Cancer Chemother Pharmacol (2010) 66:869–880
DOI 10.1007/s00280-009-1232-1

ORIGINAL ARTICLE

The Bcl-2 inhibitor ABT-263 enhances the response of multiple chemotherapeutic regimens in hematologic tumors in vivo

Scott Ackler · Michael J. Mitten · Kelly Foster · Anatol Oleksijew · Marion Refici · Stephen K. Tahir · Yu Xiao · Christin Tse · David J. Frost · Stephen W. Fesik · Saul H. Rosenberg · Steven W. Elmore · Alexander R. Shoemaker

Received: 17 September 2009 / Accepted: 24 December 2009 / Published online: 23 January 2010

Abstract
Purpose This study was designed to test the ability of the Bcl-2 family inhibitor ABT-263 to potentiate commonly used chemotherapeutic agents and regimens in hematologic tumor models.
Methods Models of B-cell lymphoma and multiple myeloma were tested in vitro and in vivo with ABT-263 in combination with standard chemotherapeutic regimens, including VAP, CHOP and R-CHOP, as well as single cytotoxic agents including etoposide, rituximab, bortezomib and cyclophosphamide. Alterations in Bcl-2 family member expression patterns were analyzed to define mechanisms of potentiation.
Results ABT-263 was additive with etoposide, vincristine and VAP in vitro in the diffuse large B-cell lymphoma line (DLBCL) DoHH-2, while rituximab potentiated its activity in SuDHL-4. Bortezomib strongly synergized with ABT-263 in the mantle cell lymphoma line Granta 519. Treatment of DoHH-2 with etoposide was associated with an increase in Puma expression, while bortezomib upregulated Noxa expression in Granta 519. Combination of ABT-263 with cytotoxic agents demonstrated superior tumor growth inhibition and delay in multiple models versus cytotoxic therapy alone, along with significant improvements in tumor response rates.
Conclusions Inhibition of the Bcl-2 family of proteins by ABT-263 enhances the cytotoxicity of multiple chemotherapeutics in hematologic tumors and represents a promising addition to the therapeutic arsenal for treatment of these diseases.

Keywords ABT-263 · Bcl-2 · Lymphoma · Myeloma · Apoptosis

Scott Ackler and Michael J. Mitten contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00280-009-1232-1) contains supplementary material, which is available to authorized users.

S. Ackler · M. J. Mitten · K. Foster · A. Oleksijew · M. Refici · S. K. Tahir · Y. Xiao · C. Tse · D. J. Frost · S. H. Rosenberg · S. W. Elmore · A. R. Shoemaker
Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, IL, USA

S. W. Fesik
Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN, USA

S. Ackler (✉)
Building AP3/105, 100 Abbott Park Road, Abbott Park, IL 60064, USA
e-mail: scott.ackler@abbott.com

Introduction

Hematologic tumors, comprising leukemias, lymphomas and myelomas, are estimated to afflict 138,530 people and lead to 52,910 deaths in 2008 [1]. The combination of cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) had been the standard of care for non-Hodgkin's lymphoma (NHL), and the addition of rituximab (R-CHOP) has improved treatment outcomes in B-cell lymphomas [2]. More recently, the proteasome inhibitor bortezomib was approved for relapsed mantle cell lymphoma (MCL) and multiple myeloma (MM) and has improved treatment outcomes in these diseases [3, 4]. However, there remains significant need for improvement in the treatment of these disorders.

A frequent event in this tumor class is the upregulation of the anti-apoptotic Bcl-2 family members [5, 6]. Indeed, the hallmark translocation in follicular lymphoma involves placement of the Bcl-2 gene under the control of the IgH enhancer region [7]. Even in the absence of this translocation, Bcl-2 and/or Bcl-xL are expressed in a high percentage of cases of diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL), MCL and MM [8–12]. Overexpression of Bcl-2 or Bcl-xL, or alteration of the Bax:Bcl-2 ratio, have been associated with poor prognosis in these tumors [5].

Chemotherapeutics can modulate the expression of Bcl-2 family members in hematologic tumors. Etoposide has been shown to upregulate Bax expression in the leukemia line HL-60 and concomitantly decreased Bcl-xL levels in K562 chronic myeloid leukemia cells [13, 14]. Belhoussine et al. [15] generated HL-60 lines resistant to either vincristine or daunorubicin and found resistance to be associated with increases in Bcl-2 or Bcl-xL expression, respectively. Bortezomib treatment stabilized mitochondria-associated Bax protein in DHL-4 DLBCL and primary CLL cells [16] and increased Noxa expression in responsive MM cells [17]. Rituximab decreased the levels of Mcl-1 and the caspase 3 inhibitory protein XIAP in circulating CLL cells [18].

ABT-263 is an orally available, potent inhibitor of Bcl-2, Bcl-xL and Bcl-w. ABT-263 competitively inhibits the binding of the pro-apoptotic Bcl-2 family members to Bcl-2, Bcl-xL and Bcl-w, allowing them to activate the intrinsic apoptotic cascade [19]. ABT-263 and the related inhibitor ABT-737 have demonstrated single-agent activity against DLBCL and acute lymphoblastic leukemias (ALL) in vivo, and CLL primary samples ex vivo [19–22]. Additionally, these agents potentiate the response to several chemotherapeutic regimens in vivo in hematologic tumors [19, 20, 23, 24]. However, an analysis of combinations of commonly used chemotherapeutics with ABT-263 in murine xenograft models has not been performed.

In this report, we analyzed ABT-263 in combination with several standard chemotherapeutic regimens in both flank and systemic models of DLBCL, MCL and MM. Significant enhancement of response was measured in vitro with combinations of ABT-263 and etoposide in DoHH-2 and rituximab in SuDHL-4 DLBCL lines, as well as bortezomib in Granta 519 MCL cells. Etoposide treatment led to increased Puma protein expression in DoHH-2, while bortezomib increased Noxa levels in Granta 519. In vivo, significant increases in tumor growth inhibition and delay and overall response rates were demonstrated using ABT-263 in combination with CHOP, rituximab, R-CHOP, etoposide, vincristine, bortezomib and cyclophosphamide in both flank and systemic models of DLBCL, MCL and MM. These data demonstrate that ABT-263 can cooperate with a broad range of cytotoxic agents and regimens in the treatment of hematologic tumors.

Materials and methods

Cell culture

DoHH-2, SuDHL-4 and WSU-DLCL2 DLBCL, Granta 519 MCL, OPM-2 MM cells were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). Cells were cultured in RPMI 1640 media (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Hyclone, Logan, UT), 1% sodium pyruvate (Invitrogen) and 4.5 g/L glucose (Sigma, St Louis, MO). Cells were maintained at 37°C in 5% CO_2 and 95% relative humidity.

Reagents

ABT-263 (N-(4-{4-[2-(4-chlorophenyl)-5,5-dimethyl-cyclohex-1-enylmethyl]-piperazin-1-yl}-benzoyl)-4-((R)-3-morpholin-4-yl-1-phenylsulfanylmethyl-propylamino)-3-trifluoromethanesulfonyl-benzenesulfonamide) was synthesized at Abbott Laboratories (Abbott Park, IL). Bortezomib was purchased from Millennium Pharmaceuticals (Cambridge, MA). Doxorubicin and etoposide were purchased from Bedford Laboratories (Bedford, OH). Vincristine was purchased from Mayne Pharmaceuticals (Paramus, NJ). Cyclophosphamide was purchased from Bristol-Myers Squibb Co. (Princeton, NJ). Rituximab was purchased from Genentech Inc. (South San Francisco, CA). Prednisolone was purchased from ETHEX Corp (St Louis, MO). Doxorubicin, vincristine, cyclophosphamide, prednisone and etoposide used in in vitro studies were purchased from Sigma. Human IgG1κ (cat # 0151K-01) was purchased from Southern Biotech (Birmingham, AL). $F(ab')_2$ fragment goat anti-human cross-linker antibody (#109-006-008) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Phosal 50 PG was purchased from American Lecithin (Oxford, CT).

Combinatorial analysis

DoHH-2, SuDHL-4 and Granta 519 cells were plated at a density of 50,000 cells per well in 96-well plates. Cells were treated with ABT-263 and cytotoxic agent(s) at half-log increments over a 3 log scale in an 8 × 8 or 8 × 10 grid, based around the IC_{50} value for each agent(s). All treatment pairs were run in duplicate. ABT-263 was tested at ranges from 20 to 0.01 μM. Etoposide was used at a dose range of 100–0.14 nM. Vincristine was used at a dose range

of 20–0.027 nM. VAP, where 1× was defined as doxorubicin (1.5 pM), vincristine (260 pM) and prednisone (1 μM), was used as described by Mohammad et al. for CHOP [25]. Relative concentrations were maintained from 300× to 0.3×. Bortezomib was used at a dose range of 100–0.01 nM. Rituximab was cross-linked as previously described [26]. ABT-263 was tested against rituximab-cross-linker (R-XL) or human IgG1κ isotype control-cross-linker (Ig-XL). All incubations were performed for 24–48 h.

Cell killing was determined using the CellTiterGlo assay (Promega, Madison, WI), and luminescence was measured on a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The combination index (CI) as described by Chou-Talalay [27] was assessed using the software program CalcuSyn (Biosoft, Ferguson, MO). CI determinations were made at the ED_{50} value.

Western analysis of treated cell lines

Tumor cells were plated at 5×10^6 cells/plate and treated with concentrations of ABT-263 and/or cytotoxic agent corresponding to maximum combination effect. Cells were treated for 4 or 24 h, and protein extraction and separation were performed as previously described [20]. Mouse anti-Bcl-2 (#610538) was purchased from BD Biosciences (San Jose, CA). Mouse anti-Bcl-xL (#2300-MC) and mouse anti-Bax (#2280-MC) were purchased from R&D Systems (Minneapolis, MN). Rabbit anti-Mcl-1 (#sc-819) was purchased from Santa Cruz (Santa Cruz, CA). Mouse anti-Noxa (#IMG-349A) was purchased from Imgenex (San Diego, CA). Rabbit anti-Bim (#AAP-330) was purchased from Assay Designs (Ann Arbor, MI). Rabbit anti-Puma (#4976), rabbit anti-caspase 3 (#9662) and rabbit anti-tubulin (#2146) were purchased from Cell Signaling (Danvers, MA). Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP were purchased from Pierce (Rockford, IL). Labeled proteins were detected and analyzed as previously described [20].

In vivo xenograft trials

All animal studies were conducted in accordance with the guidelines established by the internal Institutional Animal Care and Use Committee. C. B.-17 *scid* mice (DoHH-2, WSU-DLCL2 and Granta 519) or C. B.-17 *scid*-bg mice (SuDHL-4, OPM-2) (Charles River Laboratories, Wilmington, MA) were inoculated with 1×10^6 (DoHH-2, WSU-DLCL2), 3×10^6 (SuDHL-4) or 5×10^6 (Granta 519, OPM-2) cells subcutaneously in the right flank. For flank xenografts, inoculation volume was 0.2 mL consisting of a 50:50 mixture of cells in growth media and Matrigel (BD Biosciences, Bedford, MA). Tumor volume was estimated by 2-3 weekly measurements of the length and width of the tumor by electronic calipers and applying the following equation: $V = L \times W^2/2$. Tumors were allowed to reach approximately 225 mm^3, and mice were size matched (day 0) into treatment and control groups. For systemic Granta 519 tumor models, 2×10^6 cells were injected via the tail vein in 0.1 mL volume of cell media on day 0, and dosing initiated on either day 7 or day 14. All animals were ear-tagged and monitored individually throughout the experiment. ABT-263 was administered as previously described [19]. Marketed cytotoxic agents were administered according to the manufacturer's instructions, as described in Table 1. CHOP MTD was determined experimentally, with cyclophosphamide (25 mg/kg), doxorubicin (3 mg/kg) vincristine (0.25 mg/kg) and prednisolone (0.5 mg/kg) administered IP, IV, IV and orally, respectively, on day 1. For R-CHOP, rituximab was administered at 10 mg/kg IV, along with CHOP, on day 1. ABT-263 was administered approximately 2 h prior to cytotoxic injection, with the exception of bortezomib, which was given 4 h prior to ABT-263 to avoid combination toxicity. Individual components of CHOP and R-CHOP were injected approximately 1.5 h apart, in the following order: rituximab, cyclophosphamide, doxorubicin, vincristine, prednisolone. Tumor growth inhibition (TGI), complete response (CR), partial response (PR), overall response rate (ORR) and percent increase in life span (%ILS) of mono- and combination therapy was calculated as previously described [28]. All xenograft trials were comprised of 9 or 10 mice per group. Synergy in vivo was defined as a greater than additive effect on %TGI or %ORR or both.

Statistical analysis

Significance within in vivo experiments for tumor growth inhibition and tumor growth delay were performed by Wilcoxon rank sum analysis and Kaplan-Meier log rank analysis, respectively. Significance for CR and ORR was performed by 2-tailed Fisher's exact test.

Results

ABT-263 broadly potentiates chemotherapeutic agents in vitro

ABT-263 is a potent, orally available inhibitor of the Bcl-2 anti-apoptotic proteins Bcl-2, Bcl-xL and Bcl-w. ABT-263 has demonstrated single-agent activity in B-cell lymphoma and small cell lung cancer (SCLC) [19] and is currently being tested in phase I trials for these indications. While significant regressions were achieved by ABT-263 monotherapy in SCLC, B-cell lymphomas were more resistant to the effects of ABT-263 alone, but strongly potentiated the

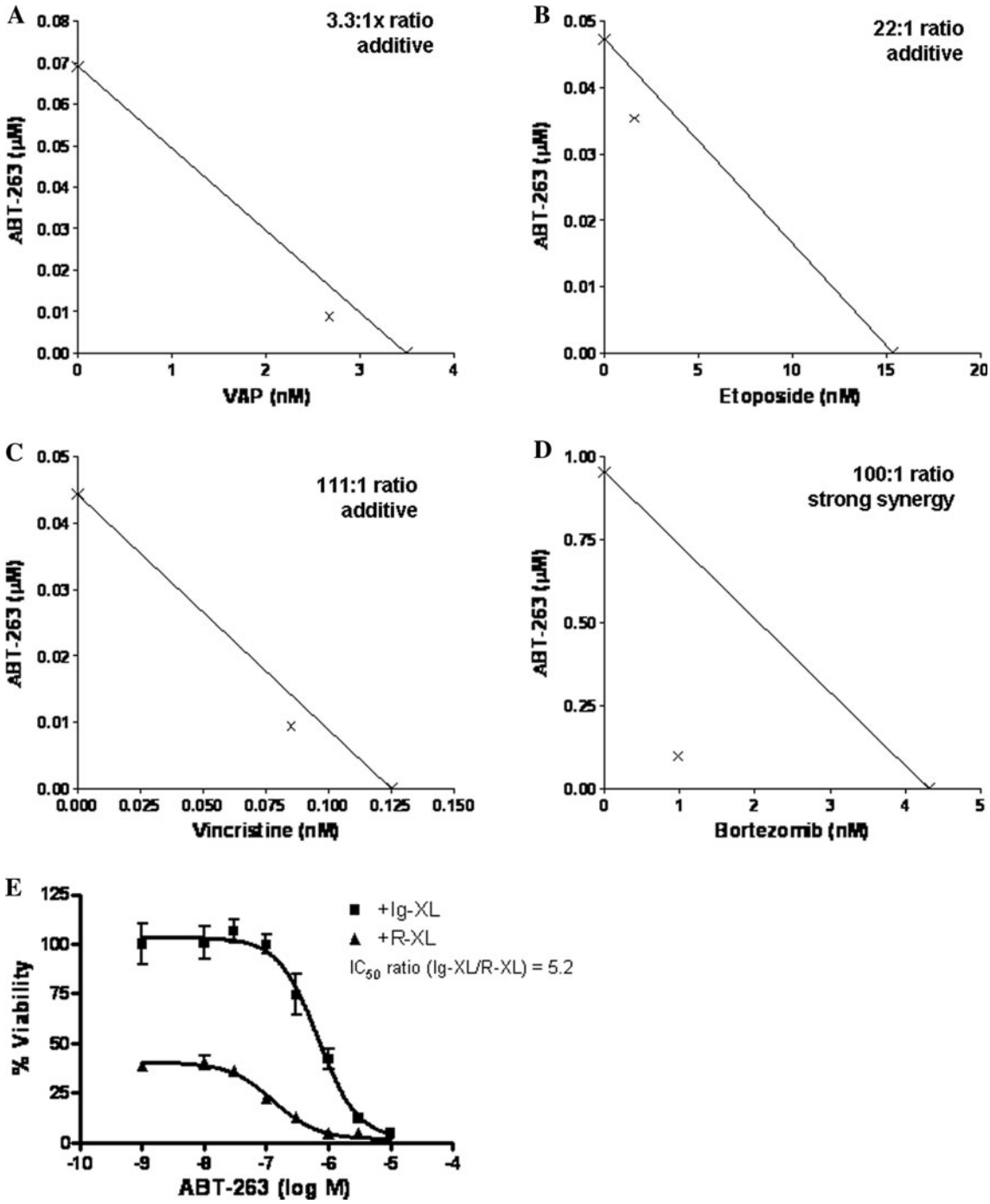


Fig. 1 In vitro combination analysis of cytotoxics with ABT-263. Cell survival was measured using the CellTiterGlo assay, and synergism was determined by the method of Chou-Talalay [27]. CI values reported are for the ED_{50} value, ratios expressed as ABT-263:cytotoxic agent or regimen. **a**–**c** DoHH-2 DLBCL cells were treated with escalating concentrations of ABT-263 in combination with varying ratios of VAP (**a**), or escalating doses of etoposide (**b**) or vincristine (**c**) for 48 h. **a** ABT-263 demonstrated additivity with VAP (CI = 0.89), etoposide (CI = 0.85) and vincristine (CI = 0.89). **d** Granta 519 cells were treated with doses of ABT-263 ranging from 10 to 0.01 μM and bortezomib ranging from 100 to 0.1 nM. Strong synergy was demonstrated with this combination (CI = 0.33). **e** SuDHL-4 killing following combination of rituximab and ABT-263. ABT-263 killing at doses ranging from 10 to 0.01 mM were measured in the presence of R-XL or Ig-XL following 48 h of exposure. R-XL alone induced significant cell killing. IC_{50} values for ABT-263 shifted from 681 nM with Ig-XL to 130 nM in the presence of R-XL, increasing the efficacy of ABT-263 5.2-fold

activity of several chemotherapeutic agents in these tumors [19, 20]. Specifically, the combination of rituximab or rapamycin with ABT-263 in DoHH-2 DLBCL tumors induced significant improvements in tumor regressions, tumor growth delay and inhibition, as well as a significant increase in ORR. In the MCL line Granta 519, combination

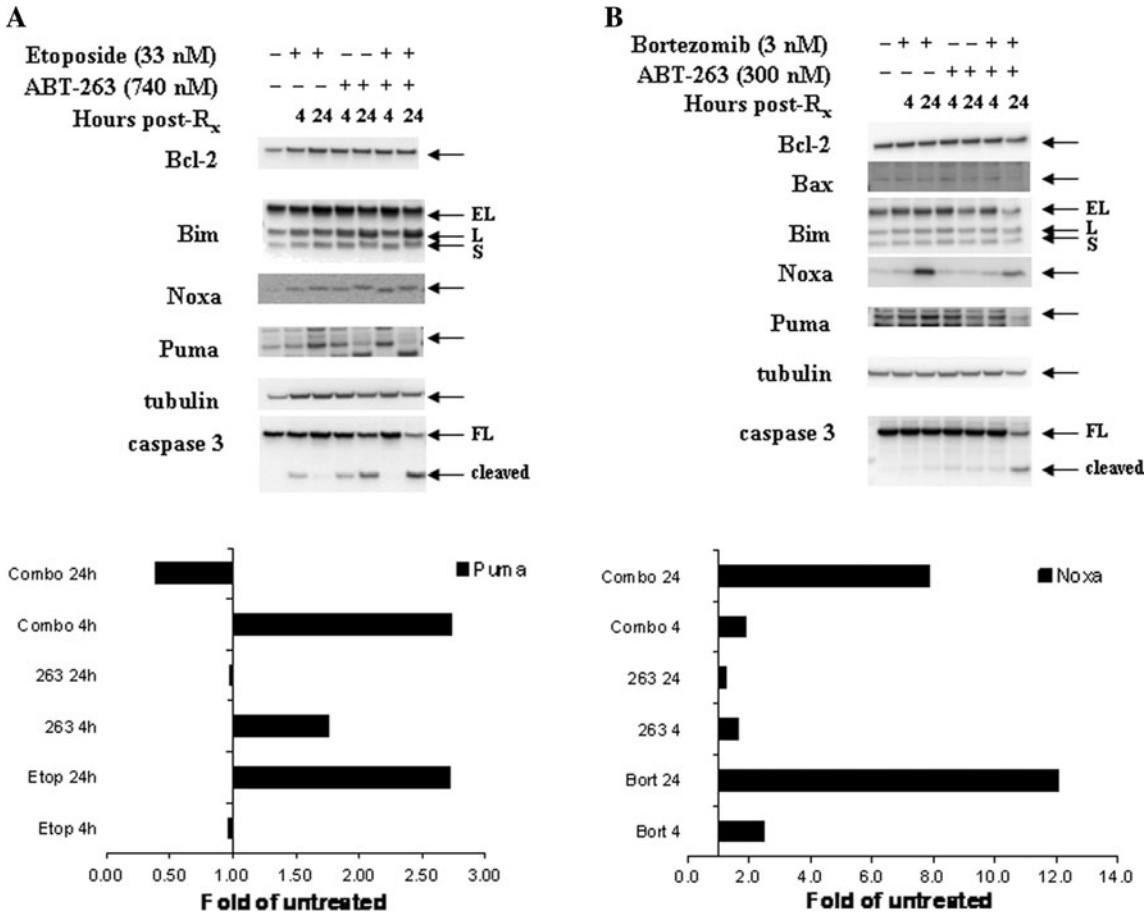
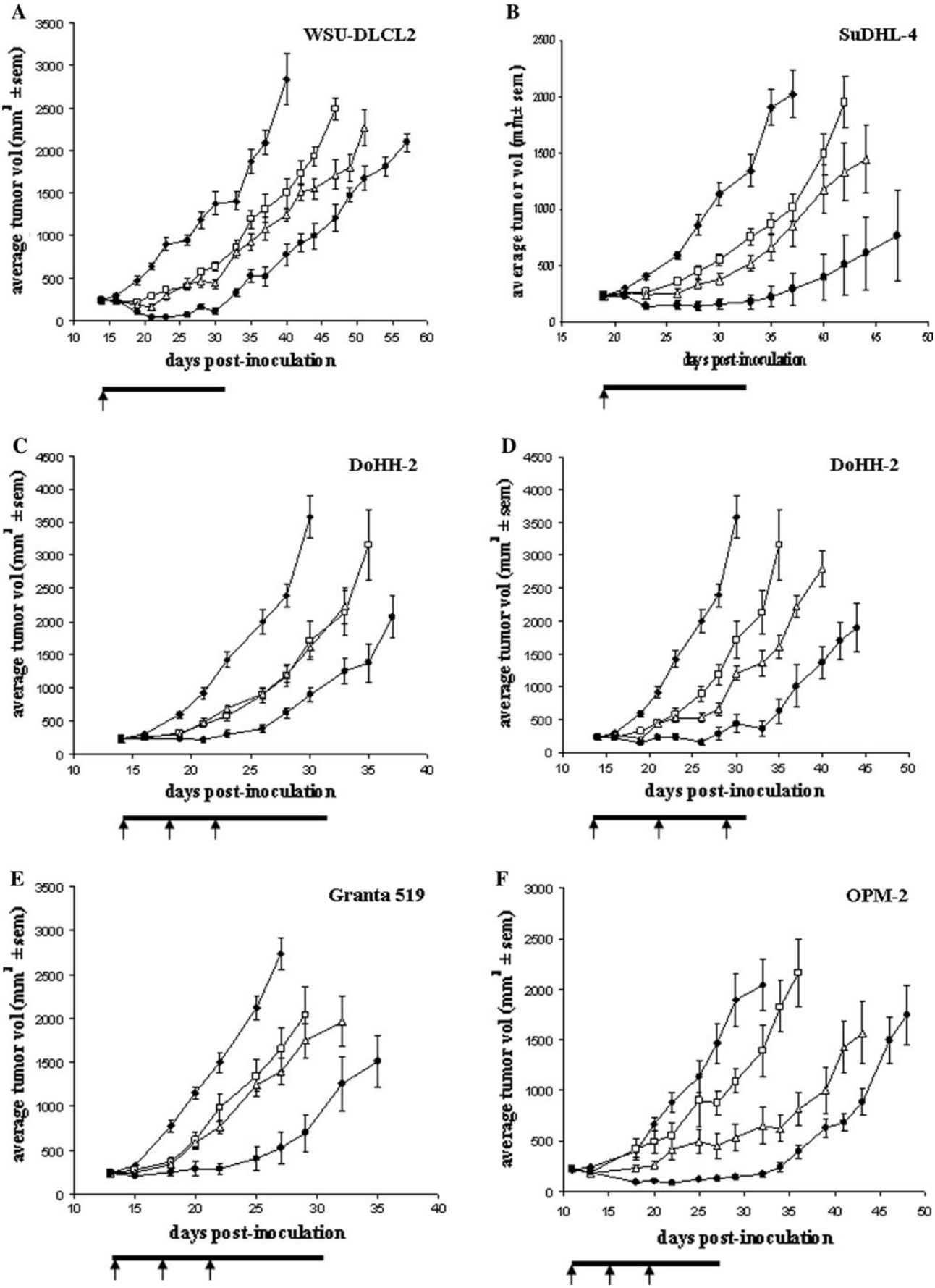


Fig. 2 Bcl-2 family member expression in hematologic tumor cells treated with cytotoxics and/or ABT-263. Expression of Bcl-2 family member proteins was examined following exposure of cells to cytotoxic agents with or without ABT-263 for 4 and 24 h. Cleavage of caspase 3 was also measured to determine level of apoptosis within cells. Concentrations used were determined by isobolographic analysis as shown in Fig. 1. Graphs depict densitometric analysis of western blots, plotted as fold of untreated cells. **a** DoHH-2 cells treated with etoposide (33 nM) with or without ABT-263 (740 nM). Treatment of DoHH-2 cells with etoposide increased Puma expression 2.5-fold following 24 h of exposure. ABT-263 induced a more modest increase in Puma following 24 h (1.5-fold). Combination of both agents increased Puma levels more rapidly, with a 2.5-fold increase observed at 4 h. No other members of the Bcl-2 family demonstrated significant alteration in expression through 24 h. Caspase activation was seen in both etoposide and ABT-263 treated groups, but was more pronounced in the combination group at 24 h. **b** Granta 519 cells were treated with bortezomib (3 nM) with or without ABT-263 (300 nM). Bortezomib increased Noxa expression levels by 12-fold following 24 h of exposure, while ABT-263 alone had no effect on Noxa levels. Combination of both agents also led to an increase in Noxa levels, albeit lower than bortezomib alone. However, caspase 3 cleavage was only significantly induced by combination treatment

of ABT-263 with R-CHOP led to a 100% CR rate and a substantial increase in survival over either monotherapy alone. ABT-263 combined with bortezomib in the MM line OPM-2 also led to complete regressions and a significant increase in survival over either monotherapy alone.

Therefore, ABT-263 was tested against several different chemotherapeutic regimens in lymphoid cell lines, to determine whether Bcl-2 family inhibition potentiated the activity of these agents. Cells were treated with ABT-263, and a cytotoxic agent or regimen in an 8 × 8 or 8 × 10 concentration grid centered approximately around the IC_{50} value of each agent for that cell line. Combination groups were as follows: DoHH-2 with VAP, etoposide and vincristine (Fig. 1a–c), and Granta 519 with bortezomib (Fig. 1d). VAP was used in place of CHOP for in vitro analysis, because cyclophosphamide requires hepatic metabolism to become activated [29]. In DoHH-2 cells, combination of ABT-263 with VAP, etoposide or vincristine was additive, with ED_{50} CI values ranging from 0.85 to 0.89. Combination of ABT-263 with bortezomib in Granta 519 cells resulted in strong synergy, with an ED_{50} CI value of 0.33 (Fig. 1d).

SuDHL-4 cells were pre-treated with rituximab or isotype control and cross-linked with a human $F(ab')_2$ fragment to induce downstream signaling and apoptosis, as previously described [26]. Modulating rituximab or cross-linker concentration was not found to be dose-responsive (data not shown). However, both rituximab and the cross-linker


A
WSU-DLCL2
average tumor vol (mm³ ± sem)
days post-inoculation
B
SuDHL-4
average tumor vol (mm³ ± sem)
days post-inoculation
C
DoHH-2
average tumor vol (mm³ ± sem)
days post-inoculation
D
DoHH-2
average tumor vol (mm³ ± sem)
days post-inoculation
E
Granta 519
average tumor vol (mm³ ± sem)
days post-inoculation
F
OPM-2
average tumor vol (mm³ ± sem)
days post-inoculation

◀ **Fig. 3** Xenograft sensitivity to ABT-263 with or without cytotoxic agent(s). Mice were grafted with WSU-DLCL2 (**a**), SuDHL-4 (**b**), DoHH-2 (**c**, **d**), Granta 519 (**e**), or OPM-2 (**F**) cells and treated following size matching at approximately 225 mm^3 (day 0). Animals were dosed with either combination vehicle (*filled diamond*), ABT-263 100 mg/kg/day (*open square*), cytotoxic agent(s) as described in Table 1 (*open triangle*) or both (*filled circle*). *Bars* represent dosing schedules for ABT-263, while *arrows* represent cytotoxic agent(s). Statistical analysis of data presented in Table 1. **a** WSU-DLCL2 tumors treated with ABT-263 and/or CHOP. Both ABT-263 and CHOP induced significant tumor growth inhibition compared to vehicle control from days 16-40 versus vehicle, with CHOP producing a 60% ORR. Combination of both agents was superior to CHOP alone, with significant decrease in tumor growth rate from days 19-51. ORR was increased to 100% with the combination *v.* CHOP alone. **b** SuDHL-4 tumors treated with ABT-263 and/or rituximab. Both rituximab and ABT-263 were superior versus vehicle control from days 23–40. Rituximab combined with ABT-263 showed significantly decreased tumor growth rate compared to rituximab alone from days 23-44. Rituximab plus ABT-263 increased ORR to 67%, all CRs. (**c**, **d**) DoHH-2 tumors treated with ABT-263 and/or etoposide (**c**) or vincristine (**d**). All single agents were superior to vehicle control from days 19–30, with vincristine producing a 10% ORR. Etoposide plus ABT-263 showed a significant decrease in tumor growth rate from days 21-37, while the vincristine/ABT-263 combination was significant from days 19-40. ABT-263 combination with etoposide significantly increased ORR to 50% with one CR, and combination with vincristine led to a 70% ORR with one CR. **e** Granta 519 tumors treated with ABT-263 and/or bortezomib. Both agents as monotherapy inhibited tumor growth rate compared to vehicle control. ABT-263 plus bortezomib was superior to bortezomib alone from days 20-32. However, increase in ORR was modest, with a 20% ORR in the combination group and one CR versus 0% ORR with bortezomib alone. (**f**) OPM-2 tumors treated with ABT-263 and/or cyclophosphamide. Tumor growth inhibition by ABT-263 was significant from vehicle control on days 13, 20, 22, 27 and 29, while cyclophosphamide was significant for days 13-29 with a 20% ORR. Combination of ABT-263 with cyclophosphamide was significantly better than cyclophosphamide monotherapy on days 18-36 and 41-43, with an increase in ORR to 100% with one CR

alone modestly induced apoptosis in SuDHL-4 following 18 h of exposure, while the combination significantly enhanced apoptosis over either agent alone (Fig. S1). Therefore, ABT-263 activity was measured in the presence of R-XL or Ig-XL. R-XL alone induced significant cell killing following 48 h of exposure (approximately 70%). The IC_{50} of ABT-263 was shifted approximately 5.2-fold in the presence of R-XL versus Ig-XL (Fig. 1e).

Cell killing with etoposide and bortezomib were associated with upregulation of pro-apoptotic BH3-only proteins

Cells were treated with cytotoxic agents and/or ABT-263 for 4 and 24 h, and protein was harvested to determine alterations in the protein levels of Bcl-2 family members. DoHH-2 cells treated with etoposide (33 nM) demonstrated a 2.5-fold upregulation of Puma protein following 24 h of exposure. While ABT-263 treatment alone at 740 nM demonstrated only a modest increase (1.5-fold) in Puma expression at 24 h, the combination of these agents accelerated the upregulation of Puma, with a 2.5-fold increase in expression following 4 h of exposure. By 24 h of exposure to both agents, Puma levels were below untreated control, but caspase 3 cleavage was significantly increased (Fig. 2a). In contrast, DoHH-2 cells treated with vincristine (250 nM) and/or ABT-263 (250 nM), or VAP (3×) and/or ABT-263 (120 nM), demonstrated no significant alterations in expression of Bcl-2 family proteins (Fig. S2).

Granta 519 MCL cells treated with bortezomib (3 nM) substantially upregulated Noxa expression approximately 12-fold after 24 h of exposure, in agreement with previous reports [17]. ABT-263 (300 nM) alone had no effect on Noxa expression. Combination of ABT-263 with bortezomib demonstrated an increase in Noxa levels which was slightly less pronounced than bortezomib alone; however, induction of caspase 3 cleavage was significantly enhanced by the combination at 24 h (Fig. 2b), most likely due to inhibition of Mcl-1 by Noxa.

ABT-263 enhances the cytotoxic activity of a broad range of chemotherapeutics in lymphoma xenografts

Based on the additivity and/or synergy seen in in vitro experiments, ABT-263 was tested in combination with several common cytotoxic agents and regimens in vivo. ABT-263 administered at 100 mg/kg/day QDx17 significantly inhibited tumor growth in all models of DLBCL tested (Fig. 3a–d; Table 1), with an approximately twofold increase in tumor growth inhibition and a significant delay in tumor growth ranging from 57 to 91%. However, outside of a single CR in the DoHH-2 group, monotherapy did not induce significant regressions in these models. In the Granta 519 MCL and OPM-2 MM models, ABT-263 monotherapy did not demonstrate a statistically significant effect on tumor growth inhibition or delay.

CHOP has been the standard of care for NHL, achieving 40% cures in these cases clinically [30]. In the DLBCL models examined here, DoHH-2 and WSU-DLCL2 were sensitive to CHOP treatment, with significant inhibition of tumor growth (76 and 46%, respectively) and tumor growth delay (122 and 92%, respectively). While CHOP therapy did not induce significant regression in DoHH-2 (20% ORR), a 60% ORR rate was achieved in WSU-DLCL2. In SuDHL-4 tumors, however, CHOP treatment was ineffective, with tumor growth inhibition of 14% and delay in tumor growth of 27%.

Combination of ABT-263 with CHOP significantly enhanced the response of all three DLBCL xenografts. Tumor growth rate inhibition was significantly increased in DoHH-2, SuDHL-4 and WSU-DLCL2 (86, 45 and 78%, respectively), along with increases in delay of tumor growth to 1 cc over CHOP alone (23, 28 and 43%, respectively).

Table 1 Combination activity of ABT-263 with various agents in hematologic flank xenograft models

Model	Cytotoxic agent[a]	Cytotoxic agent(s) monotherapy				ABT-263[a] + cytotoxic agent(s)			
		TGI[b]	%ILS[c]	%CR[d]	%ORR[e]	TGI	%ILS	%CR	%ORR
DoHH-2 (DLBCL)	None	–	–	–	–	33*	57**	10	10
	CHOP	76*	122**	0	20	86*	23	0	90†
	Etoposide	59*	33**	0	0	79*	25**	10	50†
	Vincristine	63*	43**	0	10	87*	40**	10	70†
SuDHL-4 (DLBCL)	None	–	–	–	–	50*	91**	0	0
	CHOP	16	27	0	0	45*	28**	0	11
	Rituximab	46*	109**	0	0	81	278**	67†	67†
WSU-DLCL2 (DLBCL)	None	–	–	–	–	46*	62**	0	0
	CHOP	46*	92**	0	60†	78*	43**	0	100
	Rituximab	68*	86**	20	40	85	58**	50	90
Granta 519 (MCL)	None	–	–	–	–	38	22	0	0
	R-CHOP	70*	138**	10	50	>99*	453	88†	100†
	Bortezomib	44*	71**	0	0	79*	58	10	20
OPM-2 (MM)	None	–	–	–	–	58	20	0	0
	Cyclophosphamide	72*	83**	0	20	8*	22	10	100†
	Bortezomib	89*	116**	0	90†	>99*	83**	75†	100

* $P < 0.05$ versus vehicle (vs. monotherapy for combination groups), Wilcoxon Rank Sum test

** $P < 0.05$ versus vehicle (vs. monotherapy for combination groups), Mantel–Cox log-rank test of Kaplan–Meier analysis

† $P < 0.05$ versus vehicle (vs. monotherapy for combination groups), Fisher's exact test

[a] ABT-263: 100 mg/kg, p.o., q.d. ×21 (DLBCL: q.d. ×17); CHOP: cyclophosphamide 25 mg/kg, i.p., doxorubicin 3 mg/kg, i.v., vincristine 0.25 mg/kg, i.v., prednisone 0.5 mg/kg, p.o., q.d. ×1; etoposide: 15 mg/kg, i.p., q4d x3; vincristine: 0.25 mg/kg, i.v., q7d x3; rituximab: 10 mg/kg, i.v., qd x1; R-CHOP: rituximab 10 mg/kg, i.v., cyclophosphamide 25 mg/kg, i.p., doxorubicin 3 mg/kg, i.v., vincristine 0.25 mg/kg, i.v., prednisone 0.5 mg/kg, p.o., q.d. ×1; bortezomib: 1 mg/kg, i.v., q4d ×3; cyclophosphamide: 100 mg/kg, i.p., q4d ×3

[b] Ratio of tumor volume for treated versus control (vehicle) at end of dosing period

[c] Median percentage increase compared to vehicle (vs. monotherapy for combination groups) in days to 1 cc tumor, (1st day of treatment defined as day 1)

[d] Percentage of complete responders (tumor volume below the detection limit)

[e] Overall response rate (sum of complete and partial responders)

While ORR was only slightly increased in SuDHL-4 (11%), ORR was significantly increased in DoHH-2, with a 90% response rate. Similarly, WSU-DLCL2 response rates increased from 60 to 100% (Fig. 3a). CHOP therapy alone induced minimal weight loss in all three models. While ABT-263-treated mice gained weight in DoHH-2- and WSU-DLCL2-bearing mice, SuDHL-4-bearing mice lost weight over the treatment course (7.2%). In all cases, however, weight loss was not exacerbated by combination of CHOP and ABT-263.

Rituximab is a humanized antibody to CD20 which has shown significant single-agent activity in human B-cell lymphoma, as well as enhancement of chemotherapeutic regimens [2]. It has been demonstrated previously that ABT-263 significantly enhanced the activity of rituximab in DoHH-2 flank tumors in vivo [19]. In SuDHL-4 and WSU-DLCL2, rituximab alone also demonstrated significant tumor growth inhibition (46 and 68, respectively) and tumor growth delay (109 and 86, respectively). Treatment of SuDHL-4 with rituximab alone failed to result in tumor regressions, while treatment of WSU-DLCL2 tumors with rituximab alone resulted in a 40% ORR with two CRs. Combination of rituximab with ABT-263 significantly improved tumor responses over rituximab alone in each of these tumor types. SuDHL-4 demonstrated a synergistic increase in tumor growth delay (278% *vs*. rituximab alone) with an increased inhibition of tumor growth of 81% (Fig. 3b). A 67% rate of CRs was observed. Combination efficacy was additive in WSU-DLCL2, with a 58% increase in tumor growth delay versus rituximab alone and tumor growth inhibition of 85%. A 90% ORR with five CRs was achieved in this model.

Addition of rituximab to CHOP therapy has shown significant improvement in a variety of B-cell lymphomas [2]. In the Granta 519 MCL xenograft model, R-CHOP induced significant anti-tumor activity, with a 70% decrease in tumor growth, a tumor growth delay of 138% and a 50% ORR with one CR. It was reported previously that

combination of R-CHOP with ABT-263 induced substantial tumor inhibition, with some cures [19], a result confirmed in further studies (Table 1).

Etoposide is an adjuvant drug in several therapeutic regimens for hematologic tumors [31]. In DoHH-2 DLBCL tumors, etoposide administered at 15 mg/kg/day Q4Dx3 induced significant tumor growth inhibition and delay (59 and 100%, respectively), with no tumor regressions. Concomitant ABT-263 therapy was synergistic, significantly increasing tumor growth inhibition (87%) with a 50% delay in tumor growth versus etoposide alone (Fig. 3c). ORR was significantly increased, with a 50% response rate and one CR.

Vincristine administered at 0.25 mg/kg/day Q7Dx3 induced a significant increase in tumor growth inhibition and tumor growth delay in DoHH-2 tumors (63 and 119%, respectively), with a 10% ORR and no CRs. Combination with ABT-263 led to a synergistic response, significantly enhancing tumor growth inhibition (87%), tumor growth delay (40% over vincristine alone) and induced significant tumor regression versus vincristine alone, as measured by a 70% ORR with one CR (Fig. 3d).

Bortezomib is a proteasome inhibitor approved for refractory MCL and MM [3]. It was demonstrated previously that bortezomib monotherapy had significant efficacy in OPM-2 MM cells, with a significant improvement of response when combined with ABT-263 [19]. In Granta 519, bortezomib administered at 1 mg/kg/day Q4Dx3 induced significant increases in tumor growth inhibition and delay (44 and 71%, respectively), but no tumor regressions. Combination with ABT-263 was additive, with a significant enhancement of tumor growth inhibition (79%), a moderate but not significant increase in tumor growth delay versus bortezomib alone (58%), and a 20% ORR with one CR (Fig. 3e).

Cyclophosphamide administered at 100 mg/kg/day Q4Dx3 in OPM-2 MM tumors yielded a significant increase in tumor growth inhibition and delay (72 and 83%, respectively), with a 20% ORR with no CRs. Addition of ABT-263 synergized with cyclophosphamide, with a 92% tumor growth inhibition and a moderate increase in tumor growth delay, but a significant increase in ORR to 100% with one CR (Fig. 3f).

ABT-263 synergizes with R-CHOP in systemic Granta 519 tumors

Based on the responses seen using R-CHOP and ABT-263 in the Granta 519 MCL xenograft flank model, analysis of response rates of this combination were measured in a systemic model of Granta 519. Granta 519 MCL cells were injected intravenously and treatment with R-CHOP and/or ABT-263 (100 mg/kg QDx17) starting day 7 or day 14 following inoculation. All vehicle-treated mice evidenced morbidity between days 23 and 25 following inoculation. Upon necropsy, tumors were found in the inner pelvic region (which led to hind-limb paralysis), as well as the spleen and lung.

ABT-263 monotherapy was ineffective in delaying morbidity in these mice. R-CHOP alone was able to significantly increase survival when initiated at either day 7 or day 14, although the increase in survival was larger when dosing was started earlier (97% increase in median survival, 61% for late-stage dosing). When combined with ABT-263, morbidity delay was increased to 221% above vehicle, or 63% versus R-CHOP alone, for early treatment initiation. After 130 days of monitoring, one mouse remained without overt signs of tumor burden. For the late initiation of dosing, ABT-263 plus R-CHOP remained significantly more efficacious when compared to R-CHOP alone (148% increased median survival over vehicle, or 54% versus R-CHOP alone). However, no mice survived beyond 50 days when treatment was delayed (Fig. 4). These data demonstrate that in a systemic model of MCL, a stringent model that more closely resembles human disease, ABT-263 combined with R-CHOP was still capable of significantly improving survival.

Discussion

Hematologic tumors represent a class of bone marrow cell-derived tumors encompassing leukemias, lymphomas and myelomas. CHOP has been the standard of care in DLBCL, providing a 40% cure rate [30], and addition of rituximab has significantly improved clinical outcomes [2]. Etoposide is used as an adjuvant in several existing and experimental regimens for the treatment of lymphoma and myeloma [31]. Bortezomib was recently approved for the treatment of refractory MCL and MM, where it has been found to increase ORR alone and in combination with other agents [3, 4]. ABT-263 is currently in phase I trials for the treatment of leukemia/lymphoma.

In this report, we analyzed the combination of ABT-263 with commonly used cytotoxics in models of DLBCL, MCL and MM in vitro and in vivo. In the DLBCL line DoHH-2, ABT-263 demonstrated additivity with VAP, etoposide and vincristine (Fig. 1). SuDHL-4 cells pre-treated with activated rituximab demonstrated a fivefold lower IC_{50} to ABT-263 (Fig. 1e). Synergy was observed in vivo in DoHH-2 DLBCL tumors treated with CHOP or vincristine, and SuDHL-4 DLBCL tumors treated with rituximab. Additivity was observed in DoHH-2 treated with etoposide, SuDHL-4 treated with CHOP and WSU-DLCL2 treated with rituximab (Fig. 3; Table 1). Recent reports have also demonstrated in vivo synergy with ABT-263 in DoHH-2

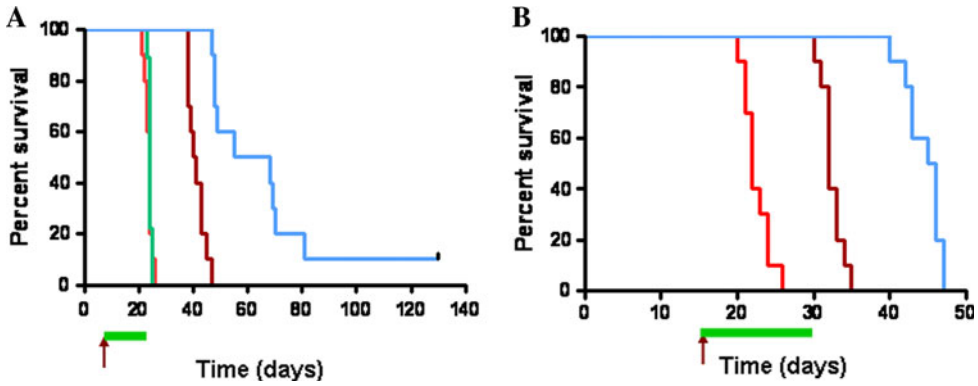


Fig. 4 Systemic Granta 519 sensitivity to ABT-263 with or without R-CHOP. Mice were inoculated IV with 2×10^6 cells, and treatment with ABT-263 and/or R-CHOP was initiated either 7 days (**a**) or 14 days (**b**) post-inoculation. Mice were treated with either combination vehicle (*red*), ABT-263 100 mg/kg/day (*green*), R-CHOP as described in "Materials and methods" (*brown*), or both (*blue*). Vehicle treated mice demonstrated morbidity between 23 and 25 days, with a median value of 24. ABT-263 monotherapy was indistinguishable from vehicle in this model. R-CHOP monotherapy demonstrated significant increase in survival in both early (97%) and late (61%) treatment groups. Combination of ABT-263 with R-CHOP led to enhanced survival when compared to R-CHOP alone in both the early and late treatment groups (63 and 54%, respectively). While no animals survived past 50 days in the late initiation group, one mouse survived through 130 days in the early initiation group receiving ABT-263 plus R-CHOP with no overt signs of disease

and SuDHL-4 using rapamycin, and in DoHH-2 using rituximab [19, 20].

In Granta 519 MCL cells, ABT-263 demonstrated robust synergy with bortezomib in vitro (Fig 1). In a flank xenograft model, combination of ABT-263 with R-CHOP was synergistic, while additivity was seen with bortezomib (Fig. 3; Table 1). A systemic model of Granta 519 also demonstrated synergism between ABT-263 and R-CHOP (Fig. 4). OPM-2 MM tumors demonstrated synergy between ABT-263 and cyclophosphamide (Fig 3; Table 1). Combination of ABT-263 with bortezomib was also found to be synergistic, confirming prior reports [19].

Mcl-1 provides resistance to ABT-263 and the related ABT-737, as it is not bound by these inhibitors, and decreasing Mcl-1 levels either pharmacologically or via RNA interference improves sensitivity to these agents [19, 32–35]. Mcl-1 has a short half-life and is regulated primarily by post-translational mechanisms. Increases in the pro-apoptotic BH3-only proteins Noxa and Puma, which are capable of binding Mcl-1, can also neutralize this factor [36]. Noxa upregulation occurred following bortezomib treatment of Granta 519 cells (Fig. 2), and this was associated with improved cell killing when combined with ABT-263 (Figs. 1, 2). While etoposide treatment of DoHH-2 induced a 2.5-fold in Puma expression, this was not sufficient to induce synergy with ABT-263. While the reason for this is unknown, Kutuk et al. [37] found that cisplatin could induce an increase in Puma and Noxa in Bcl-2 overexpressing MCF-7 cells, but Puma was not required for induction of apoptosis. Combination of ABT-263 with vincristine or VAP was additive, and alterations in Bcl-2 family members were not observed with these agents (Fig. S2), which may explain why synergy was not achieved.

As Wei et al. [5] reported a strong prognostic indicator in hematologic tumors is the Bax:Bcl-2 ratio. Rituximab was more effective when combined with agents which raise the Bax:Bcl-2 ratio [38–40]. CHOP synergized with genistein and bryostatin 1, both of which increased the Bax:Bcl-2 ratio in WSU-DLCL2 cells [30, 41]. By comparison, etoposide increased Bax levels in leukemia cells [13, 14]. ABT-263 potently binds to Bcl-2 and Bcl-xL, inducing Bax translocation to the mitochondria, release of cytochrome *c* and initiation of caspase 3 cleavage [19]. We posit that by neutralizing Bcl-2 within these cells, ABT-263 effectively increases the Bax:Bcl-2 ratio and subsequently increases cell killing induced by these agents.

Similarly, drug resistance in hematologic tumors has been associated with overexpression of Bcl-2 and/or Bcl-xL. Pre-B leukemia cell line 697 forced to overexpress Bcl-2 became resistant to methotrexate, cytarabine, etoposide, vincristine and cisplatin [42]. Bcl-xL overexpression in FL5.12 cells conferred resistance to bleomycin, cisplatin, etoposide and vincristine [43]. Finally, multiple myeloma lines RPMI-8226, IM-9 and U266 treated with doxorubicin upregulated Bcl-2, and these cells were resistant to further treatment with both doxorubicin and etoposide [44]. Based on the work presented in this report, treatment with ABT-263 should be able to restore anti-tumor activity to these upregulated lines.

In conclusion, ABT-263 potentiates a variety of approved chemotherapeutic agents in vitro and in vivo. Synergy in vitro with bortezomib was associated with the induced expression of the Mcl-1 neutralizing proteins Noxa. These data support the use of ABT-263 in combination with commonly used therapeutic regimens in the treatment of hematologic tumors.

References

1. SEER Surveillance Epidemiology and End Results [homepage on the internet]. Bethesda: National Cancer Institute; c1973-2007 [updated 18 Dec 2007; cited 2 Jan 2008]. Cancer Stats Fact Sheet, Non-Hodgkin Lymphoma [top of page]. Available from: http://seer.cancer.gov/statfacts/html/nhl.html
2. Cvetkovic RS, Perry CM (2006) Rituximab: a review of its use in non-Hodgkin's lymphoma and chronic lymphocytic leukaemia. Drugs 66:791–820
3. Orlowski RZ, Kuhn DJ (2008) Proteasome inhibitors in cancer therapy: lessons from the first decade. Clin Cancer Res 14:1649–1657
4. Utecht KN, Kolesar J (2008) Bortezomib: a novel chemotherapeutic agent for hematologic malignancies. Am J Health Syst Pharm 65:1221–1231
5. Wei MC (2004) Bcl-2-related genes in lymphoid neoplasia. Int J Hematol 80:205–209
6. Reed JC (1997) Bcl-2 family proteins: regulators of apoptosis and chemoresistance in hematologic malignancies. Semin Hematol 34:9–19
7. Tsujimoto Y, Finger LR, Yunis J, Nowell PC, Croce CM (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science 226:1097–1099
8. Agarwal B, Naresh KN (2002) Bcl-2 family of proteins in indolent B-cell non-Hodgkin's lymphoma: study of 116 cases. Am J Hematol 70:278–282
9. Harada N, Hata H, Yoshida M et al (1998) Expression of Bcl-2 family of proteins in fresh myeloma cells. Leukemia 12:1817–1820
10. Alizadeh AA, Eisen MB, Davis RE et al (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature 403:503–511
11. Packham G, Stevenson FK (2005) Bodyguards and assassins: Bcl-2 family proteins and apoptosis control in chronic lymphocytic leukaemia. Immunology 114:441–449
12. Tu Y, Renner S, Xu F et al (1998) Bcl-X expression in multiple myeloma: possible indicator of chemoresistance. Cancer Res 58:256–262
13. Fukumi S, Horiguchi-Yamada J, Nakada S, Nagai M, Ohno T, Yamada H (2000) Differential responses of Bcl-2 family genes to etoposide in chronic myeloid leukemia K562 cells. Mol Cell Biochem 206:43–50
14. Floros KV, Thomadaki H, Florou D, Talieri M, Scorilas A (2006) Alterations in mRNA expression of apoptosis-related genes BCL2, BAX, FAS, caspase-3, and the novel member BCL2L12 after treatment of human leukemic cell line HL60 with the antineoplastic agent etoposide. Ann N Y Acad Sci 1090:89–97
15. Belhoussine R, Morjani H, Gillet R, Palissot V, Manfait M (1999) Two distinct modes of oncoprotein expression during apoptosis resistance in vincristine and daunorubicin multidrug-resistant HL60 cells. Adv Exp Med Biol 457:365–381
16. Liu FT, Agrawal SG, Gribben JG et al (2008) Bortezomib blocks Bax degradation in malignant B cells during treatment with TRAIL. Blood 111:2797–2805
17. Gomez-Bougie P, Wuilleme-Toumi S, Menoret E et al (2007) Noxa up-regulation and Mcl-1 cleavage are associated to apoptosis induction by bortezomib in multiple myeloma. Cancer Res 67:5418–5424
18. Byrd JC, Kitada S, Flinn IW et al (2002) The mechanism of tumor cell clearance by rituximab in vivo in patients with B-cell chronic lymphocytic leukemia: evidence of caspase activation and apoptosis induction. Blood 99:1038–1043
19. Tse C, Shoemaker AR, Adickes J et al (2008) ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. Cancer Res 68:3421–3428
20. Ackler S, Xiao Y, Mitten MJ et al (2008) ABT-263 and rapamycin act cooperatively to kill lymphoma cells in vitro and in vivo. Mol Cancer Ther 7:3265–3274
21. Lock R, Carol H, Houghton PJ et al (2008) Initial testing (stage 1) of the BH3 mimetic ABT-263 by the pediatric preclinical testing program. Pediatr Blood Cancer 50:1181–1189
22. Del Gaizo Moore V, Brown JR, Certo M, Love TM, Novina CD, Letai A (2007) Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. J Clin Invest 117:112–121
23. Paoluzzi L, Gonen M, Bhagat G et al (2008) The BH3-only mimetic ABT-737 synergizes the antineoplastic activity of proteasome inhibitors in lymphoid malignancies. Blood 112:2906–2916
24. Kang MH, Kang YH, Szymanska B et al (2007) Activity of vincristine, L-ASP, and dexamethasone against acute lymphoblastic leukemia is enhanced by the BH3-mimetic ABT-737 in vitro and in vivo. Blood 110:2057–2066
25. Mohammad RM, Wang S, Aboukameel A et al (2005) Preclinical studies of a nonpeptidic small-molecule inhibitor of Bcl-2 and Bcl-xL [(-)-gossypol] against diffuse large cell lymphoma. Mol Cancer Ther 4:13–21
26. Stel AJ, Ten Cate B, Jacobs S et al (2007) Fas receptor clustering and involvement of the death receptor pathway in rituximab-mediated apoptosis with concomitant sensitization of lymphoma B cells to fas-induced apoptosis. J Immunol 178:2287–2295
27. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22:27–55
28. Shoemaker AR, Oleksijew A, Bauch J et al (2006) A small-molecule inhibitor of Bcl-xL potentiates the activity of cytotoxic drugs in vitro and in vivo. Cancer Res 66:8731–8739
29. Sladek NE (1988) Metabolism of oxazaphosphorines. Pharmacol Ther 37:301–355
30. Mohammad RM, Al-Katib A, Aboukameel A, Doerge DR, Sarkar F, Kucuk O (2003) Genistein sensitizes diffuse large cell lymphoma to CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy. Mol Cancer Ther 2:1361–1368
31. Greco FA (1999) Oral etoposide in lymphoma. Drugs 58(Suppl 3):35–41
32. Tahir SK, Yang X, Anderson MG et al (2007) Influence of Bcl-2 family members on the cellular response of small-cell lung cancer cell lines to ABT-737. Cancer Res 67:1176–1183
33. Lin X, Morgan-Lappe S, Huang X et al (2007) 'Seed' analysis of off-target siRNAs reveals an essential role of Mcl-1 in resistance to the small-molecule Bcl-2/Bcl-xL inhibitor ABT-737. Oncogene 26:3972–3979
34. Konopleva M, Contractor R, Tsao T et al (2006) Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. Cancer Cell 10:375–388
35. van Delft MF, Wei AH, Mason KD et al (2006) The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell 10:389–399
36. Mandelin AM II, Pope RM (2007) Myeloid cell leukemia-1 as a therapeutic target. Expert Opin Ther Targets 11:363–373
37. Kutuk O, Arisan ED, Tezil T, Shoshan MC, Basaga H (2009) Cisplatin overcomes Bcl-2-mediated resistance to apoptosis via preferential engagement of Bak: critical role of Noxa-mediated lipid peroxidation. Carcinogenesis 30:1517–1527
38. Olejniczak SH, Hernandez-Ilizaliturri FJ, Clements JL, Czuczman MS (2008) Acquired resistance to rituximab is associated with chemotherapy resistance resulting from decreased Bax and Bak expression. Clin Cancer Res 14:1550–1560
39. Jazirehi AR, Vega MI, Bonavida B (2007) Development of rituximab-resistant lymphoma clones with altered cell signaling and cross-resistance to chemotherapy. Cancer Res 67:1270–1281

40. Wobser M, Voigt H, Eggert AO et al (2007) Bcl-2 expression in rituximab refractory cutaneous B-cell lymphoma. Br J Cancer 96:1540–1543
41. Mohammad RM, Wall NR, Dutcher JA, Al-Katib AM (2000) The addition of bryostatin 1 to cyclophosphamide, doxorubicin, vincristine, and prednisone (CHOP) chemotherapy improves response in a CHOP-resistant human diffuse large cell lymphoma xenograft model. Clin Cancer Res 6:4950–4956
42. Reed JC, Kitada S, Takayama S, Miyashita T (1994) Regulation of chemoresistance by the Bcl-2 oncoprotein in non-Hodgkin's lymphoma and lymphocytic leukemia cell lines. Ann Oncol 5(Suppl 1):61–65
43. Minn AJ, Rudin CM, Boise LH, Thompson CB (1995) Expression of Bcl-xL can confer a multidrug resistance phenotype. Blood 86:1903–1910
44. Tu Y, Xu FH, Liu J et al (1996) Upregulated expression of BCL-2 in multiple myeloma cells induced by exposure to doxorubicin, etoposide, and hydrogen peroxide. Blood 88:1805–1812