ABT-263: A Potent and Orally Bioavailable Bcl-2 Family Inhibitor

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Abstract

Overexpression of the prosurvival Bcl-2 family members (Bcl-2, Bcl-xL, and Mcl-1) is commonly associated with tumor maintenance, progression, and chemoresistance. We previously reported the discovery of ABT-737, a potent, small-molecule Bcl-2 family protein inhibitor. A major limitation of ABT-737 is that it is not orally bioavailable, which would limit chronic single agent therapy and flexibility to dose in combination regimens. Here we report the biological properties of ABT-263, a potent, orally bioavailable Bad-like BH3 mimetic (K_i's of <1 nmol/L for Bcl-2, Bcl-xL, and Bcl-w). The oral bioavailability of ABT-263 in preclinical animal models is 20% to 50%, depending on formulation. ABT-263 disrupts Bcl-2/Bcl-xL interactions with pro-death proteins (e.g., Bim), leading to the initiation of apoptosis within 2 hours post-treatment. In human tumor cells, ABT-263 induces Bax translocation, cytochrome *c* release, and subsequent apoptosis. Oral administration of ABT-263 alone induces complete tumor regressions in xenograft models of small-cell lung cancer and acute lymphoblastic leukemia. In xenograft models of aggressive B-cell lymphoma and multiple myeloma where ABT-263 exhibits modest or no single agent activity, it significantly enhances the efficacy of clinically relevant therapeutic regimens. These data provide the rationale for clinical trials evaluating ABT-263 in small-cell lung cancer and B-cell malignancies. The oral efficacy of ABT-263 should provide dosing flexibility to maximize clinical utility both as a single agent and in combination regimens. [Cancer Res 2008;68(9):3421–8]

Introduction

Evasion of apoptosis is a hallmark of cancer (1). Cancer cells must overcome a continual bombardment by cellular stresses such as DNA damage, oncogene activation, aberrant cell cycle progression, and harsh microenvironments that would cause normal cells to undergo apoptosis. One of the primary means by which cancer cells evade apoptosis is by up-regulation of the prosurvival Bcl-2 family proteins such as Bcl-2, Bcl-xL, and Mcl-1 (1, 2). Thus, a variety of approaches to target these oncoproteins have been pursued to restore the natural process of programmed cell death (3).

We recently described the design of small molecules that occupy the BH3 binding groove of antiapoptotic Bcl-2 family members, which culminated in the discovery of ABT-737 (4–6). ABT-737 binds with high affinity (<1 nmol/L) to Bcl-xL, Bcl-2, and Bcl-w; exhibits single-agent activity against small-cell lung cancer (SCLC) and lymphoid malignancies; and potentiates the proapoptotic effects of several therapeutic agents in a broader context (7–16). In addition to established human tumor cell lines, ABT-737 effectively induces apoptosis in primary patient-derived lymphoma and chronic lymphocytic leukemia cells *ex vivo* (12, 17).

However, the prospects for ABT-737 as a therapeutic agent are hampered by its poor physiochemical and pharmaceutical properties. This compound is not orally bioavailable and its low aqueous solubility makes formulation for i.v. delivery challenging. In xenograft models, ABT-737 exhibits maximal monotherapy efficacy when given by i.p. injection on a continuous daily schedule. Because continuous administration of a parenteral agent in the clinical setting is problematic, we sought an orally bioavailable agent with the pharmacologic properties of ABT-737. We believe that such an agent will maximize clinical utility as a single agent and more readily enable combination with standard chemotherapeutics.

Here, we report the biological properties of our second-generation, orally bioavailable BH3 mimetic, ABT-263. ABT-263 exhibits selective cytotoxicity to Bcl-2/Bcl-xL–dependent cells *in vitro*. When dosed p.o. *in vivo*, ABT-263 elicits complete tumor regressions in SCLC and ALL xenograft models as well as potentiates the therapeutic efficacy of commonly used chemotherapeutic regimens in B-cell lymphoma and multiple myeloma xenograft models. These results have provided the rationale and strategy for ongoing clinical studies.

Materials and Methods

Compound synthesis and affinity determination. ABT-737 and ABT-263 were synthesized as previously described (12, 18). The enantiomer and BH3-only peptides were synthesized at Abbott (Abbott Park, IL). Binding affinities (K_i or IC_{50}) were determined with competitive fluorescence polarization assays (19). The following peptide probe/protein pairs were used: f-bad (1 nmol/L) and Bcl-xL (6 nmol/L), f-Bax (1 nmol/L) and Bcl-2 (10 nmol/L), f-Bax (1 nmol/L) and Bcl-w (40 nmol/L), f-Noxa (2 nmol/L) and Mcl-1 (40 nmol/L), and f-Bax (1 nmol/L) and Bcl-2-A1 (15 nmol/L). Binding affinities for Bcl-xL were also determined using a time-resolved fluorescence resonance energy transfer assay. Bcl-xL (1 nmol/L, His tagged) was mixed with 200 nmol/L f-Bak, 1 nmol/L Tb-labeled anti-His antibody, and compound at room temperature for 30 min. Fluorescence was measured on an Envision plate reader (Perkin-Elmer) using a 340/35 nm excitation filter and 520/525 (f-Bak) and 495/510 nm (Tb-labeled anti-His antibody) emission filters. Dissociation constants (K_i) were determined using Wang's equation (20).

Cell culture and *in vitro* cytotoxicity assays. Human tumor cell lines [American Type Culture Collection or Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ)] were maintained at 37°C

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-07-5836

containing 5% CO_2. SCLC cell lines were cultured in RPMI 1640 (Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen), 1% sodium pyruvate, 25 mmol/L HEPES, 4.5 g/L glucose, and 1% penicillin/streptomycin (Sigma). Leukemia and lymphoma cell lines were cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin.

Cells (1×10^4–5×10^4) were treated for 48 h in 96-well culture plates in a final volume of 100 μL and cytotoxicity was assessed with the CellTiter Glo assay (Promega).

***In vivo* tumor xenograft studies.** All animal studies were conducted according to guidelines established by the internal Institutional Animal Care and Use Committee. C.B.-17 *scid*-bg or C.B.-17 *scid* mice (Charles River Laboratories) were implanted with 5×10^6 (1×10^6 for DoHH2) cells in 0.2 mL 50% Matrigel (BD Biosciences) s.c. into the right flank. Tumor-bearing mice were size matched ($\sim$235 mm^3; day 0) into treatment and control groups, ear tagged, and monitored individually. Tumor volume was measured two to three times weekly by electronic calipers (volume = length $\times$ width^2 / 2). Tumor growth inhibition was calculated based on the difference in mean tumor volumes between treated and appropriate vehicle control groups. Partial response (PR) is defined as $\geq$50% tumor growth inhibition, and complete response (CR) is defined as nonpalpable tumor. All studies used 8 to 10 mice per group. Statistical comparisons of tumor growth rate and tumor growth delay used the Wilcoxon rank sum test and the Mantle-Cox log-rank test, respectively.

ABT-263 was formulated in 10% ethanol, 30% polyethylene glycol 400, and 60% Phosal 50 PG and administered p.o. The other agents used [rituximab (Genentech, Inc.), doxorubicin (Bedford Laboratories), cyclophosphamide (Bristol-Myers Squibb), vincristine (GensiaSicor Pharmaceuticals), bortezomib (Millenium Pharmaceuticals), and prednisone (Aero Pharmaceuticals)] were administered i.p., p.o., or i.v. and formulated according to the manufacturers' recommendations. For combination studies, ABT-263 was given $\sim$2 h before the other agents, except bortezomib, which was given $\sim$4 h before ABT-263.

Additional details on materials and methods can be found in Supplementary data.

Results

ABT-263 is an orally bioavailable Bcl-2 family inhibitor. The structural features in ABT-737 that impart undesirable drug properties result from design elements essential for inhibition of a large surface area, hydrophobic protein-protein interaction, and reduction of serum albumin binding (4–6). To improve the oral efficacy of this relatively large (MW >800) molecule, a careful balance was required between target affinity, cellular potency, and oral absorption. Three key sites along the backbone of ABT-737 were identified that affect charge balance, metabolism, and oral absorption (Fig. 1). Analogues incorporating modifications at these positions were optimized to maximize the pharmacokinetic/pharmacodynamic relationship between oral exposure in animals and efficacy in human tumor cell lines. These efforts resulted in the identification of ABT-263.

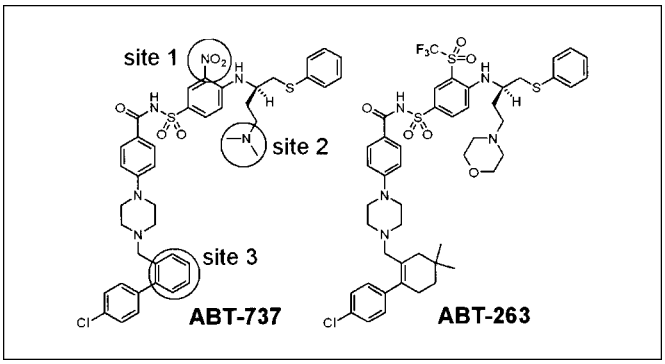

Figure 1. Chemical structures of ABT-737 (*left*) and ABT-263 (*right*).

ABT-263 maintains high affinity for Bcl-xL, Bcl-2, and Bcl-w, which is below the detection limit of the fluorescence polarization assay ($K_i \leq 1$ nmol/L), but binds more weakly to Mcl-1 and A1 (Table 1). This selectivity pattern is similar to that of its predecessor ABT-737 and the BH3-only protein Bad (12). Subnanomolar affinity of ABT-263 for Bcl-xL was confirmed by a more sensitive time-resolved fluorescence resonance energy transfer assay that shows the enantiomer (a stereoisomer with the opposite configuration of the morpholinoethyl group, used as a less active control) to be $\sim$40-fold less potent.

The pharmacokinetic profile of ABT-263 is characterized by low plasma clearance values and low volumes of distribution in mouse, rat, dog, and monkey, with plasma elimination half-lives after i.v. dose of 4.6 to 8.4 hours (Supplementary Table S1). Bioavailability after oral gavage was $\sim$20% in all four species. Due to its low aqueous solubility, ABT-263 displays a prolonged dissolution rate-limited oral absorption. P.o. administration in lipid-based formulations, in which the compound is significantly more soluble, yields enhanced absorption with bioavailability near 50% and an oral elimination half-life of 8.9 hours in dogs. A representative plasma drug concentration curve after i.v. or p.o. dosing in dog is shown in Supplementary Fig. S1.

ABT-263 exhibits mechanism-based cytotoxicity. A number of BH3 mimetic small molecules have been reported to bind Bcl-2 family members with modest affinity and induce apoptosis in tumor cell lines (21–23). However, many of these agents were recently shown to kill cells in a Bax/Bak–independent manner, calling into question the functional relevance of their weak affinity for Bcl-2 proteins and the true mechanisms of action for these molecules (14). Therefore, we felt it important to robustly demonstrate that apoptosis induced by ABT-263 was the direct result of inhibition of Bcl-2 family proteins. First, the cellular activity of ABT-263 was evaluated in the interleukin-3 (IL-3)–dependent prolymphocytic FL5.12 murine cell line. Withdrawal of IL-3 induces FL5.12 apoptosis, in part by up-regulation of the proapoptotic factors Bim and Puma (24, 25). Overexpression of Bcl-2 (FL5.12-Bcl-2) or Bcl-xL (FL5.12-Bcl-xL) protects against the effects of IL-3 withdrawal by sequestration of Bim and Puma (25). ABT-263, but not the enantiomer, reversed the protection afforded by overexpression of Bcl-2 or Bcl-xL (EC_{50} = 60 and 20 nmol/L, respectively; Fig. 2*A*). ABT-263 was ineffective in eliciting cell death in the presence of IL-3 where FL5.12 cells were not subject to proapoptotic stimuli. The ability of ABT-263 to kill FL5.12-Bcl-2 or FL5.12-Bcl-xL cells under IL-3 withdrawal was significantly attenuated in the presence of the caspase inhibitor ZVAD, indicating that cell killing is caspase dependent (Supplementary Fig. S2).

To determine if ABT-263–induced cytotoxicity can be attributed to the disruption of intracellular Bcl-2 family protein-protein interactions, coimmunoprecipitation studies were done. ABT-263 induced a dose-dependent decrease in Bim:Bcl-xL interactions within 2 hours posttreatment in FL5.12-Bcl-xL cells (Supplementary Fig. S3). Similar patterns were also observed for the disruption of Bim:Bcl-2 complexes in FL5.12-Bcl-2 cells (data not shown), indicating that ABT-263 restores IL-3–dependent cell death by attenuating the ability of Bcl-xL and Bcl-2 to sequester proapoptotic factors such as Bim. The ability of ABT-263 to disrupt Bcl-2 family protein-protein interactions was confirmed in a mammalian two-hybrid system (Fig. 2*B*). ABT-263 inhibited the interaction of

Table 1. Binding affinities to Bcl-2 family proteins

	FPA K_i (nmol/L)					TR-FRET K_i (nmol/L)
	Bcl-xL	Bcl-2	Bcl-w	Mcl-1*	A1*	Bcl-xL
hBad	0.5 ± 0.1	15 ± 4	22 ± 10	>1,000	>1,000	0.12
hNoxa	>1,000	>1,000	>1,000	64 ± 6	107 ± 43	>93
ABT-737	≤0.5	<1	0.9	>1000	—	0.08
ABT-263	≤0.5	≤1	≤1	550 ± 40	354 ± 63	0.40
Enantiomer	3 ± 2	≤1	79 ± 17	370 ± 90	444 ± 12	17.4

NOTE: Binding affinity of ABT-263, its enantiomer, ABT-737, Bad-BH3 (Ac-NLWAAQRYGRELRRMSDK(FITC)FVD-amide, and Noxa-BH3 (6-FAM-GELEVEFATQLRRFGDKLNF-amide) for Bcl-2 family proteins. Shown is the K_i ± SE ($n \geq 3$). For TR-FRET, shown is the mean K_i ($n = 2$).
Abbreviations: FPA, fluorescence polarization assay; TR-FRET, time-resolved fluorescence resonance energy transfer assay.
*IC_{50} values reported for Mcl-1 and A1.

VP16-Bcl-xS with Gal4-Bcl-xL (apparent EC_{50} ~50 nmol/L), whereas the enantiomer was much less effective.

To further interrogate the mechanism of action, the activity of ABT-263 was evaluated in a series of mouse embryonic fibroblast (MEF) cells that included wild-type (WT) as well as cells deficient in Bcl-x, Mcl-1, and Bax/Bak (DKO). ABT-263 potently induced cell death in *Mcl-1*$^{-/-}$ (EC_{50} ~50 nmol/L) but not *Bcl-x*$^{-/-}$ (EC_{50} ≥10 μmol/L) MEF cells (Supplementary Fig. S4*A*). This confirms

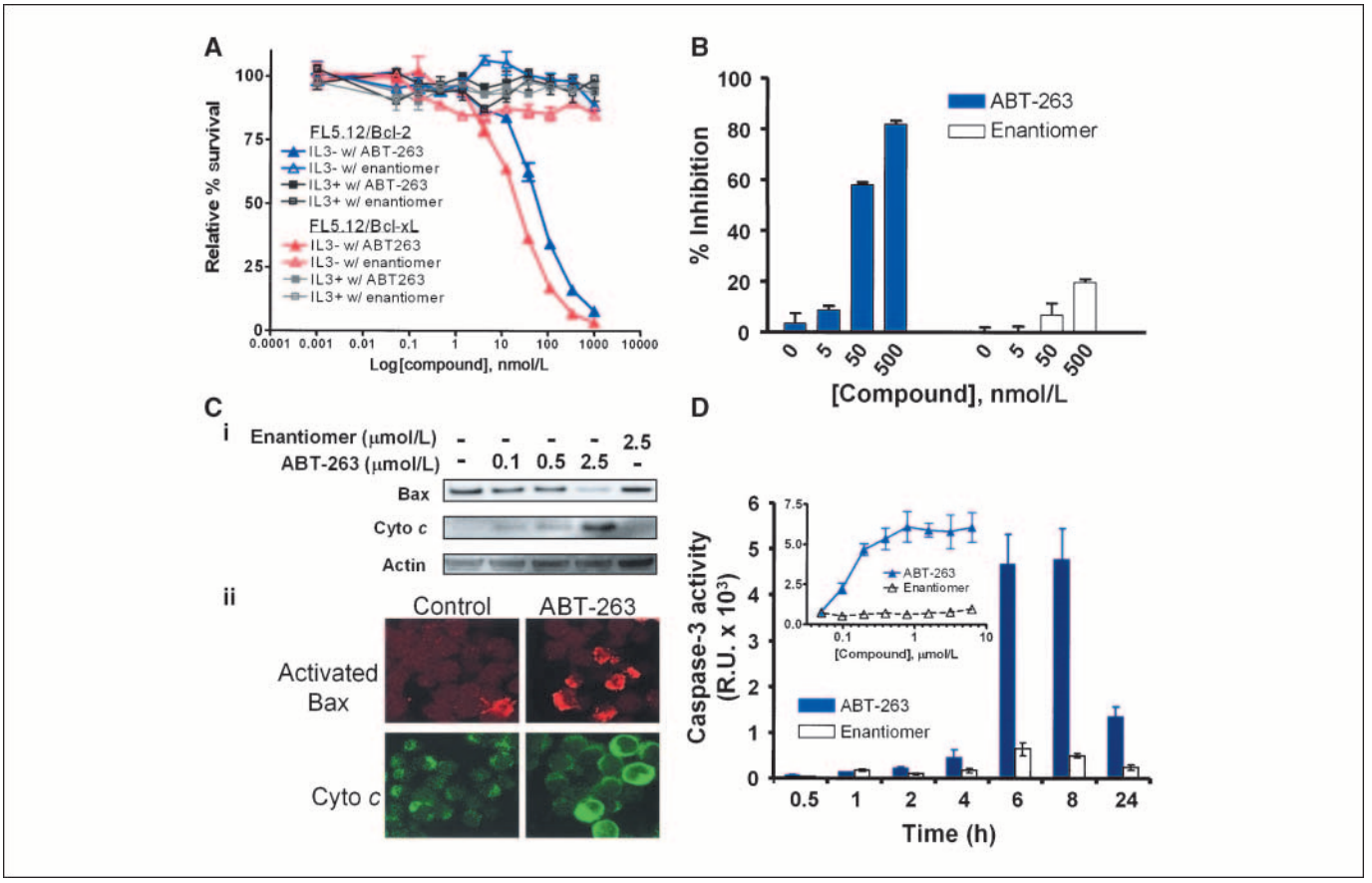

Figure 2. Inhibition of the antiapoptotic Bcl-2 family proteins by ABT-263 induces mitochondria-dependent apoptosis. *A,* restoration of IL-3 dependence in FL5.12 cells overexpressing Bcl-xL or Bcl-2. Cells ± IL-3 were treated with 0 to 1,000 nmol/L of ABT-263 or the enantiomer control and viability was assessed with CellTiter Glo. *Points,* mean ($n = 3$); *bars,* SD. *B,* disruption of Bcl-xL/Bcl-xS interactions assessed by the mammalian two-hybrid system in HeLa cells. Cells were treated with 0 to 500 nmol/L of ABT-263 (*closed columns*) or the enantiomer (*open columns*) and disruption was assessed with BrightGlo. *Columns,* mean ($n = 3$); *bars,* SD. *C,* activation of Bax and cytochrome *c* (*Cyto c*) release on inhibition of Bcl-2 and Bcl-xL in H146 cells. *i,* immunoblots of Bax and cytochrome *c* in cytosolic fractions isolated 2 h posttreatment with various concentrations of ABT-263 or the enantiomer. *ii,* immunohistochemistry of cells with anti–activated Bax (*red*) and anti–cytochrome *c* (*green*) antibodies. *D,* time-dependent and dose-dependent [*inset*; ABT-263 (*closed squares*) or enantiomer (*open triangles*)] activation of caspase-3 induced by 195 nmol/L ABT-263 (*closed columns*) or 195 nmol/L enantiomer (*open columns*) in H146 cells. *Columns,* mean ($n = 3$); *bars,* SD. *R.U.,* relative units.

the ability of ABT-263 to functionally inhibit Bcl-xL, but not Mcl-1, in a cellular context and is consistent with previous observations with ABT-737 (7, 11, 13, 14, 26). The enantiomer was ineffective in both systems. ABT-263 was also ineffective (EC_{50} $\geq$10 μmol/L) in killing either WT or DKO MEF cells, consistent with previous reports (14). In contrast, etoposide, a general cytotoxic agent, as well as two reported small-molecule BH3 mimetics [(−)-gossypol and GX15-070], induced cell death with similar EC_{50}s across all four MEF cell types, suggesting that these compounds induce cell death, at least in part, by Bcl-2 family–independent mechanisms (Supplementary Fig. S4*C*).

To assess the ability of ABT-263 to directly induce apoptosis in a human tumor cell line, Bax translocation and release of cytochrome *c* were monitored in the SCLC cell line H146. H146 has been shown to be dependent on Bcl-2 for survival (25). ABT-263 induced a dose-dependent decrease in cytosolic Bax coupled with an increase in cytosolic cytochrome *c* within 2 hours posttreatment (Fig. 2*C-i*). The enantiomer was unable to elicit a similar response. Bax activation and cytochrome *c* release were confirmed by microscopy (Fig. 2*C-ii*). The 6A7 anti-Bax antibody, which specifically recognizes the conformationally active form of Bax, was used to detect activated Bax in H146 cells. Consistent with the fractionation studies, ABT-263 treatment was associated with a substantial increase in activated Bax. This corresponded to release of cytochrome *c* from the mitochondria to the cytosol as evidenced by decreased punctate structures and increased cytosolic staining. ABT-263, but not its enantiomer, also induced a time-dependent increase in caspase-3 activity that was detectable at 2 hours and which peaked at ~6 hours (Fig. 2*D*). This effect was concentration dependent, with an EC_{50} ~100 nmol/L at the 6-hour time point (Fig. 2*D, inset*). In contrast, camptothecin, a topoisomerase I inhibitor reported to induce mitochondrial apoptosis and subsequent caspase activation (27, 28), was only able to elicit an increase in caspase-3 activity after 24 hours (data not shown). These data indicate that ABT-263 acts directly to inhibit Bcl-2 and Bcl-xL, releasing proapoptotic factors such as Bim to induce a rapid activation of the mitochondrial apoptotic pathway.

To evaluate the breadth of cellular activity of ABT-263, a panel of human tumor cell lines spanning a range of tumor types was examined. Consistent with previous reports on ABT-737 (12), ABT-263 exhibited single-agent activity in SCLC and hematologic malignancies but not in the majority of other tumor types (data not shown). To more closely investigate the activity within these sensitive tumor types, ABT-263 was examined in panels of cell lines derived from SCLC (n = 22) and hematologic malignancies (n = 23). In each panel, ABT-263 exhibited a range of potency with 32% (SCLC) and 48% (hematologic) of cell lines being highly sensitive (EC_{50} <1 μmol/L; Fig. 3). The enantiomer was >20-fold less active in these sensitive cells (Supplementary Table S2).

Oral dosing of ABT-263 results in the regression of SCLC and ALL xenograft tumors *in vivo*. To extend these observations *in vivo*, ABT-263 was evaluated in flank xenograft models established from some of the sensitive SCLC and hematologic cell lines. When dosed p.o., once a day at 100 mg/kg for 21 consecutive days, ABT-263 induced rapid and complete tumor responses (CR) that were durable for several weeks after the end of treatment in all animals bearing H889 (SCLC) or RS4;11 (ALL) tumors (Fig. 4*A*). Similar treatment of mice bearing H146 SCLC tumors induced rapid regressions resulting in CR in 60% and PR in 40% of the animals (Fig. 4*A*). Gradual tumor rebound was observed beginning several weeks after the end of dosing in this model. Activity was dose dependent in the H146 xenograft model. Treatment with ABT-263 at 50 mg/kg for 21 consecutive days induced CR in 22% and PR in 44% of the animals, whereas 25 mg/kg induced a statistically significant but modest tumor growth inhibition with no observed tumor regressions. ABT-263 was well tolerated (<5% weight loss) at all doses.

To correlate the exposure of ABT-263 with antitumor efficacy, a steady-state pharmacokinetic study was done in which non–tumor-bearing mice were dosed p.o. once a day for 3 days, and plasma drug concentrations determined after the third dose. Both peak plasma concentration (C_{max}) and area under the plasma concentration curve (AUC) were proportional to dose and increased in a roughly linear fashion (Supplementary Table S3). A dose of 100 mg/kg, which produced a 100% overall response rate (overall response rate = CR + PR), gave C_{max} and AUC values of 7.7 μmol/L and 90 μmol/L h, respectively. The exposure resulting from a 50 mg/kg dose, which elicited a 66% overall response rate, was 5.4 μmol/L (C_{max}) and 54 μmol/L h (AUC). These data indicate that peak plasma drug concentrations of ABT-263 ranging from ~5.4 to 7.7 μmol/L are highly efficacious.

ABT-263 enhances the activity of chemotherapeutic agents *in vivo*. The *in vivo* efficacy of ABT-263 was investigated in combination with commonly used therapeutic agents in several aggressive models of hematologic malignancies. ABT-263 and rituximab were examined in the DoHH2 B-cell lymphoma flank xenograft model (Fig. 4*B*). When dosed daily at 100 mg/kg p.o. for 17 days, ABT-263 exhibited 44% tumor growth inhibition. A single 10 mg/kg dose of rituximab elicited 84% tumor growth inhibition. Neither ABT-263 nor rituximab alone achieved sustained tumor regression; however, the combination exhibited a superior effect, achieving 70% CR and 10% PR. This combination also resulted in a significant improvement in tumor growth delay (>700%) versus rituximab alone (300%).

The efficacy of ABT-263 alone and in combination with a modified R-CHOP regimen was also evaluated in a GRANTA-519 flank xenograft model of mantle cell lymphoma (Fig. 4*B*). ABT-263 delivered at 100 mg/kg p.o. for 21 consecutive days elicited 40% tumor growth inhibition. In comparison, the R-CHOP regimen inhibited tumor growth by 68% with 20% CR. The combination resulted in dramatic tumor regressions and complete tumor responses in all animals tested with no evidence of tumor regrowth in four of nine evaluable tumors.

Finally, we examined the ability of ABT-263 to potentiate the effects of chemotherapy in a model resistant to ABT-263. In the OPM-2 flank xenograft model of multiple myeloma (*in vitro* EC_{50} = 6.7 μmol/L), ABT-263 delivered daily for 21 days at 100 mg/kg did not significantly inhibit tumor growth (Fig. 4*B*). Bortezomib delivered at its maximum tolerated dose (1 mg/kg i.v., qd ×3) inhibited tumor growth by 70% with no CRs. ABT-263 enhanced the efficacy of bortezomib with the combination resulting in 95% tumor growth inhibition and a 40% CR rate.

ABT-263 induces a rapid but reversible thrombocytopenia. We recently reported that ABT-737 induces a rapid and reversible thrombocytopenia in animals resulting from inhibition of Bcl-2 family proteins and induction of apoptosis in circulating platelets without bone marrow toxicity (29). As expected, ABT-263 also induces thrombocytopenia. After a single p.o. dose in dogs, circulating platelet counts decreased within 2 hours with a platelet nadir at 6 hours and evidence of rebound within 24 hours (Fig. 5*A*). To determine the effects of prolonged dosing, we examined the pharmacokinetic/pharmacodynamic relationship following multiple daily doses in dog (Fig. 5*B*). ABT-263 was dosed p.o. at

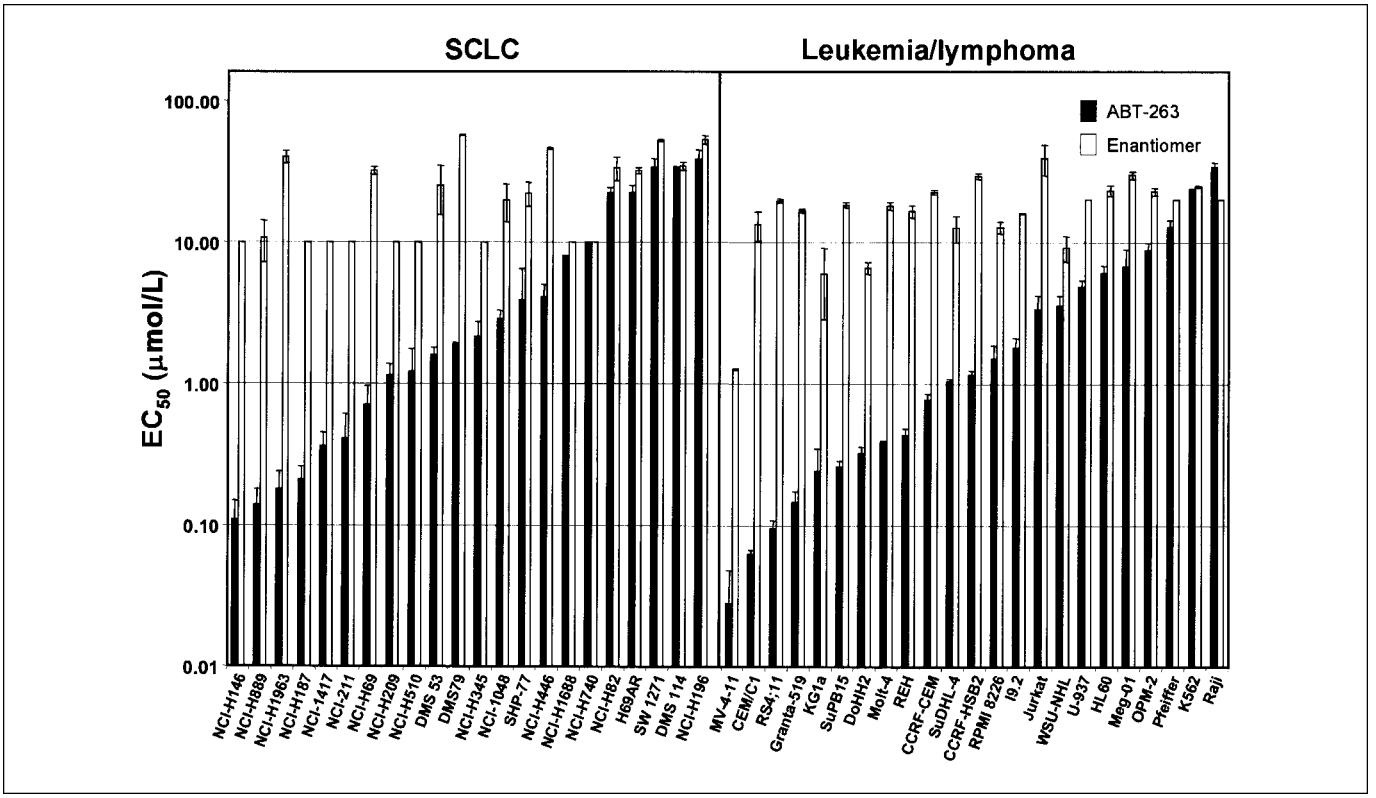

Figure 3. Cellular activity of ABT-263 *in vitro*. EC_{50} values of ABT-263 (*closed columns*) or the enantiomer (*open columns*) against a panel of SCLC (*left*) or leukemia/lymphoma (*right*) human tumor cell lines in the presence of 10% human serum. *Columns,* mean ($n \geq 3$); *bars,* SD.

2 mg/kg/d for 6 days, and then increased to 6 mg/kg/d for an additional 6 days. Platelet counts and plasma drug concentrations were evaluated 6 hours posttreatment to capture the likely platelet nadirs on days 1 to 4 and days 7 to 11. At 2 mg/kg, platelet counts decreased from an initial value of ~250,000/μL to ~125,000/μL by the second dose, thereafter holding steady for the remaining doses. Plasma drug concentration obtained 6 hours posttreatment (C_{6h}) remained constant at values ranging from 3.6 to 4.2 μmol/L. Based on the pharmacokinetic profile of ABT-263 (Supplementary Fig. S1), the C_{6h} is roughly 2-fold lower than the C_{max}, indicating that the peak plasma drug concentrations achieved after multiple 2 mg/kg doses are ~7 to 8 μmol/L. These levels are similar to those obtained after a 100 mg/kg p.o. dose in mice. When the dose of ABT-263 was increased to 6 mg/kg/d, platelet counts decreased to ~50,000/μL by the second day of this higher dose and remained relatively constant for the duration of the study. The C_{6h} values at this dose level ranged from 10.2 to 14.8 μmol/L (C_{max}, 20–30 μmol/L). ABT-263 was well tolerated in this study with no mortality or adverse clinical signs. However, some hematomas were apparent at the site of venipuncture, consistent with alterations in hemostasis. These data indicate that repeated daily exposure of ABT-263 at levels shown to be highly efficacious in murine models results in ~50% reduction in circulating platelets in dogs. Furthermore, plasma concentrations severalfold higher than the efficacious level are well tolerated, with circulating platelet counts reduced to ~50,000/μL.

Discussion

Tumor initiation, progression, and maintenance commonly involve alterations in apoptosis, which lead to enhanced cell survival and resistance to therapy (30). The prosurvival Bcl-2 family proteins act by sequestering and neutralizing proapoptotic proteins and play a pivotal role in determining the ability of a cancer cell to undergo apoptosis. Consequently, Bcl-2 and its prosurvival homologues are attractive targets for therapeutic intervention. ABT-737 was the first small-molecule mimetic of the proapoptotic BH3 binding domain that exhibits high affinity to multiple Bcl-2 family members (12). Studies from numerous laboratories have established the activity of ABT-737 in a variety of single agent and combination settings (8–10, 12, 14, 16, 17, 25, 31). However, the physical properties of ABT-737 will likely prohibit its full potential as a clinical agent. To overcome this, we report here the development of ABT-263, a second-generation, orally bioavailable small-molecule Bcl-2 family protein inhibitor that is currently in phase I clinical trials.

Analogous to its predecessor ABT-737, ABT-263 possesses high affinity for Bcl-xL, Bcl-2, and Bcl-w, but not for Mcl-1 or A1 (Fig. 1). In contrast to ABT-737, however, ABT-263 is orally bioavailable in mouse, rat, dog, and monkey. ABT-263 exhibits a low volume of distribution consistent with high serum protein binding, resulting in an oral half-life of ~9 hours in dog. Furthermore, ABT-263 dosed p.o. reaches exposures similar to those attained by ABT-737 when given at the same dose by i.p. injection (Supplementary Table S3).

In cells, ABT-263 inhibits the interaction between proapoptotic and antiapoptotic Bcl-2 family proteins in both a mammalian two-hybrid system and in FL5.12 cells. IL-3 withdrawal in FL5.12 cells has previously been shown to dramatically increase Bim and reduce Mcl-1 levels, resulting in the induction of apoptosis (24, 32). Bcl-2 overexpression protects the cells from cytokine withdrawal by

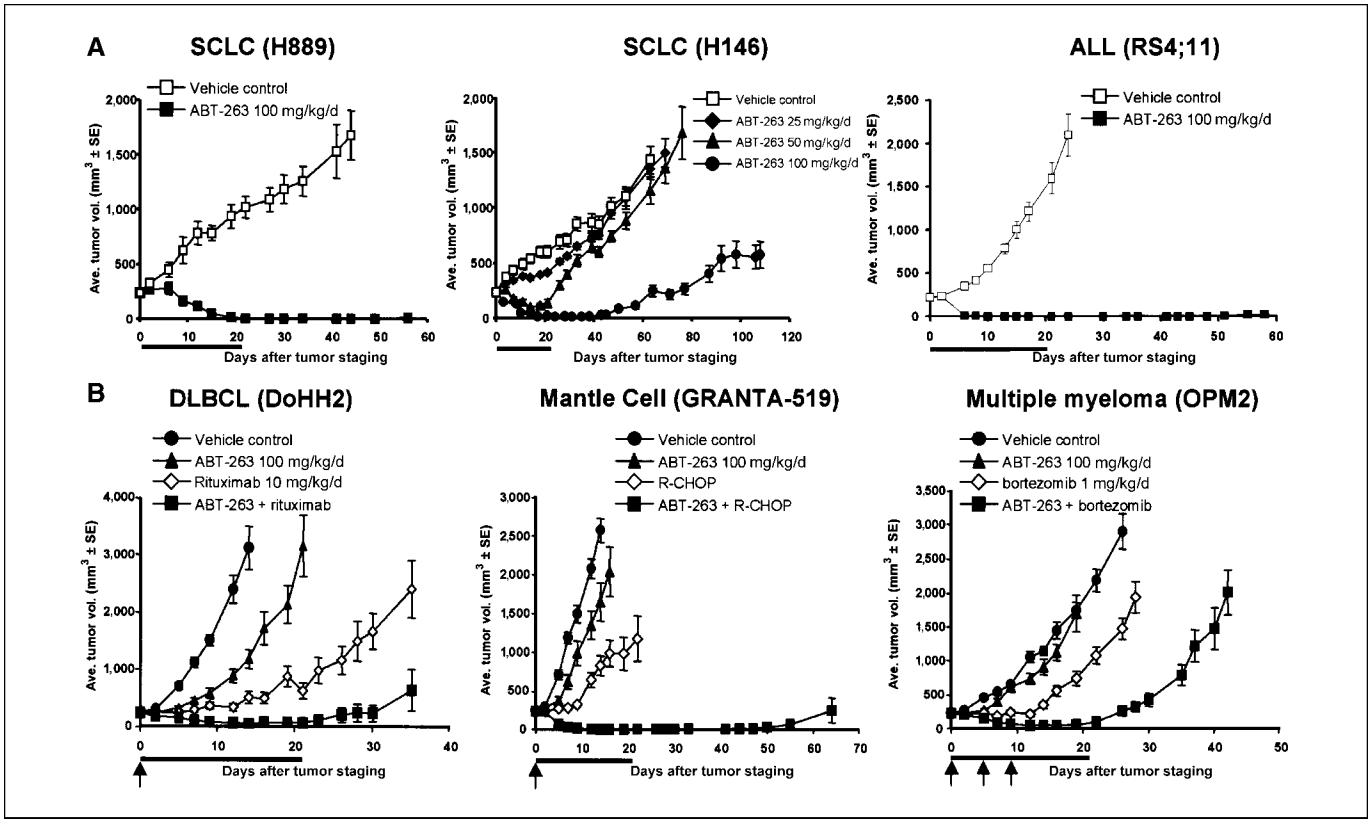

Figure 4. *In vivo* activity of ABT-263. Significant inhibition of tumor growth relative to vehicle control determined with the Wilcoxon rank sum test ($P < 0.05$). Significant improvement in tumor growth delay (median time to 1-mm^3 tumor end point) determined with the Mantle-Cox log-rank test; $P < 0.001$. *A,* ABT-263 is highly efficacious in xenograft models of SCLC and ALL. *Left,* H889. *Closed squares,* ABT-263 given p.o. once daily for 21 d; *open squares,* vehicle. *Middle,* H146; dose response of ABT-263. *Closed circles,* 100 mg/kg/d; *closed triangles,* 50 mg/kg/d; *closed diamonds,* 25 mg/kg/d; *open squares,* vehicle. *Right,* RS4;11. *Closed squares,* ABT-263 given p.o. once daily for 21 d; *open squares,* vehicle. *B,* ABT-263 in combination with other agents. *Left,* DoHH2 B-cell lymphoma xenograft model. *Closed circles,* combination vehicle; *closed triangles,* ABT-263 at 100 mg/kg, p.o., qd ×17; *open diamonds,* rituximab at 10 mg/kg, i.v., qd ×1; *closed squares,* ABT-263 + rituximab. *Middle,* GRANTA-519 mantle cell lymphoma xenograft model. *Closed circles,* combination vehicle; *closed triangles,* ABT-263 at 100 mg/kg, p.o., qd ×21; *open diamonds,* R-CHOP (rituximab at 10 mg/kg, i.v., qd ×1; cyclophosphamide at 25 mg/kg, i.p., qd ×1; doxorubicin at 3 mg/kg, i.v., qd ×1; vincristine at 0.25 mg/kg, i.v., qd ×1; prednisone at 0.5 mg/kg, p.o., qd ×1); *closed squares,* ABT-263 + R-CHOP. *Right,* OPM-2 multiple myeloma xenograft model. *Closed circles,* combination vehicle; *closed triangles,* ABT-263 at 100 mg/kg, p.o., qd ×21; *open diamonds,* bortezomib at 1 mg/kg, i.v., qd ×3; *closed squares,* ABT-263 + bortezomib.

sequestering Bim in a Bim:Bcl-2 complex and compensating for the loss of Mcl-1 (25). Disruption of this complex and release of Bim either to directly activate Bax and/or Bak (direct model) or to further antagonize antiapoptotic proteins such as Mcl-1 to release activated Bax and Bak (indirect model) is sufficient to induce cell death. On the other hand, ABT-263 is ineffective in cells that have not previously been primed with proapoptotic stimuli (i.e., FL5.12 cells in the presence of IL-3; Fig. 2).

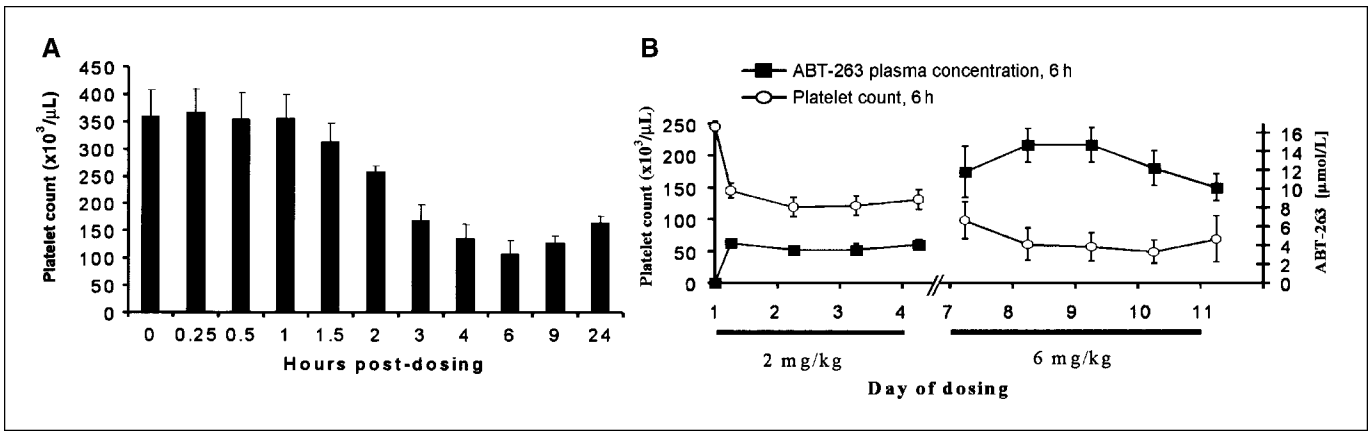

Figure 5. Effect of ABT-263 on circulating platelets in dogs. *A,* circulating platelet count levels after a single p.o. dose of ABT-263 (5 mg/kg) in dogs. *Columns,* mean ($n = 3$); *bars,* SE. *B,* platelet count and ABT-263 plasma concentrations after multiple daily doses (2 mg/kg, days 1–6; 6 mg/kg, days 7–11) in dog. *Closed squares,* ABT-263 plasma concentrations; *open circles,* platelet count on days 1 to 4 and days 7 to 11. *Points,* mean ($n = 3$); *bars,* SD.

The induction of apoptosis by the proapoptotic BH3-only proteins is dependent on Bax or Bak (33, 34). As such, Bax/Bak–deficient cells are immune to BH3-only protein–mediated cell death. It was previously shown that the induction of apoptosis by ABT-737 in MEF cells requires the neutralization of Mcl-1 and is dependent on Bax and Bak (14). Similarly, we observe that neutralization of Mcl-1 (i.e., *Mcl-1*$^{-/-}$ MEFs) is required for ABT-263 to induce apoptosis (Supplementary Fig. S4*A*). This is indicative of cell killing mediated through inhibition of Bcl-xL (and Bcl-2). In contrast, other reported Bcl-2 family inhibitors exhibit cytotoxicity independent of Bax and Bak (14). Consistent with these observations, both reported BH3 mimetics tested in this study [GX15-070 and (−)-gossypol] displayed a similar spectrum of cell killing in WT and DKO MEF cells to the general cytotoxic agent etoposide (Supplementary Fig. S4*C*).

In contrast to nonmalignant cells, cancer cells are continually bombarded by multiple stress signals that predispose them to die (1, 25). Cancer cells overcome these death signals through alterations in the apoptotic pathway (e.g., up-regulation of antiapoptotic Bcl-2 family members). Human tumor cell lines such as H146 that have been shown to be "addicted" to Bcl-2 are, in fact, quite sensitive to treatment with ABT-263, leading to rapid Bax activation, cytochrome *c* release, and caspase activation. In a broader sense, the protection afforded to cancer cells by Bcl-xL and Bcl-2 is illustrated by the single-agent activity of ABT-263 in ~30% to 50% of SCLC and hematologic malignant cell lines (Fig. 3).

From gene expression microarray analysis, cells overexpressing Mcl-1 are resistant to ABT-263, similar to observations made with ABT-737 (Supplementary Fig. S5). Furthermore, siRNA knockdown of Mcl-1 in the resistant SCLC cell line H196 restores sensitivity to ABT-263 (Supplementary Fig. S6). The role of Mcl-1 as a resistance factor for ABT-263 is also supported by the fact that ABT-263 is only effective in MEF cells in which Mcl-1 has been deleted (Supplementary Fig. S4*A*). This is consistent with recent studies with ABT-737, showing that attenuation of Mcl-1 in resistant cells leads to restoration of single-agent activity (7, 11, 13, 14, 26). Interestingly, ABT-263 does exhibit measurable (550 nmol/L) affinity for Mcl-1 (Table 1), suggesting that affinity <500 nmol/L is necessary to functionally inhibit this protein.

In vivo, ABT-263 exhibited pronounced oral activity in multiple xenograft models, both as a single agent and in combination with chemotherapy (Fig. 4). ABT-263 treatment of mice bearing established SCLC and ALL tumors resulted in rapid and complete tumor regression, suggesting that these tumor types are attractive areas for clinical investigation. Furthermore, ABT-263 dramatically enhanced the efficacy of several approved oncology agents in B-cell malignant xenograft models. Recent reports have described the role of both Bcl-2 and Bcl-xL in conferring resistance to rituximab (35–37). In the DoHH2 diffuse large B-cell xenograft, ABT-263 dramatically enhanced rituximab treatment above what was observed with each agent alone and induced complete regression of the majority of treated tumors. ABT-263 also significantly enhanced the activity of R-CHOP in a mantle cell lymphoma xenograft model, increasing the CR from 20% to 100%, with 44% of the mice exhibiting apparent cures. These are significant findings given that rituximab is routinely administered either alone or in combination with CHOP (i.e., R-CHOP) as first- and second-line therapy in non-Hodgkin's lymphoma patients (38). The activity of bortezomib was also significantly enhanced by ABT-263 in the OPM-2 multiple myeloma xenograft model, a tumor type that is typically associated with high Mcl-1 expression. Bortezomib-induced cell death is mediated, in part, by the neutralization of Mcl-1 via up-regulation of Noxa (39–44). Indeed, we observe the up-regulation of Noxa in OPM-2 cells treated with bortezomib (Supplementary Fig. S7). This suggests that the pronounced synergy between bortezomib and ABT-263 may be due to net neutralization of Bcl-xL/Bcl-2 (by ABT-263) and Mcl-1 (by bortezomib). These data show that, in addition to its single agent activity, ABT-263 can significantly enhance the efficacy of common chemotherapies to improve tumor regressions and overall response rates. This is true even in cases such as the OPM-2 multiple myeloma model, where ABT-263 alone displays little monotherapy activity.

Chronic daily dosing of ABT-263 is well tolerated in murine xenograft models with minimal weight loss and no overt toxicities. However, we previously reported that inhibition of prosurvival Bcl-2 family proteins with ABT-737 induces apoptosis in circulating platelets that results in their hepatic clearance without bone marrow toxicity (29). Recent reports have also described hypomorphic mutations in *Bcl-x*, which lead to thrombocytopenia in mice similar to that observed with ABT-737 treatment (45). The platelet effects of ABT-737 are also completely Bak/Bax dependent, indicating that ABT-737 acts upstream of these important mediators in the intrinsic apoptotic pathway. These data suggest that inhibition of Bcl-xL directly results in thrombocytopenia. Here we examined the effects of multiple daily dosing that would more closely mimic the clinical setting. Because dogs exhibit platelet levels and hemostasis similar to humans, and Bcl-2 and Bcl-xL proteins are highly conserved between these species, evaluation of the ABT-263–induced thrombocytopenia was done in the dog (Fig. 5).

Similar to ABT-737, ABT-263 induced a rapid reduction in circulating platelets in dogs. However, multiple daily dosing that achieves sustained plasma levels of ABT-263 does not significantly reduce platelet counts below that observed after the first dose. This suggests that either there are distinct populations within the circulating platelet pool that display differential sensitivities to ABT-263 or the animals are able to rapidly compensate by producing more platelets to maintain a steady-state platelet count. Although dose escalation results in a further decrease in platelets, continued dosing at this level produces a similar steady-state effect. Importantly, these studies suggest that a therapeutic window exists. In murine efficacy models, peak plasma concentrations of ABT-263 from 5.4 to 7.7 μmol/L were highly efficacious. Plasma drug concentrations severalfold above these efficacious levels were achieved in dogs after repeated daily p.o. dosing without decreasing circulating platelet count below 50,000/μL, which would constitute clinical grade 3 thrombocytopenia. Bcl-xL inhibition by ABT-263 seems to only affect circulating platelets, which is fundamentally different than thrombocytopenia observed with most cytotoxic chemotherapies that act through myelosuppression. It is important to point out that the platelet effects observed with ABT-263 are mechanism based, reversible, easily monitored, and well tolerated in these studies. In fact, it has been suggested that monitoring circulating platelet counts may represent a convenient surrogate marker for Bcl-xL inhibition in the clinic (45).

In summary, we describe here the properties of ABT-263, an orally bioavailable Bcl-2 family protein inhibitor that not only exhibits robust single-agent activity but also significantly enhances the antitumor activity of common chemotherapeutics. The ability to orally dose ABT-263 provides the flexibility to dose chronically to

maximize efficacy as a single agent, as well as to develop practical and effective combination-dosing regimens with parenteral chemotherapeutics. These studies provide a strong rationale for the clinical development of ABT-263 in both SCLC and hematologic malignancies.

Acknowledgments

Received 10/12/2007; revised 1/24/2008; accepted 2/15/2008.

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Conflict of interest: All of the authors are employees of Abbott.

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Cancer Res 2008;68:3421-3428.