that Noxa expression increases on acute exposure to ABT-737 in some cell lines (Fig. 6B and C). Although we are actively pursuing therapeutically relevant methods to decrease Mcl-1 and increase Noxa expression in SCLC, we believe the most important application of our findings may be in the identification of

relevant biomarkers that will best predict sensitivity to ABT-263, the highly related successor to ABT-737 currently in early-phase clinical trials for SCLC and other malignancies (33). Amplification of chromosomal region 18q21-23, containing both *BCL-2* and *NOXA*, is an excellent predictor of sensitivity to ABT-737 (24). However, because we have shown that elevated Noxa is the primary determinant of sensitivity, immunohistochemical detection of Noxa may be a better clinical biomarker for several reasons. First, immunohistochemical staining is currently applicable in most clinical pathology laboratories, whereas genomic analysis is not. Second, protein expression is regulated at multiple levels, and thus, for a given copy number there could still be disparate relative levels of protein expression even for two closely linked genes. For example, although the WBA and H526 cell lines express similar levels of Bcl-2 (Fig. 1A), they have differing sensitivities to ABT-737, likely caused at least in part by their differing expression of Noxa. The latter observation, along with our studies of enforced expression of Noxa and Bcl-2, suggests that Noxa expression levels may be the best predictor of SCLC sensitivity to Bcl-2/Bcl-X_L antagonists, especially if combined with an analysis of Mcl-1 expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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