Activity of the Bcl-2 Family Inhibitor ABT-263 in a Panel of Small Cell Lung Cancer Xenograft Models

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Abstract

Purpose: The purpose of this study was to characterize the activity of the Bcl-2 protein family inhibitor ABT-263 in a panel of small cell lung cancer (SCLC) xenograft models.

Experimental Design: A panel of 11 SCLC xenograft models was established to evaluate the efficacy of ABT-263. Single agent activity was examined on a continuous dosing schedule in each of these models. The H146 model was used to further evaluate dose and schedule, comparison to standard cytotoxic agents, and induction of apoptosis.

Results: ABT-263 exhibited a range of antitumor activity, leading to complete tumor regression in several models. Significant regressions of tumors as large as 1 cc were also observed. The efficacy of ABT-263 was also quite durable; in several cases, minimal tumor regrowth was noted several weeks after the cessation of treatment. Antitumor effects were equal or superior to that of several clinically approved cytotoxic agents. Regression of large established tumors was observed through several cycles of therapy and efficacy was retained in a Pgp-1 overexpressing line. Significant efficacy was observed on several dose and therapeutic schedules and was associated with significant induction of apoptosis.

Conclusions: ABT-263 is a potent, orally bioavailable inhibitor of Bcl-2 family proteins that has recently entered clinical trials. The efficacy data reported here suggest that SCLC is a promising area of clinical investigation with this agent.

With >170,000 new cases and 160,000 deaths in 2006, lung cancer represents the most deadly malignancy in the United States (1). Small cell lung cancer (SCLC) is a neuroendocrine-derived subtype of this disease that accounts for 15% to 20% of these totals (2). Standard therapy for SCLC typically involves a combined regimen of etoposide and cisplatin plus radiotherapy, and although a relatively robust initial response is often observed, a high percentage of patients ultimately develop highly refractory disease (3, 4). Indeed, median survival for patients diagnosed with extensive disease is <1 year, and fewer than 5% of individuals survive beyond 2 years from diagnosis (3). Among the genetic defects implicated in disease initiation and progression are loss of the *p53* and *RB* tumor suppressor genes, and activation of the c-*kit* and *MYC* oncogenes and their downstream effectors, such as *AKT* (5–9). Less well-understood are the potential contributions of apoptotic signaling molecules to the progression of this disease (10–12). However, recent reports have shown that several Bcl-2 family proteins are frequently overexpressed and/or amplified in SCLC tumor lines (13–15). Furthermore, Phase I studies using antisense oligonucleotides directed against Bcl-2 in SCLC showed that this therapeutic intervention was well-tolerated and generated encouraging response rates (16). Other reported small molecule inhibitors of Bcl-2 family proteins have also recently entered clinical trials (17, 18).

We have reported previously on the activity of ABT-737, a small molecule inhibitor of Bcl-2 family proteins that binds with high affinity to Bcl-2, Bcl-X_L, and Bcl-w (K_i, <1 nmol/L for all three proteins; refs. 19, 20). This compound shows robust single agent activity in models of SCLC as well as in several hematologic tumor models. Synergistic activity with several cytotoxic and targeted agents has also been shown (19, 21–27). Importantly, ABT-737 also kills cells via a BAX/BAK-dependent mechanism (19, 25, 28).

Although ABT-737 has proven to be very important for proof-of-concept evaluation of inhibitors of Bcl-2 family proteins, the clinical utility of this agent is limited due to its poor solubility and lack of oral bioavailability. We have described recently the discovery of a next generation Bcl-2 family inhibitor, ABT-263 (29). Here, we report in detail the *in vivo* efficacy of ABT-263 in a large panel of SCLC xenograft models. ABT-263 and ABT-737 exhibit very similar binding profiles to Bcl-2 family proteins; both compounds exhibit

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K_is of <1 nmol/L for Bcl-2, Bcl-X_L, and Bcl-w and exhibit much weaker binding to Mcl-1 and A1 (K_is ranging from ~0.5->1 μmol/L). However, whereas ABT-737 is a parenteral agent, ABT-263 is orally bioavailable and more suited for continuous daily dosing. ABT-263 caused complete regression of established tumors in multiple models, even when therapy was initiated on large tumors of 1,000 mm^3. Antitumor efficacy was durable with minimal tumor regrowth noted after termination of dosing in several cases. Efficacy was equal to or superior to that of several standard cytotoxic agents and was accompanied by a robust induction of apoptosis *in vivo*.

Materials and Methods

In vitro *activity*. All SCLC cell lines were obtained from the American Type Culture Collection. Cells were cultured using standard techniques in RPMI 1640 supplemented with 10% fetal bovine serum. Cell survival was analyzed as described previously (24). For Western analysis, 20 μg of total protein was loaded on 4% to 12% Bis-Tris NuPage Gel (Invitrogen). Gels were transferred to Protran nitrocellulose membranes (Schleicher & Schuell BioScience) and blocked with PBS containing Tween 20 and polyvinylpyrrolidine (Sigma) as previously described (30). Primary antibodies used were rabbit anti – Bcl-2 at 1:500 (BD Pharminogen) and goat antiactin at 1:200 (sc-1615; Santa Cruz Biotechnology).

In vivo *xenograft modeling*. All animal studies were conducted in accordance with the guidelines established by the internal Institutional Animal Care and Use Committee. C.B.-17 *scid*-bg (*scid*-bg) or C.B.-17 *scid* (*scid*) mice (Charles River Laboratories) were implanted with 5×10^6 cells (H146, H1963, H211, H510, H345, H1048, and H82) or 1×10^6 cells (H526) of each line s.c. The H889, H1417, and H128 lines are maintained by *in vivo* propagation. Inoculation volume was 0.2 mL consisting of 50% Matrigel (BD Biosciences). When tumors reached the appropriate tumor volume, mice were size matched (day 0) into treatment and control groups. Tumor volume was estimated by twice weekly measurements of the length and width of the tumor by electronic calipers and applying the following equation: $V = L \times W^2/2$. Tumor growth inhibition (%TGI) was calculated based on the difference in mean tumor volumes for the treated group relative to the appropriate vehicle control. Partial response (%PR) was defined as regression of an established tumor by at least 50% relative to its starting volume. Complete response (%CR) was defined as regression of established tumor beyond the level of detection by palpation. Overall response rate (%ORR) is the sum of %PR plus %CR rates. Effects on tumor growth delay were assessed by determining percent increase in life span as described previously (31). Statistical comparisons of tumor growth rate and tumor growth delay were conducted using the Wilcoxon rank sum test and Kaplan-Meier log-rank analysis, respectively.

ABT-263 ((R)-4-(3-Morpholin-4-yl-1-phenylsulfanylmethyl-propylamino)-N-(4-{4-[2-(4-chlorophenyl)-5,5-dimethylcyclohex-1-enylmethyl]-piperazin-1-yl}-benzoyl)-3-trifluoromethanesulfonylbenzenesulfonamide) was formulated in 10% ethanol, 30% polyethylene glycol 400, and 60% Phosal 50 PG (American Lecithin Company). The synthesis of this compound is described elsewhere (32). Cytotoxic drugs (paclitaxel, vincristine, carboplatin, cisplatin, etoposide, and cyclophosphamide) were administered i.p. or i.v. and formulated according to the manufacturers recommendations. Paclitaxel, carboplatin, and cyclophosphamide were obtained from Bristol-Myers Squibb. Cisplatin and etoposide were obtained from Bedford Laboratories. Vincristine was obtained from GensiaSicor Pharmaceuticals.

Immunohistochemistry. Tumor samples were collected into Streck Tissue Fixative (Streck Laboratories, Inc.) or 10% neutral buffered formalin (Richard Allen Scientific). Primary antibodies used were mouse anti-human Bcl-2 at 1:150 (M0887; DAKO Corp.), mouse anti-human Pgp-1 at 1:200 (MAB4334; Chemicon), and rabbit anti-human directed against the activated form of caspase-3 (BD PharMingen) at 1:400. Image analysis of the caspase-3 staining was conducted using AxioVision software (Zeiss, Inc.).

Results

Activity of ABT-263 in a panel of SCLC tumor lines. The cellular potency of ABT-263 in a panel of SCLC cell lines is shown in Table 1. A subset of these SCLC lines was developed as xenograft models for evaluation of antitumor efficacy. The cell lines used are classified mostly as classic SCLC and

Table 1. Activity of ABT-263 in 11 SCLC tumor models

Cell line	Cellular EC_{50} (nmol/L)*	Fold over enantiomer †	TGI ‡	%PR§	%CR‖	%ORR¶
H146	110 ± 39 (3)	>91	94	40	60	100
H889	140 ± 40 (3)	76	100	0	100	100
H1963	180 ± 60 (3)	223	100	0	100	100
H1417	360 ± 90 (3)	>28	60	13	13	26
H211	410 ± 200 (3)	>24	55	0	30	30
H128	N.D.	N.D.	41	0	0	0
H526	N.D.	N.D.	20	0	0	0
H510	1,220 ± 540 (3)	>8	30	0	0	0
H345	2,160 ± 580 (3)	>4	83	20	0	20
H1048	2,860 ± 430 (6)	7	56	0	0	0
H82	22,400 ± 2,800 (3)	1	13	0	0	0

NOTE: *In vitro* and *in vivo* experiments were conducted as described in Materials and Methods. For *in vivo* studies, tumors were size matched to 200 to 250 mm^3 before therapy. ABT-263 was given at 100 mg/kg/d, p.o., q.d. ×21 d. n = 8 to 10 mice per group.
Abbreviation: ND, not determined.
*Mean EC_{50} ± SE in 10% human serum (n).
† Cellular activity compared with the less active enantiomer of ABT-263.
‡ % TGI as measured at the end of the dosing period.
§ %PR, 50% < tumor shrinkage < 100%, relative to starting tumor volume.
‖ %CR, no palpable tumor present.
¶ %ORR, %PR + %CR.

represent tumors isolated from both the lung and sites of metastasis (33). Most of the patients had extensive disease at the time of cell isolation and had undergone various chemotherapy regimens and/or radiation (Supplementary Table S1). A wide range of cellular activity was observed with ABT-263 having a 50% growth inhibition (EC_{50}) of 110 nmol/L against the most sensitive line (H146), whereas its activity in the least sensitive line (H82) resulted in an EC_{50} at 22 μmol/L (Table 1). In all cell lines where the EC_{50} was <1 μmol/L, ABT-263 was more potent than its enantiomer by a factor of >20, suggesting mechanism-based cell killing. The mechanism-based nature of the activity of ABT-263 has been analyzed in detail (34). ABT-263 treatment of tumor cell lines results in potent, dose-dependent induction of Bax translocation, cytochrome *c* release, and activation of caspase-3. Compound treatment also disrupts interactions between Bim and antiapoptotic Bcl-2 family proteins and results in selective killing of FL5.12 cells engineered to be dependent on either Bcl-2 or Bcl-X_L for survival. Importantly, the enantiomer of ABT-263 exhibits dramatically less activity in each of these assays. In general, there was good correlation in cell line sensitivity/resistance for ABT-263 and that reported previously for ABT-737 (24). All four cell lines with EC_{50}s of <400 nmol/L (H146, H889, H1963, and H1417) were also highly sensitive to ABT-737, and the two most resistant lines (H1048 and H82) were similarly resistant to ABT-263.

To evaluate the *in vivo* activity of ABT-263, nine of the cell lines evaluated for *in vitro* activity, plus two additional lines, were developed as xenograft models. For initial evaluation, ABT-263 was administered at a dose of 100 mg/kg/day given p.o. once daily for 21 days. This dose generates peak drug plasma levels of ~7 μmol/L and is well-tolerated in mice with modest weight loss (typically <5%) noted. As reported previously for ABT-737 (19, 35, 36), administration of ABT-263 at 100 mg/kg resulted in a significant decrease in circulating platelets and lymphocytes. Six hours after a single 100 mg/kg dose, platelets were reduced by ~90% relative to baseline values in CF-1 mice. As observed with ABT-737, platelet counts returned to baseline values by 72 hours.

ABT-263 was highly efficacious in three of the SCLC tumor lines tested (H146, H889, and H1963; Table 1). In each of these models, a 100% ORR was achieved with rapid and complete regression of all tumors for the H889 and H1963 models. Tumor response was also durable; there were numerous tumors that failed to show significant tumor regrowth (tumor volume, <100 mm^3) at the end of the study (H146, 1 of 10 evaluable tumors at day 134; H889, 3 of 6 tumors at day 70; H1963, 6 of 10 tumors at day 62). The absence of viable tumor cells was confirmed in several cases by analyzing serial sections of tissue surrounding the tumor injection site (data not shown). Significant antitumor activity was observed in three additional SCLC models (H1417, H211, and H345) with robust inhibition of tumor growth rate and a smaller fraction of tumor regression (Table 1). In the remaining five models, modest inhibition of tumor growth rate (40-60% TGI) was observed in two models (H128 and H1048), but little to no activity was apparent in the other three models (H526, H510, and H82; Table 1). In general, *in vivo* efficacy correlated well with cellular activity. Interestingly, efficacy was observed in two models (H345 and H1048) where relatively weak *in vitro* activity was observed (see below).

Efficacy of ABT-263 relative to standard cytotoxic agents. The H146 xenograft model was used to gauge the chemotherapeutic effect of ABT-263 relative to clinically approved agents. Each cytotoxic agent was used at or near the maximum tolerated dose in mice. H146 xenograft tumors were found to be highly responsive to treatment with paclitaxel or vincristine (Fig. 1A). Paclitaxel given at 30 mg/kg/day, q4d × 3 resulted in PR and CR in 4 of 6 and 2 of 6 tumors, respectively. Vincristine given at 0.5 mg/kg/day, q4d × 4 resulted in PR and CR in 5 of 9 and 1 of 9 tumors, respectively. Carboplatin, cisplatin, cyclophosphamide, and etoposide all showed antitumor activity in the H146 model; however, tumor regression was not observed for any of these agents. Carboplatin, cisplatin, and etoposide treatment resulted in 40% to 60% TGI that was statistically significant throughout the course of the trial, whereas cyclophosphamide treatment resulted in a transiently significant 20% to 30% inhibition of tumor growth (Fig. 1A). The efficacy of ABT-263 at doses at or above 100 mg/kg/day was comparable or superior to all of the cytotoxic agents tested (Fig. 1A; Table 2). Highly significant inhibition of tumor growth rate was achieved with partial or complete tumor regression observed in the majority of tumors.

To further evaluate the efficacy of ABT-263, a separate experiment was conducted in which therapy was initiated in H146 tumors of ~1,000 mm^3. As shown in Fig. 1B, treatment with ABT-263 at 100 mg/kg/day led to significant regression of all treated tumors. By the end of treatment, the average tumor volume was reduced by >80% relative to the starting value. The rate of response was faster and the magnitude of effect was similar to that observed for docetaxel given at 30 mg/kg, i.v., q7d × 2. This dose of docetaxel was not tolerated, however, as treated mice had to be terminated after the second dose of drug due to >20% weight loss.

Effective chemotherapeutic treatment of SCLC is related directly to previous treatment history. Objective response rates of >50% can be achieved with agents such as etoposide in first-line therapy (37). However, response rates decrease significantly in second-line or later settings. The induction of resistance factors such as an increase in expression of drug efflux pumps contributes to this loss of efficacy. Similarly, we found H146 tumors respond well to initial therapy with paclitaxel, but resistance quickly developed with subsequent rounds of treatment. H146 tumors treated with paclitaxel at 30 mg/kg/day q4d × 4 were propagated into new hosts after relapse. These tumors were passaged in mice four times (in the absence of paclitaxel treatment) to establish an H146 variant line. This line was then expanded and retested with paclitaxel at 30 mg/kg/day, q4d × 3. As shown in Fig. 1C, this variant line was considerably more resistant to paclitaxel than the parental H146 line (Fig. 1A). Resistance to vincristine was also observed in this line (data not shown). Concomitant with this acquired resistance to paclitaxel was a significant increase in expression of the Pgp-1 drug efflux pump protein (Fig. 1C, *insert*). Importantly, ABT-263 was still highly efficacious in this H146 variant line (68% TGI and 63% PR). These results suggest that Pgp-1 expression may not be a primary mechanism of resistance to ABT-263 in the clinic.

To investigate further potential resistance to ABT-263, parental H146 tumors were treated with multiple cycles of ABT-263 (Fig. 1D). Tumors were size matched at ~200 mm^3 and a subset were treated with ABT-263 at 100 mg/kg/day for 5 days. All

tumors regressed in response to this initial treatment but eventually began to regrow. However, H146 tumors consistently responded robustly (regressed) after treatment with ABT-263 (Fig. 1D). Response included large tumors (1,000-2,000 mm^3) that received as many as six previous cycles of therapy.

Analysis of dose and schedule of ABT-263. ABT-263 was examined in the H146 model at doses ranging from 25 to 300 mg/kg/day given on a continuous daily dosing regimen. Each of these doses provided statistically significant inhibition of tumor growth (Table 2). Doses of 100 mg/kg/day and higher resulted in a 100% ORR with complete tumor response in the majority of animals. One death due to unknown cause was noted during dosing in the 300 mg/kg group. Treatment at 50 mg/kg/day resulted in ~80% TGI with CR in 4 of 9 and PR

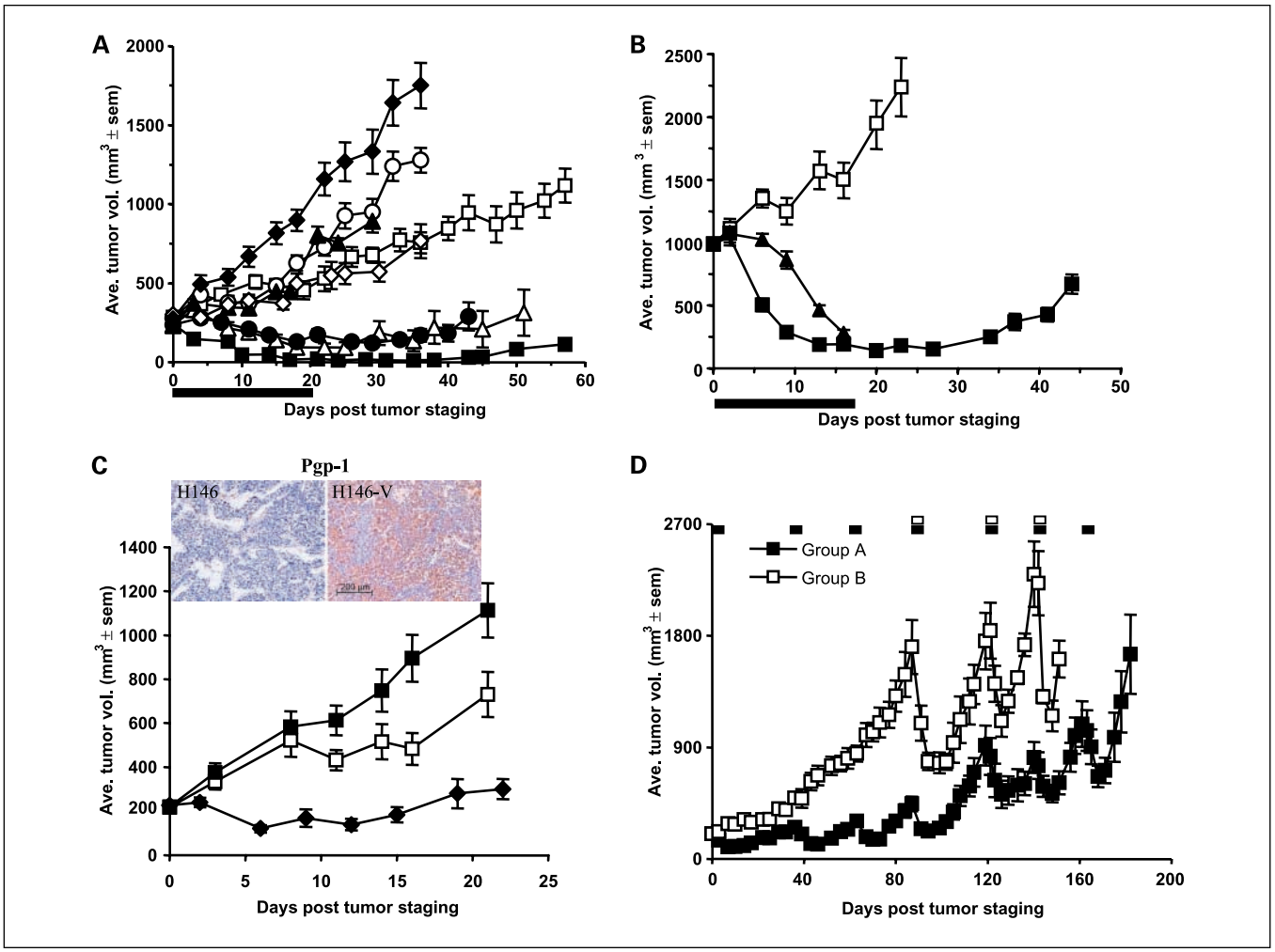

Fig. 1. *A,* efficacy of ABT-263 in the H146 SCLC xenograft model relative to several standard cytotoxic agents. Shown is data compiled from seven independent experiments. In each trial, tumors were size matched to 240 to 300 mm^3 (day 0) and therapy was initiated the following day. *Open circles,* cisplatin given at 3 mg/kg, i.p., thrice every 4 d; *closed triangles,* etoposide given at 25 mg/kg, i.p., q4d × 3; *open squares,* carboplatin given at 50 mg/kg, i.p., q4d × 4; *open diamonds,* cyclophosphamide given at 100 mg/kg, i.p., q4d × 3; *closed circles,* vincristine given at 0.5 mg/kg, i.v., q7d × 4; *open triangles,* paclitaxel given at 30 mg/kg, i.p., q4d × 3; *closed squares,* ABT-263 given at 100 mg/kg, p.o., 21 doses daily (*black bar*); *closed diamonds,* cisplatin vehicle. For simplicity, only one vehicle group has been plotted. However, all statistics and analyses of efficacy were conducted by comparing to the vehicle control specific for each agent. All cytotoxic agents were given at or near their maximum tolerated doses. All drugs exhibited a statistically significant inhibition of tumor growth throughout the study except for cyclophosphamide, which was significant only on days 4, 8, and 16 postdose initiation ($P < 0.05$, Wilcoxon rank sum test). *B,* treatment with ABT-263 causes regression of large, established H146 xenograft tumors. Tumors were allowed to reach an average tumor volume of ~1,000 mm^3 before initiation of therapy. *Closed squares,* ABT-263 was given at 100 mg/kg, p.o., 17 doses daily (*black bar*); *closed triangles,* docetaxel given at 30 mg/kg, i.v., q7d × 2; *open squares,* vehicle. ABT-263 treatment resulted in 92% TGI at the end of therapy with all tumors showing at least an 80% reduction in tumor volume relative to starting size ($n = 10$ mice per group). *C,* analysis of paclitaxel-resistant variant of H146. Parental H146 tumors were initially treated with four doses of paclitaxel at 30 mg/kg/d. Tumors that relapsed after treatment were propagated into new hosts and expanded into new lines. The H146 variant line (H146-V) shown here was significantly more resistant to paclitaxel treatment of 30 mg/kg/d compared with the parental line. *Closed diamonds,* ABT-263 given at 100 mg/kg, p.o., 21 doses daily; *open squares,* paclitaxel given at 30 mg/kg, i.p., q4d × 3; *closed squares,* paclitaxel vehicle. Immunohistochemical analysis of parental H146 and H146-V tumors showed that the variant line expresses significantly higher levels of Pgp-1 (*inset*; magnification, ×100). ABT-263 given at 100 mg/kg/d still showed significant efficacy in the H146-V line ($P < 0.01$, Wilcoxon rank sum test). *D,* efficacy of ABT-263 in the H146 xenograft model after multiple cycles of therapy. Tumors were randomized into groups of equal tumor volume (~200 mm^3) on day 0 with group A receiving ABT-263 at 100 mg/kg/d, p.o., from day 0 to 4 and group B receiving vehicle. Additional 5-d cycles of treatment with ABT-263 at 100 mg/kg/d were administered as follows: group A, d36-40, 63-67, 87-91, 119-123, 140-145, and 161-165; group B, d87-91, 119-123, and 140-144. Group B was also treated with vehicle on days 36 to 40 and 63 to 67. *Black boxes,* dosing periods for group A; *white boxes,* dosing periods for group B. ABT-263 treatment resulted in significant tumor regression, even after six previous cycles of therapy. Regression of large (>2,000 mm^3) tumors was also seen after multiple therapy cycles.

Table 2. Dose response of ABT-263 in the H146 SCLC xenograft model

Dose (mg/kg/d)	Ave. tumor volume (± SE)	%TGI*	%PR	%CR	ORR
300	20 ± 7	94 †	13	87	100
200	25 ± 8	93 †	10	90	100
100	20 ± 6	94 †	40	60	100
50	140 ± 31	77 †	44	22	66
25	414 ± 16	31 †	0	0	0
0	601 ± 52				

NOTE: Tumors were size matched (day 0) to ~230 mm^3 before therapy (day 1), n = 10 mice per group. ABT-263 given p.o., q.d., d 1-21. Data compiled from two studies.
Abbreviations: Ave. tumor volume, average tumor volume; q.d. d 1-21, daily from day 1 to 21.
*% TGI as measured at the end of the dosing period.
† $P < 0.01$ versus vehicle (Wilcoxon Rank Sum test).

(tumor shrinkage of at least 50% relative to starting volume) in 2 of 9 evaluable mice, respectively (Table 2). Based on these results, a dose of 50 mg/kg/day was deemed to be the minimally efficacious dose in the H146 model. Pharmacokinetic analysis of plasma blood levels indicated a C_{max} of ~5 μmol/L and area under the plasma concentration curve of ~50 μmol/L·hr at the 50 mg/kg dose.

To evaluate further the dose/efficacy relationship, a variety of alternative dosing schedules were evaluated. Continuous dosing for 21 days provided the most robust efficacy with 100% ORR, including complete regressions and one maintained complete regression (no detectable tumor at the end of the study) in both the once daily and twice daily every 21 days dosing groups (Fig. 2). Twice daily dosing at 100 mg/kg/day (i.e., 50 mg/kg per dose) provided equivalent efficacy to once daily dosing at 100 mg/kg. Significant antitumor activity, including a high percentage of partial regressions, was observed when ABT-263 was administered for 14 days or as few as 7 consecutive days at 100 mg/kg/day.

ABT-263 was also efficacious when administered on intermittent dosing schedules. Treatment at 50 or 100 mg/kg every 3 days or 100 mg/kg every 7 days resulted in significant inhibition of tumor growth rate as well as causing a significant tumor growth delay (Fig. 2). The 100 mg/kg dose given q3d × 24 resulted in a 47% delay in median time for tumors to reach 800 mm^3 ($P < 0.001$, Kaplan-Meier log-rank test). These results indicate that acute administration of ABT-263 is sufficient to result in significant antitumor activity; however, chronic therapy provides a stronger effect on tumor growth delay and more pronounced regression. The fact that robust activity is observed with intermittent administration of ABT-263 suggests that a significant acute apoptotic response occurs in tumors after treatment. Histologic analysis of H146 tumors after a single dose of ABT-263 revealed extensive apoptosis within hours of treatment (Fig. 3). In contrast to generalized tumor necrosis that typically is most pronounced in the center of xenograft tumors, H146 tumors treated with a single dose of ABT-263 showed significant numbers of dead and dying cells throughout, including the well-vascularized tumor periphery. Within 24 hours of treatment at 100 mg/kg the number of caspase-3–positive cells was elevated by >15-fold relative to vehicle controls.

H1048 and H345 tumors are more sensitive to ABT-263 than predicted by in vitro potency. We have shown previously that sensitivity to ABT-737 and ABT-263 in a variety of cell lines correlates with relative expression of Bcl-2 family member proteins (24, 29, 34). Sensitive cell lines tend to express higher levels of Bcl-2, Bcl-X_L, Noxa, and/or Bim and lower levels of Mcl-1 compared with resistant lines. For most of the cell lines shown in Table 1, there is good correlation between *in vitro* cellular potency and *in vivo* tumor efficacy. Cell lines with an EC_{50} of <200 nmol/L were associated with 100% ORR *in vivo*. The two cell lines with an EC_{50} of ~400 nmol/L (H1417 and H211) showed significant inhibition of tumor growth rate and some evidence of tumor regression *in vivo*. Minimal *in vivo* efficacy was observed in two models where the cellular EC_{50} was >1 μmol/L (H510 and H82). A similar pattern of activity was observed for ABT-737 with >75% TGI and >33% ORR observed for all cell lines with an EC_{50} of <300 nmol/L (data not shown; refs. 19, 24).

Interestingly, ABT-263 was active in two models (H1048 and H345) despite EC_{50}s of >2 μmol/L in these two cell lines. When ABT-263 was administered at 100 mg/kg/day in the H345 xenograft model, significant antitumor efficacy was observed with 80% TGI and 20% of treated tumors showing at least a 50% reduction in tumor volume (Table 1). Similarly, nearly 60% TGI (roughly equivalent to the maximally tolerated dose of etoposide) was observed in the H1048 model (Table 1; Fig. 4A). When examined by immunohistochemistry, the level of Bcl-2 expression in H345 and H1048 tumors was among the highest observed in our panel of SCLC xenograft models (Fig. 4B). In contrast, the H82 line (resistant to ABT-263 both *in vitro* and *in vivo*) showed no detectable expression of Bcl-2 by IHC. To accurately compare the levels of Bcl-2 expression in tumors compared with cells grown in culture, lysates from both sources were compared concomitantly by Western blot. Interestingly, analysis of Bcl-2 levels in H1048 tumors versus H1048 cells grown in culture revealed that Bcl-2 seemed to be up-regulated in the xenograft tumors relative to cells grown in culture (Fig. 4C). Only a slight increase was noted for H345 tumor versus cell lysates (data not shown). A similar effect was noted with ABT-737 in the H1048 line ($EC_{50} \approx 8$ μmol/L *in vitro*; 89% TGI and 60% PR *in vivo*). No significant differences in Mcl-1 expression between cell lines and xenograft tumors were observed (data not shown). These results suggest that robust growth of H1048 cells *in vivo* may be associated with an increased resistance to apoptosis, perhaps resulting from a reliance on up-regulation of Bcl-2 family proteins, and this may contribute to the enhanced sensitivity to ABT-263/737 observed *in vivo*.

Discussion

Stimulation of tumor-specific apoptotic response as a means of chemotherapeutic intervention is an area of active drug discovery being explored by a variety of approaches (38–40). One of the more promising lines of investigation centers on the development of small molecule inhibitors of Bcl-2 family proteins (19, 28, 41). In addition to the inherent challenge of trying to inhibit these protein-protein interactions, it is critical to show that the inhibitor is acting on mechanism. Merely

A

Dose (mg/kg/day)	Route/ Schedule[a]	Ave. Tumor Volume (± SEM)	%TGI[b]	%ILS[c]	%PR[d]	%CR[e]	ORR[f]
100	p.o., q,d., d 1-7	187 ± 13	45*	46*	40	0	40
100	p.o., q,d., d 1-14	109 ± 7	68*	66*	100	0	100
100	p.o., q,d., d 0-20	22 ± 5	96*	124*	80	20	100
100	p.o., q,d., d 0-20	27 ± 4	95*	111*	80	20	100
0	p.o., q,d., d 0-20	520 ± 39					
100	p.o., q7d x 11	528 ± 54	46*	79*	0	0	0
50	p.o., q3d x 24	473 ± 51	40*	29*	10	0	10
100	p.o., q3d x 24	226 ± 24	71*	47*	10	0	10
0	p.o., q3d x 24	790 ± 47					

[a] Tumors were size matched (day 0) to 205-230 mm^3, n = 10 mice per group. Data compiled from three trials.
[b] % Tumor growth inhibition as measured 22 (d1-7, d1-14 dosing), 23 (q.d. vs. b.i.d. dosing), or 43 (q7d and q3d dosing) days after initiation of dosing. TGI calculated based on comparison to relevant vehicle from each trial.
[c] Median % increase in time for tumors to reach 0.8 cc end point compared to vehicle control.
[d] % Partial response.
[e] % Complete response.
[f] Overall response rate (%PR + %CR).
*P < 0.01 vs. vehicle (Wilcoxon Rank Sum test for TGI, Kaplan-Meier logrank test for ILS).

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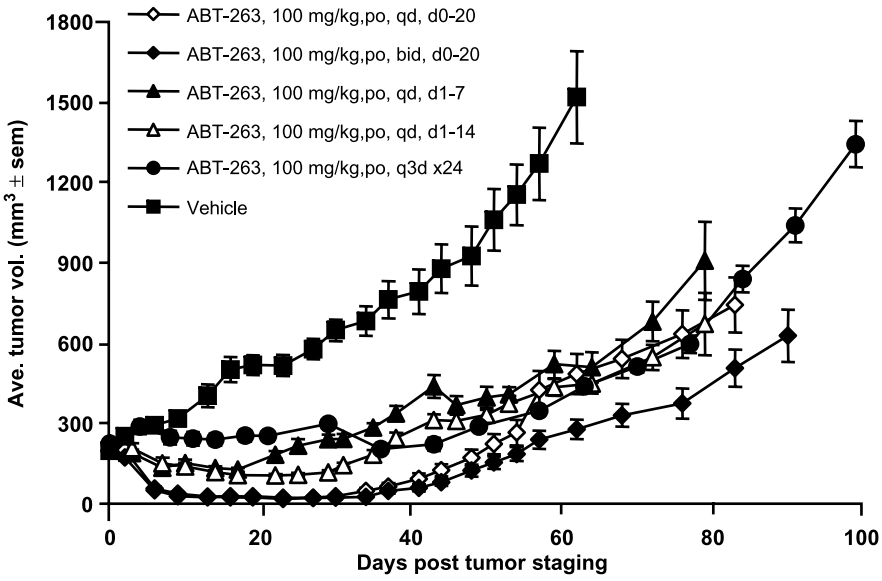

Fig. 2. Efficacy of ABT-263 in the H146 xenograft model when administered on various dosing schedules. *A,* comparison of efficacy for ABT-263 given on various continuous dosing schedules and on intermittent dosing schedules. Continuous dosing was more efficacious than intermittent dosing with a high percentage of complete and/or partial tumor regressions observed even with dosing as short as 1 wk. The efficacy observed with once daily dosing was equivalent to that observed with twice daily dosing. Intermittent dosing of ABT-263 (dosing every 3 or 7 d) led to significant inhibition of tumor growth. However, significant tumor regression was only observed with continuous dosing. *B,* growth curve of representative dosing schedules. All dosing schedules examined (continuous and intermittent) resulted in significant inhibition of tumor growth rate throughout the study ($P < 0.01$, Wilcoxon rank sum test). Plotting was terminated when individual mice from a group were removed due to tumors reaching end point. Q.d., daily; b.i.d., twice daily; q7d ×11, 1 dose every 7 d for 11 total doses; q3d ×24, 1 dose every 3 d for 24 total doses; q7d, once every 7 d; q3d, once every 3 d; ILS, increase in life span.

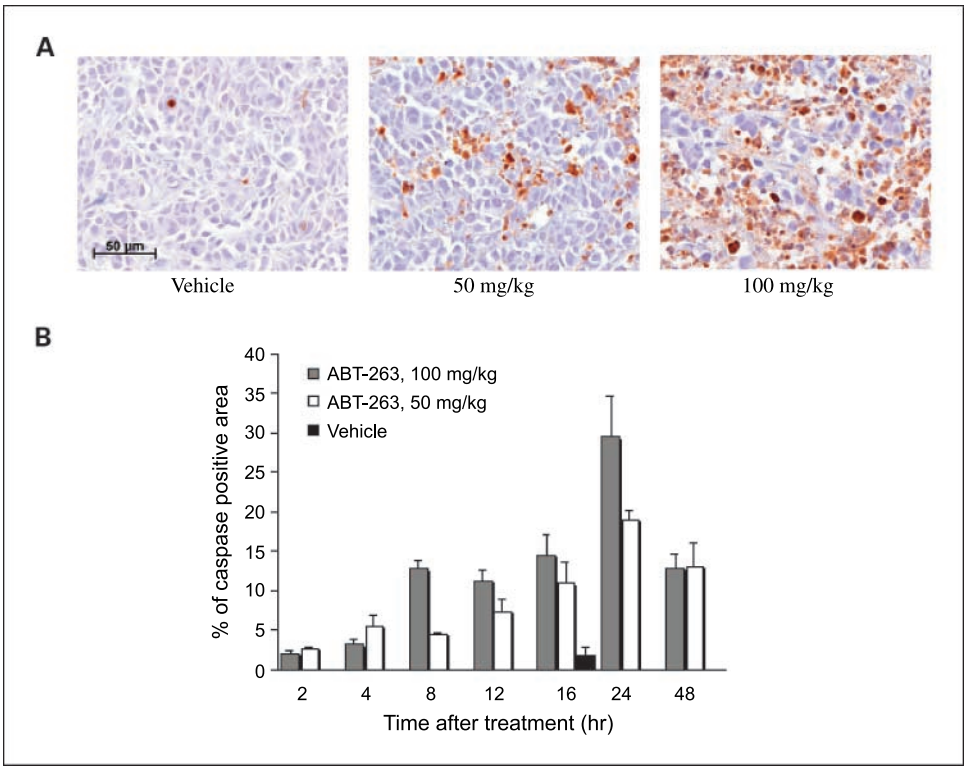

Fig. 3. Histologic analysis of caspase-3 activation in H146 xenograft tumors treated with ABT-263. H146 cells (5×10^6) were inoculated s.c. into the flank of *scid*-bg mice. Once tumors reached ~500 mm^3, mice were given a single p.o. administration of ABT-263. Tumors were harvested at various times after treatment ($n = 3$ mice per group), and tissue sections were stained with H&E or with an antibody specific for activated caspase-3. *A,* representative photos of H146 tumors stained with an anti – caspase-3 antibody. Tumors were collected 24 h after a single dose of ABT-263. Magnification, ×400. *B,* quantitative analysis of caspase-3 staining in H146 tumors. Mosaic images of entire cross-sections at ×100 magnification from three independent tumors from each treatment group were analyzed using AxioVision image analysis software. The percentage of total sectional area positive for caspase-3 staining (±SE) is presented. Significant differences were observed for both doses of ABT-263 compared with vehicle at all time points from 8 h onward ($P < 0.05$, student's t test).

demonstrating that a compound can kill tumor cells, and in doing so, displays one or more of the hallmarks of apoptosis such as caspase activation or phosphatidylserine externalization, can be misleading as many cytotoxic agents would be expected to elicit such a response. Recently, ABT-737 has emerged as a promising chemotherapeutic agent that functions as a *bona fide* inhibitor of Bcl-2 family proteins (19, 25, 42). ABT-737 has subnanomolar affinity for Bcl-2, Bcl-X_L, and Bcl-w but does not have significant affinity for other antiapoptotic Bcl-2 family proteins. Likewise, ABT-263 also binds to Bcl-2, Bcl-X_L, and Bcl-w with an affinity of <1 nmol/L and to A1 with an affinity of >1 μmol/L. ABT-263 shows a somewhat higher affinity than ABT-737 for Mcl-1 (K_is of 0.55 and >1 μmol/L, respectively). However, these and previous results (31) suggest that significantly higher binding affinities are required to achieve biologically meaningful inhibition. Importantly, these activities are specific as the enantiomer of ABT-263 shows highly diminished (~40×) binding and is dramatically less active than ABT-263 in a variety of mechanistic and cell viability assays (Table 1, ref. 34). As with ABT-737, ABT-263 potently inhibits interactions between Bim and antiapoptotic proteins, induces Bax translocation, cytochrome *c* release, and caspase activation. Furthermore, ABT-263 can induce cell killing in cytokine-deprived cells dependent on Bcl-X_L or Bcl-2 for survival.

Here, we have extended the *in vivo* evaluation of ABT-263 by describing detailed efficacy analysis in a panel of SCLC xenograft models. ABT-263 exhibits a wide range of potency against these cell lines, ranging from exquisitely potent (100 nmol/L) to inactive (22 μmol/L; Table 1). Characterization of experimental cancer compounds typically involves a fairly small number of xenograft models. Analysis of efficacy in multiple models of a single tumor type is not commonly reported. Use of limited models carries with it significant risk in that it is difficult to determine how representative such a sample set will be relative to the corresponding clinical population. Indeed, our evaluation of 11 different SCLC models shows that sensitivity to ABT-263 can vary significantly in this tumor population. Furthermore, the use of this larger sample set has enabled careful characterization of factors likely to influence clinical response to this compound.

As observed for ABT-737, ABT-263 treatment resulted in transient thrombocytopenia and lymphopenia but was otherwise well-tolerated in mice. ABT-263 exhibited dramatic antitumor efficacy in several SCLC models, with complete regressions observed in all tumors for two different models (Table 1). Highly significant activity was observed in an additional four SCLC models with 20% to 100% ORR (Table 1). ABT-263 caused regression not only of relatively small (200-250 mm^3) tumors but also in large, 1,000 mm^3 H146 tumors (Fig. 1B). In comparing efficacy to a variety of clinically approved cytotoxic agents, we found the activity of ABT-263 was comparable with or slightly better than the maximally tolerated doses of paclitaxel, docetaxel, and vincristine and clearly superior to the efficacy of etoposide, cisplatin, carboplatin, and cyclophosphamide (Fig. 1). Efficacy evaluation of experimental cancer compounds in xenograft models is frequently criticized for failing to predict clinical activity (43, 44). In this regard, it is important that rigorous parameters are used for evaluation of preclinical activity. Inhibition of tumor growth by 30% to 40% is often cited as a significant effect in xenograft models. Although this level of activity may be indicative of interesting biological activity, it would most likely equate to progressive disease in a clinical setting. The ability of ABT-263 to achieve partial and, in many cases, durable complete tumor responses in these SCLC models

argues that this efficacy is more likely to translate into a meaningful clinical effect (45). Another important variable that often limits the utility of cancer therapies is the development of resistance. We generated an H146 variant that was resistant to treatment with paclitaxel and showed that Pgp-1 was overexpressed in this variant (Fig. 1C). ABT-263 retained activity against this line, suggesting that the common resistance mechanism of Pgp-1 mediated drug efflux may not result in acquired resistance to ABT-263. Furthermore, we showed that SCLC xenograft tumors could be treated with multiple cycles of ABT-263 and continue to effectively respond to treatment, suggesting that it is difficult for these tumors to become resistant to this agent (Fig. 1D).

Dose-response studies showed that a dose of 50 mg/kg/day was the minimally efficacious dose, resulting in ~75% TGI (Table 2). Pharmacokinetic analysis of plasma from *scid* mice indicated that this dose results in a C_{max} and area under the plasma concentration curve of ~5.4 μg/mL and 54 μmol/L/h, respectively. Although the most robust antitumor activity was observed when ABT-263 was delivered on a prolonged continuous dosing schedule, significant activity, including tumor regression, was also obtained with shorter duration treatment and on intermittent dosing schedules (Fig. 2). These results are consistent with the finding that acute administration of ABT-263 results in dramatic *in vivo* activation of caspase-3 in tumors (Fig. 3) and that a higher incidence of complete regression is achieved with continuous dosing (Fig. 2).

The efficacy observed with ABT-263 *in vivo* was generally well-correlated with *in vitro* sensitivity. Two exceptions to this trend were the H1048 and H345 cell lines where the activity in xenograft tumors was better than was predicted by the cellular EC_{50} values of >2 μmol/L in these lines (Table 1). The H1048 line showed significant up-regulation of Bcl-2 protein levels in xenograft tumors relative to cells grown in culture (Fig. 4C). These results suggest that some tumor cell lines may rely on up-regulation of Bcl-2 (and hence resistance to apoptosis) for growth *in vivo* and, therefore, be acutely sensitive to treatment with Bcl-2 family inhibitors such as ABT-263. The exact cause of Bcl-2 up-regulation will require further investigation. Preliminary evaluation of H1048 cells grown under hypoxic conditions did not indicate an up-regulation of Bcl-2 expression (data not shown). The phenomenon we observed here is similar to results recently reported by Benimetskaya et al. (46) in 518A2 melanoma cells. They showed that knockdown of Bcl-2 levels in these cells significantly impaired the ability of these cells to grow *in vivo* but did affect growth properties *in vitro*.

Numerous studies have shown the importance of the Mcl-1 protein in affecting tumor apoptosis (47, 48). Mcl-1 stability

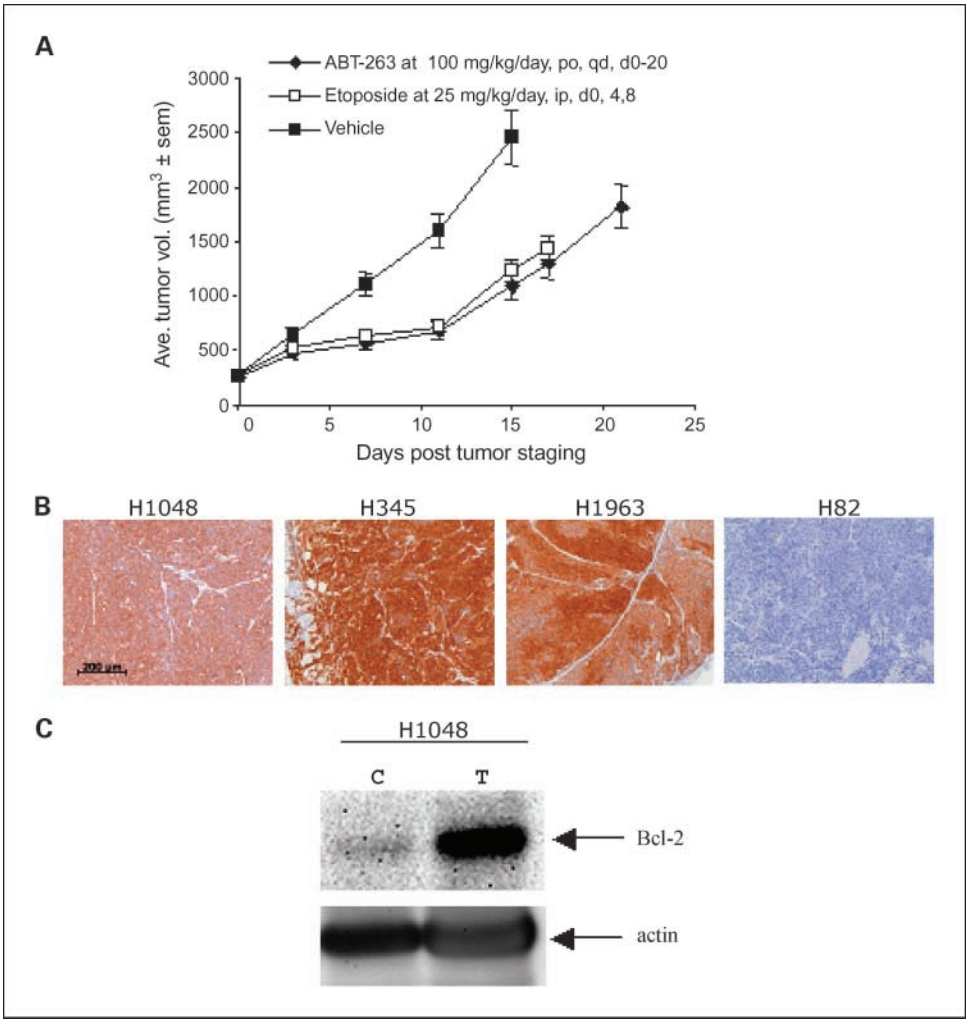

Fig. 4. Efficacy of ABT-263 in the H1048 xenograft model. *A,* ABT-263 administered at 100 mg/kg/d of daily for 21 d (q.d. × 21) led to significant inhibition of H1048 tumor growth throughout the study ($P < 0.01$, Wilcoxon rank sum test). The efficacy of ABT-263 was comparable with that observed with etoposide delivered at its maximum tolerated dose. *B,* immunohistochemical analysis of Bcl-2 expression in H82, H345, H1048, and H1963 tumors. Expression of Bcl-2 in H345 and H1048 tumors was comparable with H1963 tumors, a line that is highly sensitive to ABT-263 and contains amplification of the Bcl-2 locus. Magnification, ×100. *C,* comparison of Bcl-2 expression levels by Western blotting in H1048 lysates from tumors grown *in vivo* (*T*) versus cells in culture. *C,* 20 μg of total protein was loaded in each case. Equal loading of protein was confirmed by reprobing with actin.

is significantly influenced by factors such as availability of growth factors and exposure to DNA-damaging agents, and this dynamic serves as an important regulator of apoptotic response (49, 50). Consistent with these findings and with the binding profile of ABT-263, cell lines such as H889 and H146 that express Bcl-2 and/or Bcl-X_L and little Mcl-1 are sensitive, and cell lines such as H82 that express little Bcl-2/Bcl-X_L and abundant Mcl-1 are resistant. These results suggest that a logical approach to broaden the efficacy of ABT-263 would be to combine it with agents that down-regulate or interfere with Mcl-1 activity. Indeed, several recent reports have shown that combining ABT-737 with various agents that modulate Mcl-1 activity results in significant antitumor activity (21, 24, 25). We have also shown recently that ABT-263 shows significant *in vivo* activity in combination with bortezomib in a multiple myeloma xenograft model that expresses high levels of Mcl-1 (34). Bortezomib treatment was shown to lead to increased NOXA and decreased Mcl-1 expression in these cells, providing a mechanistic rationale for this combination activity.

Although SCLC represents a relatively small fraction of total lung cancer, diagnosis of late stage disease is still essentially a death sentence. Very few new treatment options have been developed despite the fact that 2-year survival rates are <5% with current therapy options (2). Therapeutic intervention at the Bcl-2 axis represents an attractive new approach for the treatment of SCLC. Bcl-2 is overexpressed and, in some cases, genetically amplified in SCLC and is also believed to be an important drug resistance factor (5, 10, 11, 15, 51). Here, we have shown that ABT-263 shows impressive activity, including durable complete regression of established tumors, in a large panel of xenograft SCLC tumor lines. Importantly, we have coupled this with a strong mechanistic rationale for understanding potential differential sensitivity. ABT-263 has recently entered phase I clinical testing and the results presented here suggest clinical investigation of the therapeutic utility in SCLC is warranted.

Disclosure of Potential Conflicts of Interest

All authors are current or former employees of Abbott Laboratories. All authors are stockholders in Abbott Laboratories.

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Activity of the Bcl-2 Family Inhibitor ABT-263 in a Panel of Small Cell Lung Cancer Xenograft Models

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