Actinomycin D Decreases Mcl-1 Expression and Acts Synergistically with ABT-737 against Small Cell Lung Cancer Cell Lines

Haishan Xu and Geoffrey W. Krystal

Abstract

Purpose: ABT-737, which blocks the function of Bcl-2 and Bcl-X\textsubscript{L} but not Mcl-1, has shown single-agent activity in preclinical models of small cell lung cancer (SCLC). Elevated expression of Mcl-1 induces resistance to ABT-737 in SCLC. Based on the short half-life of Mcl-1 mRNA and protein, we hypothesized that the actinomycin D could reverse Mcl-1–induced resistance to ABT-737.

Experimental Design: The dose-response of multiple SCLC cell lines to actinomycin D in the absence and presence of ABT-737 was followed by the assessment of Bcl-2 family expression and poly ADP ribose polymerase cleavage by Western blot, viability by tetrazolium dye reduction and clonogenic assay, and cell cycle kinetics by flow cytometry.

Results: Actinomycin D decreased Mcl-1 expression and resulted in a cell line–dependent increase in Noxa expression. Clinically relevant concentrations of actinomycin D from 0.4 to 4 ng/mL showed single-agent activity across a panel of SCLC cell lines. When combined with low micromolar doses of ABT-737, near complete loss of viability was seen with synergistic combination indices of 0.5 to 0.7. Exposure to 4 ng/mL actinomycin was only required for the first 24 hours of the combined incubation, mimicking a clinically achievable area under the curve, but the presence of ABT-737 was required for an additional 48 hours to obtain maximal effect.

Conclusions: Clinically relevant concentrations of actinomycin D act synergistically with ABT-737 to induce SCLC apoptosis, which can be at least partially attributed to the actinomycin D–induced decrease in Mcl-1 and increase in Noxa expression. Taken together, these data suggest the feasibility of combining actinomycin D with BH3-mimetic drugs in the clinical setting.

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 due to the rapid development of chemotherapy-resistant disease, generally following an initial response to therapy (1). One important factor in the development of chemotherapy resistance is the overexpression of prosurvival members of the Bcl-2 family (2). In SCLC, overexpression of Bcl-2 has been shown (3), and Bcl-X\textsubscript{L} and Mcl-1 are also expressed at varying levels as well (4, 5). BH3-mimetic drugs represent an exciting new development in cancer therapeutics designed to counteract the effects of prosurvival Bcl-2 family proteins by blocking their ability to bind and inactivate the BH3 domain–containing proapoptotic Bcl-2 family members, thus allowing the proapoptotic proteins to directly or indirectly induce permeabilization of the outer mitochondrial membrane and activation of the intrinsic apoptotic caspase cascade (6). ABT-737, designed based on structural data to fit into the BH3 domain–binding groove of Bcl-X\textsubscript{L}, efficiently blocks Bcl-2 and Bcl-X\textsubscript{L} function, but does not effectively bind Mcl-1 (7). ABT-737 and the related orally bioavailable compound ABT-263 have single-agent activity in preclinical models of SCLC, although sensitivities of individual cell lines differ, with IC\textsubscript{50}s ranging from <0.1 to >10 μmol/L (7, 8).

Relative resistance to the effects of ABT-737 has been attributed to high levels of Mcl-1 expression and low levels of Noxa expression (4). Noxa, a selective BH3-containing binding partner of Mcl-1, competes with the BH3-containing multidomain proapoptotic effector proteins Bak and Bak, which directly induce outer mitochondrial membrane permeability, for binding to Mcl-1. In many cell types, Noxa has also been shown to mediate proteasome degradation of Mcl-1 (9, 10). However, in SCLC, we have shown that Noxa-mediated degradation of Mcl-1 was lost in all four cell lines examined, either because Mcl-1 levels were insensitive to changes in Noxa expression or because endogenous Noxa expression was absent (5). This observation suggests that
Actinomycin D Acts Synergistically with ABT-737

Translational Relevance

ABT-737 is the prototype BH3-mimetic drug designed to block the function of the prosurvival Bcl-2 and Bcl-X_L proteins. We and others have characterized elevated expression of Mcl-1 and decreased expression of its cognate proapoptotic binding partner Noxa as the predominant mechanism of small cell lung cancer (SCLC) resistance to ABT-737 and ABT-263, a related compound currently in clinical trials. In this report, we show that actinomycin D, a component of early combination chemotherapy regimens for SCLC, decreases Mcl-1 and increases Noxa expression, and acts synergistically with ABT-737 against SCLC cell lines. We also define the schedule dependency of this interaction. These findings could have direct effect on the design of future clinical trials combining BH3-mimetic drugs with chemotherapeutic agents active against SCLC and other malignancies.

Materials and Methods

Compounds

ABT-737 [(R)-4-[(3-dimethylamino-1-phenylsulfonyl methyl)-propylamino]-N-(4-[(4-chloro-biphenyl-2-ylmethyl)-piperazin-1-yl]-benzoyl]-3-nitro-benzenesulfonamide] was synthesized and kindly provided by Abbott. ABT-737 stock solutions were made in 100% DMSO (Sigma) and diluted with culture media before use. Actinomycin D (Sigma) and dilute with culture media before use. The final DMSO and ethanol 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 U/mL penicillin/streptomycin (Life Technologies) in a humidified chamber at 37°C containing 5% CO2. H526 subclones containing the pCEP4 (Invitrogen) or pCEP4-NOXA expression vectors were produced as previously described (5) and maintained in the above medium containing hygromycin (EMD/Calbiochem) and then cultured without hygromycin for 48 hours before and during experimentation.

Cell growth and viability assay

Relative viable cell number was measured using the MTT (Sigma) colorimetric dye reduction method (15). Duplicate plates containing eight replicate wells per assay condition were seeded at a density of 1 x 10^4 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 hours compared with initial values obtained 3 hours after plating. For each experiment, a dose-response curve was generated by plotting the change in absorbance compared with the vehicle control. Median dose effect analysis for synergy was done using the method of Chou and Talalay (16) after conversion of absorbance data to the fraction affected [1 − (experimental/control absorbance at 72 h)] and analyzed using the CalcuSyn software (Biosoft).

Clonogenic assays

H526 cells (5 x 10^3) were treated with vehicle (control), 4 ng/mL actinomycin D, 3.3 μmol/L ABT-737, and the drug combination for 24 hours in complete medium. The cells were then washed, and ABT-737 incubation was continued for an additional 48 hours for the experimental groups exposed to ABT-737. For groups not exposed to ABT-737, incubation was continued for 48 hours in vehicle-containing medium. The cells were then washed and resuspended in 3 mL 0.25% low-melting-point agarose (Life Technologies) in complete medium and plated over a layer of 0.5% agarose in medium in 60-mm plates. Plates were incubated for 8 days, and viable macroscopic colonies were counted 2 hours after overlay with MTT dye.

Western blotting

Whole-cell lysates were prepared by resuspending 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 (BCA, Pierce); 75 μg of protein were resolved on either a 10% SDS polyacrylamide gel or a 10% to 20% gradient gel specifically for the detection of Noxa. Bromophenyl 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-2 monoclonal (DAKO), β-actin monoclonal (Sigma), and poly ADP ribose polymerase (PARP) polyclonal (Cell Signaling Technology). Detection was achieved using the West Pico Chemiluminescent System (Pierce) and a Raytest cooled charge-coupled device camera imaging system equipped with the AIDA 4.15 software package for quantitation.

Flow cytometric cell cycle analysis

Nuclear DNA content was determined by propidium iodide staining of nuclei isolated using the BD CycleTEST PLUS kit according to the supplier’s directions. Fluorescence was quantitated using a BD FACSCalibur flow cytometer equipped with CellQuest software. Ten thousand events were counted with gates set for the less than G1, G1, S, and G2-M DNA content of nuclei derived from the vehicle control cells.

Results

Actinomycin D decreases Mcl-1 and increases Noxa expression in a dose- and time-dependent fashion

To determine if actinomycin D had an affect on Mcl-1 expression, H526 cells were exposed for 24 hours to a range of drug concentrations beginning at 4 ng/mL, which is three logs less than the standard concentration used in the laboratory to completely inhibit general transcription (17). Figure 1A illustrates that actinomycin D decreases Mcl-1 expression to nearly undetectable levels in a dose-dependent fashion, with the effect peaking at 110 ng/mL, which approximates maximal achievable peak plasma concentration after bolus administration (18, 19). Induction of PARP cleavage, a biochemical marker of apoptosis, correlated well with the decrease in Mcl-1 expression. Marked reduction in Mcl-1 expression occurred approximately 4 to 6 hours after exposure to 110 ng/mL actinomycin and progressed to ∼10% of baseline expression 16 to 24 hours after drug exposure (Fig. 1B). Interestingly, an increase in Noxa expression was also seen in the H526 cell line, beginning between 6 and 16 hours after drug exposure and peaking at 24 hours after exposure. Induction of PARP cleavage occurred concomitantly with induction of Noxa. This decrease in Mcl-1 and increase in Noxa expression would predict sensitization to ABT-737 based on our earlier study (5). However, it is important to point out that Noxa expression was not required to see the reduction in Mcl-1 expression because it occurred (at slightly higher drug concentrations) in the H209 cell line, which does not express detectable levels of Noxa (Fig. 2).

Actinomycin D synergistically induces apoptosis in combination with ABT-737

To confirm that the actinomycin D–induced decrease in Mcl-1 expression correlated with enhanced PARP cleavage in the presence of ABT-737, we exposed multiple cell lines to both drugs singly and in combination for 16 hours. Figure 2 illustrates that, although the magnitude of the effect varied modestly among cell lines, in general, the reduction in Mcl-1 expression correlated well with enhanced PARP cleavage, which was further enhanced by low micromolar concentrations of ABT-737. The induction of Noxa expression by actinomycin seen in all cell lines other than H209 also correlated well with induction of PARP cleavage.

To determine whether the noted changes in Mcl-1 and Noxa expression, as well as PARP cleavage, correlated with loss of viability, MTT assays were done in the continuous presence of varying concentrations of both drugs over the course of the 72-hour assay. Figure 3 illustrates that low concentrations of actinomycin D induced significant loss of viability in the H526, H209, and WBA cell lines, and that the presence of ABT-737 enhanced the effect. H209 required ∼3-fold higher concentrations of ABT-737 for an equivalent effect, presumably at least partly because of the absence of Noxa expression (Fig. 2). Median dose-effect analyses (16) of dose-response data covering a broad range of concentrations including those graphically illustrated in Fig. 3 documented combination indices (CIs) in the 0.5 to 0.7 range, indicating synergy. To determine whether the actinomycin D/ABT-737 combination affected nonmalignant cells, cultures of MRC-5 diploid pulmonary

![Figure 1. Actinomycin D induces a decrease in Mcl-1 expression and an increase in Noxa expression. H526 cells were treated with increasing concentrations of actinomycin D for 24 h (A) or with 110 ng/mL of actinomycin D for the indicated time periods (B). Expression of the indicated Bcl-2 family members and apoptosis, as determined by PARP cleavage, was monitored by Western blot. Mcl-1 and Noxa expression ratios relative to actin were determined by densitometry and are indicated below each lane. Data are representative of three independent determinations.](cancergenes.aacrjournals.orgزيارة2010. © 2010 American Association for Cancer Research.)
fibroblasts were also exposed to similar drug concentrations, and as Fig. 3 illustrates, viability was ≥80% at actinomycin D concentrations of ≤4 ng/mL, without any dose response to ABT-737.

In addition to expression of prosurvival members of the Bcl-2 family, another common mechanism of multidrug resistance in SCLC is overexpression of the ABC transporter MRP1 (20). To determine the effect of MRP1 overexpression on the efficacy of the actinomycin D/ABT-737 combination, we compared the response of the parental H69 cell line to that of the MRP-amplified H69AR subclone derived by selection in doxorubicin (20). Figure 4A illustrates that H69 is moderately sensitive to ABT-737, and combinations with actinomycin D induce synergistic loss of viability (CI, 0.5). Interestingly, H69AR, while showing a high level of resistance to ABT-737, showed enhanced sensitivity to actinomycin D such that the dose response of H69AR to actinomycin alone resembled that of H69 treated with the combination including 1.1 μmol/L ABT-737 (Fig. 4B). These results suggest that although MRP1 overexpression induces resistance to ABT-737, it does not induce resistance to actinomycin D in SCLC.

The actinomycin D–induced decrease in Mcl-1 and increase in Noxa expression mediates synergy with ABT-737

The correlation between the actinomycin D–induced decrease in Mcl-1 and increase in Noxa expression with apoptosis, along with prior studies elucidating determinants of sensitivity to ABT-737 (4, 5), would suggest that these alterations in expression are at least in part responsible for the synergistic interaction between these drugs. To strengthen the correlation between actinomycin-induced changes in Mcl-1 and Noxa expression and enhanced sensitivity to ABT-737, we studied the dose response of the H526-NOXA 1A3–transfected cell line, which expresses elevated levels of Noxa without a change in Mcl-1 expression (5), to low concentrations of ABT-737 and actinomycin D. We have previously shown that excess Noxa in this cell line effectively reduces the amount of Mcl-1 free to bind proapoptotic Bcl-2 proteins released from Bcl-2 and Bcl-XL by ABT-737. Therefore, with decreased levels of free Mcl-1, the NOXA 1A3 cell line should be more sensitive to a further decrease in Mcl-1 induced by actinomycin D, which should be reflected in enhanced synergy with low doses of ABT-737. Figure 4C illustrates that the Noxa-expressing cell line exposed to ABT-737 concentrations between 37 to 370 nmol/L exhibits CIs consistent with synergy at various dose effect levels, whereas the vector control only exhibits CIs approximating additivity (CI, 1) at these low drug concentrations. These results further support the hypothesis that the actinomycin D–induced increase in Noxa and the decrease in Mcl-1 is an important factor involved in the synergistic interaction between ABT-737 and actinomycin D, although it may not be the only factor given the potential for actinomycin D to alter expression of other genes.

Optimal sequencing of actinomycin D and ABT-737 is necessary for maximal loss of viability and clonogenic potential

Actinomycin D is generally administered as an i.v. bolus, the pharmacokinetics for which have recently been elucidated (18, 19). The mean area under the curve using a standard sarcoma/Wilms tumor dosing regimen was ∼100 ng.h/mL, which can be approximated in vitro by incubation in 4 ng/mL actinomycin D for 24 hours. To determine whether incubation times shorter than the
72 hours used in the initial MTT assays would produce similar cytotoxicity, we incubated cell lines in 4 ng/mL of actinomycin and 3.3 μmol/L ABT-737 for 24 hours, followed by an additional 48-hour incubation in ABT-737 alone. Figure 5A illustrates that a limited 24-hour incubation in actinomycin D was sufficient to induce 80% to 90% loss of viability, similar to that obtained with incubation in both drugs for 72 hours (Figs. 3 and 4). To determine if the timing of the combined incubation affected cytotoxicity, H526 cells were treated with the combination of actinomycin D (4 ng/mL) and ABT-737 (3.3 μmol/L) for a 24-hour period, with that period varying during the 72-hour MTT assay as indicated in Table 1. Maximal cytotoxicity was obtained if coincubation was during the initial 24 hours followed by 48 hours in ABT-737 alone. If the coincubation was delayed until the terminal 24 hours, loss of viability was not different than cells incubated in ABT-737 alone for 72 hours. Failure to continue incubation in ABT-737 after the initial 24-hour coincubation resulted in a 50% increase in viable cells relative to continuous exposure and was only marginally more effective than a 24-hour incubation in actinomycin D alone. Taken together, these data indicate that ABT-737 must be present for at least 48 hours following exposure to actinomycin for maximum effect.

To determine if changes in Mcl-1 and Noxa expression could explain the requirement for an additional 48-hour incubation in ABT-737, we studied expression of these proteins at 72 hours following the initiation of a 24-hour exposure to actinomycin D, a 72-hour incubation in ABT-737, or the combination. Figure 5B illustrates that Mcl-1 expression remained lower than control values even after washout of actinomycin D. Interestingly, although Noxa expression was elevated after a 24-hours incubation in actinomycin D (data not shown), 48 hours following washout Noxa levels dropped below control basal levels. As previously described (5), prolonged incubation in ABT-737 resulted in increased Noxa expression. Coincubation with actinomycin D and ABT-737 resulted in lower levels of Mcl-1 and higher levels of Noxa expression than incubation with actinomycin alone, potentially explaining the requirement for continued incubation in ABT-737. We also noted that in spite of reduced viability consistent with data illustrated in Fig. 5A and Table 1, actinomycin D alone produced minimal PARP cleavage, an observation that we cannot readily explain at this time.

To determine the effects of a limited exposure to the drug combination on longer term viability and replicative potential, following 24 hours of combined drug exposure and an additional 48 hours in ABT-737, H526 cells

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Fig. 3. The combination of actinomycin D and ABT-737 selectively induces synergistic loss of viability in SCLC cell lines. The H526, H209, and WBA SCLC cell lines and the MRC-5 diploid pulmonary fibroblast cell line were exposed to the indicated concentration of actinomycin D and ABT-737 for 72 h, and viability was determined using an MTT assay. CIs, determined on expanded data sets by the method of Chou and Talalay, indicate synergistic results in all three SCLC cell lines. ABT, ABT-737. Data are representative of three independent determinations. Points, mean; bars, SD.
were washed and plated in soft agarose and viable colonies were assessed after 8 days. Whereas prior incubation in ABT-737 alone resulted in only a modest reduction in colonies, prior coincubation in the combination of ABT-737 and actinomycin D resulted in a >85% reduction in colony number (Fig. 5C). These data are consistent with the MTT data obtained immediately after drug exposure (Fig. 5A), showing that reduced viability after 72 hours accurately reflects reduced clonogenic potential. In addition, these data confirm the loss of viability seen with actinomycin D treatment (Fig. 5B) despite minimal PARP cleavage.

**Low-dose actinomycin D induces G2-M arrest**

Actinomycin D, used at doses that inhibit general transcription (1-10 μg/mL), is known to induce dual cell cycle actinomycin D–induced changes in Mcl-1 and Noxa expression mediate synergy with ABT-737, and this drug combination circumvents MRP1-mediated drug resistance. The parental H69 SCLC cell line (A) and the MRP1-amplified H69AR derivative (B) were exposed to the indicated concentrations of actinomycin D and ABT-737 for 72 h, and viability was determined using an MTT assay. MRP1 expression induced relative resistance to ABT-737 in the H69AR cell line but also was associated with enhanced sensitivity to actinomycin D. C, Chou and Talalay median dose effect analyses were done through MTT assay on H526-NOXA 1A3 cells and the corresponding vector control using combinations of actinomycin D (0.13-1.3 ng/mL) and ABT-737 (37-370 nmol/L). At low drug concentrations, the Noxa-expressing cell line consistently showed stronger synergy at all dose effect levels (P = 0.02 at FA50, FA75, P = 0.07 at FA90) compared with the vector control. Data are representative of three independent determinations. Columns, mean; bars, SD.

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

**Fig. 4.** Actinomycin D–induced changes in Mcl-1 and Noxa expression mediate synergy with ABT-737, and this drug combination circumvents MRP1-mediated drug resistance. The parental H69 SCLC cell line (A) and the MRP1-amplified H69AR derivative (B) were exposed to the indicated concentrations of actinomycin D and ABT-737 for 72 h, and viability was determined using an MTT assay. MRP1 expression induced relative resistance to ABT-737 in the H69AR cell line but also was associated with enhanced sensitivity to actinomycin D. C, Chou and Talalay median dose effect analyses were done through MTT assay on H526-NOXA 1A3 cells and the corresponding vector control using combinations of actinomycin D (0.13-1.3 ng/mL) and ABT-737 (37-370 nmol/L). At low drug concentrations, the Noxa-expressing cell line consistently showed stronger synergy at all dose effect levels (P = 0.02 at FA50, FA75, P = 0.07 at FA90) compared with the vector control. Data are representative of three independent determinations. Columns, mean; bars, SD.

![Graph A](image4)

![Graph B](image5)

![Graph C](image6)

**Fig. 5.** Clinically relevant exposure to actinomycin D is sufficient to induce maximal cytotoxicity and loss of clonogenic potential in combination with ABT-737. A, SCLC cell lines were exposed to 4 ng/mL actinomycin D for 24 h, 3.3 μmol/L ABT-737 (1.1 μmol/L for H69) for 72 h, or the combined treatment for 24 h followed by an additional 48 h in ABT-737, with viability analyzed by MTT assay at 72 h. B, H526 cells were treated with 4 ng/mL actinomycin D for 24 h, 3.3 μmol/L ABT-737 for 72 h, or the drug combination as described above, and lysed 72 h after initiation of actinomycin treatment. Mcl-1 and Noxa expression ratios were determined by densitometry relative to actin and are displayed below each lane along with viability determined by MTT assay in parallel to the Western blot. C, the above 72-h treatment protocol was done on H526 cells, which were then washed free of drugs, dispersed as a single-cell suspension, and plated in soft agarose. Colonies were stained with MTT and counted after 8 d. Data are representative of three independent determinations. Columns, mean; bars, SD.
arrest at the G1-S and G2-M interfaces, likely secondary to inhibition of cyclin synthesis (21, 22). To investigate the effect of clinically relevant actinomycin D exposure on cell cycle kinetics, we exposed asynchronous H1526 cells to 4 ng/mL actinomycin for 24 hours in the absence or presence of 3.3 μmol/L ABT-737 and analyzed their cell cycle profile after 48 hours using propidium iodide flow cytometry (Fig. 6). Actinomycin treatment alone induced a high-grade G2-M arrest, with a doubling in the percent of cells in G2-M compared with controls. ABT-737 treatment alone produced a cell cycle profile indistinguishable from control except for a slight increase in G1 fraction that was subject to interexperimental variation. Interestingly, the drug combination resulted in a composite of these two effects, with the cell cycle profile most resembling that of control cells except for a 9- to 10-fold increase in the percentage of hypodiploid cells relative to that seen with either drug alone. It is worth noting that the hypodiploid cells likely underestimate the degree of apoptosis, not only because DNA fragmentation is a late event in ABT-737-induced apoptosis (23) but also because DNA fragmentation in cells arrested in G2 could result in DNA content indistinguishable from S-phase cells.

**Discussion**

In this report, we have shown that clinically relevant concentrations of actinomycin D decrease Mcl-1 expression, increase Noxa expression, and act synergistically with the BH3-mimetic drug ABT-737 to cause loss of viability and clonogenic potential of SCLC cell lines. BH3-mimetic drugs have the potential to dramatically alter cancer treatment, not only because of their single-agent activity in malignancies that are highly dependent on prosurvival Bcl-2 family function, but also because of their much broader applicability in sensitizing tumor cells to the effects of a wide variety of chemotherapeutic and biologically targeted agents (6). Although SCLC is one of the few solid tumors to show responsiveness to single-agent ABT-737 (7) and the closely related ABT-263 (24), currently in early phase clinical trials, the fact remains that SCLC has shown responsiveness to a wide variety of single agents (1). It was recognized approximately four decades ago that to make progress beyond the small improvements in survival produced by single agents, combination therapy would be required. Among the first studies to test this hypothesis was conducted by Hansen et al. (25), who used alternating therapy with cyclophosphamide/methotrexate and actinomycin D/vincristine, with radiation administered to those patients with limited stage concomitant with the actinomycin D/vincristine therapy. This pioneering study was also remarkable for characterizing response by histologic subtype and by careful staging, devising a staging system for SCLC that is still essentially unchanged. Interestingly, for the four extensive stage SCLC patients treated with this protocol, median survival was ~9 months, which is similar to modern combination regimens (1). This observation not only suggests that actinomycin D has some clinical activity in SCLC, but also underscores the limited progress made in the treatment of extensive stage SCLC in the ensuing years. However, considerable progress has been made in understanding the molecular pathogenesis of SCLC (26), and our current challenge is to use this information to derive better combination regimens.

One possible approach to deriving more active combination regimens is to combine agents that are not only synergistic but also block the major mechanisms of resistance to the partner drugs. We and others have shown that high-level expression of Mcl-1 or low expression of Noxa is a common mechanism of SCLC resistance to ABT-737 (4, 5). We have shown that actinomycin D, in addition to decreasing Mcl-1 expression at clinically relevant concentrations, also had the surprising effect of increasing Noxa expression (Fig. 1B), the mechanism for which has yet to be elucidated. Based on our previous data (5), these expression changes should cooperatively increase sensitivity to ABT-737. We have shown (Figs. 3 and 4) that the combination with actinomycin D is synergistic, and that alteration of the Mcl-1/Noxa axis by overexpression of Noxa enhances that synergy at low ABT-737 concentrations, implicating the actinomycin-induced changes in Mcl-1 and Noxa expression in the mechanism for that synergy. Interestingly, although expression of MRP1 in the H69AR cell line provided some resistance to ABT-737, confirming data previously obtained using ABT-263 (8), this cell line was more sensitive to actinomycin D, resulting

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**Table 1. ABT-737 must be present for 48 h following combined drug exposure for optimal activity**

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>% Viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>ActD</td>
<td>33 ± 1.3</td>
</tr>
<tr>
<td>ABT-737</td>
<td>78 ± 3.1</td>
</tr>
<tr>
<td>ActD</td>
<td>19 ± 1.0</td>
</tr>
<tr>
<td>ABT-737</td>
<td>28 ± 1.4</td>
</tr>
<tr>
<td>ActD</td>
<td>41 ± 1.2</td>
</tr>
<tr>
<td>ABT-737</td>
<td>84 ± 3.4</td>
</tr>
</tbody>
</table>

ActD (4 ng/mL) and ABT-737 (3.3 μmol/L) were present (+) during the indicated 24-h intervals. Viability was measured by MTT assay after 72 h.
in an identical response to the combination of actinomycin D plus 1.1 μmol/L ABT-737 as did the H69 parental cell line (Fig. 4B). These data suggest that actinomycin D may also compensate for the specific type of ABC transporter drug resistance commonly seen in SCLC.

To explore the clinical feasibility of the combination of actinomycin D and BH3-mimetics, we studied the effects of scheduling using a clinical achievable area under the curve for actinomycin. Exposure to an actinomycin D concentration of 4 ng/mL for 24 hours was as effective as 72 hours, provided that ABT-737 was continuously present during the 72-hour incubation (Fig. 5A). This finding is not surprising, given that actinomycin D is an avid DNA-intercalating agent and concentrations within leukocytes remain constant for days relative to rapid disappearance from plasma and RBC (14). It is interesting that the presence of ABT-737 was required for at least 48 hours after the initial coincubation, suggesting that although the biological effect of actinomycin D lingered after removal from drug, to obtain maximal loss of viability, Bcl-2 and Bcl-XL function also had to be suppressed for this period of time (Table 1). Loss of viability after 72 hours of incubation correlated well with the loss of clonogenic potential (Fig. 5C), suggesting that the sequence of 24 hours coincubation with actinomycin and ABT-737 followed by 48 hours in ABT-737 was sufficient to eliminate the self-renewing cell population.

Although we believe sufficient data exist to implicate changes in Mcl-1 and Noxa expression in synergism between actinomycin D and ABT-737, given the potential effects on expression of a broad range of genes, it is possible that other effects also contribute to synergy. A recent report has described alterations in the expression of several hundred genes as a result of treatment of HCT116 colon carcinoma cells with actinomycin D doses comparable with those we have used (27). One important effect that we have noted, which is potentially unrelated to alterations in Mcl-1 and Noxa expression, is that exposure to low-dose actinomycin D induced a high-grade G2-M cell cycle arrest in SCLC cells (Fig. 6). Although a prior study using labeling and mitotic indices showed dual arrest at the G1-S and G2-M interfaces in Chinese hamster ovary cells, that study did show that at concentrations of actinomycin D <1 μg/mL, the G2 arrest predominated (21), in good agreement with our flow cytometry data. It is interesting to speculate whether the effects on cell cycle progression, likely mediated through interference with the cyclin/cyclin-dependent kinase cascade (22), contributed to the synergistic induction of apoptosis.

In summary, this study has shown that clinically relevant actinomycin D exposure, in combination with the BH3-mimetic drug ABT-737 synergistically induced apoptosis and loss of clonogenic potential in multiple SCLC cell lines displaying moderate to high-grade resistance.
to ABT-737 as a single agent. The mechanism for synergy likely involves an actinomycin D–induced decrease in Mcl-1 and increase in Noxa expression, but other mechanisms including opposing effects on cell cycle progression could possibly also contribute to the effect. Based on these data, as well as prior clinical data indicating potential utility of actinomycin D in SCLC treatment, we believe that consideration of the inclusion of actinomycin D and a BH3-mimetic drug in future salvage and potentially first-line SCLC treatment protocols is warranted. It can be speculated that the use of this combination in malignancies such as sarcomas and Wilms tumor, in which actinomycin D continues to play a cornerstone role in chemotherapeutic regimens, may be advantageous, but this conclusion awaits the development of appropriate preclinical data.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Haishan Xu and Geoffrey W. Krystal


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