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The Bcl-2 Homology Domain 3 Mimetic ABT-737 Targets the Apoptotic Machinery in Acute Lymphoblastic Leukemia Resulting in Synergistic in Vitro and in Vivo Interactions with Established Drugs[S]

Laura M. High, Barbara Szymanska, Urszula Wilczynska-Kalak, Nicole Barber, Rosemary O'Brien, Seong Lin Khaw, Ingela B. Vikstrom, Andrew W. Roberts, and Richard B. Lock

Children's Cancer Institute Australia for Medical Research, University of New South Wales, Sydney, New South Wales, Australia (L.M.H., B.S., U.W-K., N.B., R.O., R.B.L.); and the Walter and Eliza Hall Institute, Parkville, Victoria, Australia (S.L.K., I.B.V., A.W.R.)

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ABSTRACT

Antiapoptotic Bcl-2 proteins are overexpressed in a number of cancers, including leukemias, and are frequently associated with resistance to conventional chemotherapeutic drugs. ABT-737, a Bcl-2 homology domain 3 mimetic (for structure, see *Nature* **435:** 677–681, 2005) inhibits the prosurvival function of Bcl-2, Bcl-X_L, and Bcl-w. We show that ABT-737 was effective as a single agent against a panel of pediatric acute lymphoblastic leukemia (ALL) xenografts, previously established, from patient biopsies, in immunodeficient mice. Although in vitro resistance of leukemia cell lines correlated with expression of the prosurvival protein Mcl-1, there was no relationship between Mcl-1 expression and in vivo xenograft response to ABT-737. However, expression of the proapoptotic protein Bim, and the extent of its association with Bcl-2, significantly correlated with in vivo ABT-737 sensitivity. ABT-737 potentiated the antileukemic effects of L-asparaginase, topotecan, vincristine, and etoposide against drug-resistant xenografts in vitro and in vivo. Finally, we show that the combination of L-asparaginase (by specifically down-regulating Mcl-1 protein levels), topotecan (by activating p53 via DNA damage), and ABT-737 (by inhibiting antiapoptotic Bcl-2 family members) caused profound synergistic antileukemic efficacy both in vitro and in vivo. Rational targeting of specific components of the apoptotic pathway may be a useful approach to improve the treatment of refractory or relapsed pediatric ALL. Overall, this study supports the inclusion of the clinical derivative of ABT-737, ABT-263 (for structure, see *Cancer Res* **68:**3421–3428, 2008), into clinical trials against relapsed/refractory pediatric ALL.

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The introduction of combination chemotherapy regimens for childhood ALL, along with advances in supportive care, have dramatically improved survival in this disease to a rate now approaching 80% in developed countries (Pui and Evans, 2006). Despite this success, the overall survival of the 15 to 20% of patients who relapse is poor, and most patients succumb to their disease (Bailey et al., 2008). Relapse is frequently associated with acquired resistance to central components of induction therapy protocols, including glucocorticoids and L-asparaginase (L-asp) (Bailey et al., 2008).

ABBREVIATIONS: 4-HPR, fenretinide; A1, Bcl-2-related protein A1a; ALL, acute lymphoblastic leukemia; Bad, Bcl-2-associated death promoter; Bak, Bcl-2 homologous antagonist/killer; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma-leukemia gene 2; BCP, B-cell precursor; BH, Bcl-2 homology; BH3, Bcl-2 homology domain 3; Bid, BH3-interacting domain death agonist; Bik, Bcl-2-interacting killer; Bim, Bcl-2 interacting mediator of cell death; Bmf, Bcl-2 modifying factor; CI, combination index; DEX, dexamethasone; EFS, event-free survival; ETO, etoposide; ETO, etoposide; FBS, fetal bovine serum; Hrk, harakiri; huCD45$^+$, human CD45-positive; L-asp, L-asparaginase; %huCD45$^+$, proportion of huCD45$^+$ cells versus total murine CD45$^+$ and huCD45$^+$ cells in the murine peripheral blood; LGD, leukemia growth delay; Mcl-1, myeloid cell leukemia sequence 1; MTD, maximal tolerated dose; MTP, mitochondrial transmembrane potential; MT-PBS, mouse tonicity-phosphate-buffered saline; NOD, nonobese diabetic; PI, propidium iodide; pNA, *para*-nitroaniline; PS, phosphatidylserine; Puma, p53 up-regulated modulator of apoptosis; SCID, severe combined immunodeficient; TPT, topotecan; VCR, vincristine; z-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp fluoromethyl ketone.

The majority of conventional cytotoxic agents indirectly induce apoptosis through DNA damage and cell cycle arrest. However, malignant cells frequently acquire defects, including oncogene activation and deregulation of apoptotic signaling pathways, thereby allowing them to evade apoptosis (Hanahan and Weinberg, 2000). For these reasons, and the high levels of toxicity frequently observed with traditional treatment, recent approaches to cancer therapy have focused on targeting key components of pathways shown to be fundamental to tumor survival and disease progression (Dai and Grant, 2007). This approach is intended to circumvent acquired drug resistance pathways and resensitize the malignant cell to apoptosis.

The Bcl-2 family of proteins consists of central regulators of apoptosis, and cell survival is determined by the interaction and balance between proapoptotic and antiapoptotic family members (Adams and Cory, 1998). The Bcl-2 family consists of at least 20 proteins, each of which contains at least one of the four conserved Bcl-2 homology (BH) domains, and is divided into three subclasses. Multidomain proapoptotic proteins Bax and Bak are essential for apoptosis, and they oligomerize at the mitochondria to disrupt the outer mitochondrial membrane and facilitate the release of proapoptotic proteins, including cytochrome *c* (Adams and Cory, 1998). Antiapoptotic family members (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and A1) maintain outer mitochondrial membrane integrity by suppressing the function of Bax and Bak (Zhou et al., 1997). Another subclass of the Bcl-2 family (including Bim, Bid, Bad, Hrk, Bik, Bmf, Puma, and Noxa) are referred to as "BH3-only" proteins and share only the BH3 domain with other family members (Huang and Strasser, 2000). There are two proposed mechanisms by which BH3-only proteins function. The "indirect" model proposes that the BH3 family of proteins unleash Bax and Bak suppression by prosurvival Bcl-2 family proteins (Willis et al., 2007). Alternatively, the "direct" action model suggests that Bid and Bim can also interact with proapoptotic Bax and Bak, inducing their oligomerization and subsequent apoptosis (Letai et al., 2002).

An imbalance of pro- and antiapoptotic Bcl-2 family proteins is a common feature of malignancy, including ALL, and can render tumor cells refractory to chemotherapy (Campana et al., 1993). The ability of prosurvival members of the Bcl-2 family to facilitate evasion of cell death signals has made them attractive targets for cancer drug discovery (Zhang et al., 2007). A number of small-molecule inhibitors of prosurvival Bcl-2 family members are at various stages of preclinical and clinical development (Becattini et al., 2004; Oltersdorf et al., 2005). ABT-737 and the closely related orally available homolog ABT-263 have shown potent single-agent in vitro and in vivo activity against cancer cell lines and primary cells, including ALL (Oltersdorf et al., 2005; Del Gaizo Moore et al., 2008; Lock et al., 2008). Moreover, both compounds significantly potentiate the efficacy of established and novel chemotherapeutic drugs, indicating a high priority for clinical trials using novel drug combinations (Kang et al., 2007; Kuroda et al., 2008). ABT-737 exhibits low-affinity binding to the antiapoptotic Mcl-1 and A1 proteins, and resistance to ABT-737 in cancer cells lines has been attributed to high levels of Mcl-1 and A1 expression (Deng et al., 2007; Lin et al., 2007). Nevertheless, the determinants of in vivo sensitivity to ABT-737/263 remain poorly understood.

In this study, we examined the in vitro ABT-737 sensitivity of a panel of leukemia cell lines and the in vivo and ex vivo sensitivity of a panel of B-cell precursor ALL (BCP-ALL) xenografts established in nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice directly from patient explants (Liem et al., 2004), in relation to Bcl-2 family protein expression. Although Mcl-1 expression is significantly correlated with ABT-737 sensitivity in leukemia cell lines, Bim protein levels seemed the most important determinant of in vivo ABT-737 sensitivity in BCP-ALL xenografts. Moreover, ABT-737 showed broad ex vivo and in vivo synergy with established chemotherapeutic drugs used to treat pediatric ALL, indicating that rational targeting of components of the apoptotic machinery may be an effective approach to salvage relapsed patients.

Materials and Methods

In Vitro Cell Culture. Jurkat, REH, and HeLa cell lines were obtained from the American Type Culture Collection (Manassas, VA), and Hal-01 and Raji cell lines were kindly provided by Dr A. Thomas Look (Dana-Farber Cancer Institute, Boston, MA) and Professor Richard Christopherson (School of Molecular and Microbial Biosciences, University of Sydney), respectively. CEM, Nalm-6, Molt-4, K562, and HL-60 cells used in the study were laboratory stock cell lines. Cell lines were maintained in static suspension culture in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM).

Procedures by which we previously established continuous xenografts from childhood ALL biopsies in immune-deficient NOD/SCID (NOD/LtSz-scid/scid) mice are described in detail elsewhere (Liem et al., 2004). Xenograft characteristics are presented in Table 1. For all ex vivo experiments, xenograft cells were retrieved from cryostorage and resuspended in QBSF-60 medium (Quality Biological, Gaithersburg, MD) supplemented with Flt-3 ligand (20 ng/ml; a gift kindly provided by Amgen, Thousand Oaks, CA), penicillin (100 U/ml), streptomycin (100 μg/ml), and L-glutamine (2 mM). Viability was determined by exclusion of 0.2% trypan blue. For cytotoxicity experiments, cells were equilibrated in medium in a humidified atmosphere overnight at 37°C, 5% CO_2 before drug treatment. An equivalent volume of an appropriate vehicle control was added to control cells. Cells were harvested by centrifugation at 490*g* for 10 min and washed twice with PBS.

In some experiments, xenograft cells were cocultured on a confluent layer of murine MS-5 stromal cells overnight and then treated with 10^{-12} to 10^{-6} M ABT-737 for up to 48 h. Before harvesting, 2×10^4 10-μm latex beads (Beckman Coulter, Fullerton, CA) were added to each well. Each sample was stained with allophycocyanin-conjugated anti-human CD45 antibody (BD Biosciences, San Jose, CA), washed, and resuspended in flow buffer containing 5 μg/ml propidium iodide (PI; Sigma Aldrich, Castle Hill, NSW, Australia). Using CellQuest software, viable human leukocytes were enumerated using a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Diego, CA) and quantified with reference to the bead control, as described previously (Liem et al., 2004).

In vitro cytotoxicity assays using primary murine lymphoid cells. Femurs and tibias were harvested from multiple wild-type, Bim(−/−), and Puma(−/−) C57BL/6 mice. Generation of the knockout mice has been described previously (Michalak et al., 2009). Marrow was flushed from the bones with MT-PBS/2% FBS. Tissue from syngeneic mice was pooled, pelleted, subjected to red cell lysis, and then washed and resuspended in MT-PBS/2% FBS, and filtered through a nylon mesh. Small aliquots of one sample were removed and stained with either anti-B220-5,6-carboxyfluorescein (clone RA3-6B2) or anti-IgM-phycoerythrin (clone 331.12)

to serve as controls for fluorescence compensation during flow cytometry. Both antibodies were grown and conjugated in-house. The remaining cells were stained with a mixture containing the same antibodies. After incubation on ice for 15 min, the cells were washed with MT-PBS/2% FBS, pelleted and resuspended at 30×10^6 cells/ml in MT-PBS/2% FBS containing PI (5 μg/ml). Using a FACSAria cell sorter (BD Biosciences), pro- and pre-B cells ($B220^+IgM^-$) were collected into sterile tubes containing B-cell media (RPMI with 5% FBS and 0.1% 2-β-mercaptoethanol) supplemented with 50% FBS. The cells were then pelleted, resuspended in B cell media at 10^6 cells/ml, and incubated in a 96-well plate with concentrations of ABT-737 ranging from 10^{-9} to 10^{-6} M, in a humidified atmosphere with 10% CO_2 at 37°C for 24 h. Cell viability was quantified using PI staining as described above.

MTT Colorimetric Assay. Procedures by which leukemia cell lines and xenograft cells were assessed for ABT-737 sensitivity by MTT assay have been described in detail previously (Bachmann et al., 2007). Cell survival was expressed as a percentage of solvent-treated controls. For combination cytotoxicity experiments, cells were exposed to fixed-ratios of drugs around the IC_{50} value (0.25, 0.5, 1, 2, and 4 times the IC_{50} value). After a 48-h drug exposure, the fraction of cells affected by each drug and the combination was calculated. The nature of interactions between drugs was assessed by calculating a combination index (CI) using the method described by Chou et al. (1994) with CalcuSyn software (Biosoft, Ferguson, MO). With this method, a CI $<$ 0.1 indicates very strong synergism, 0.1 to 0.3 strong synergism, 0.3 to 0.7 synergism, 0.7 to 0.85 moderate synergism, 0.85 to 0.9 slight synergism, 0.9 to 1.1 nearly additive, 1.1 to 1.2 slight antagonism, 1.2 to 1.45 moderate antagonism, 1.45 to 3.3 antagonism, 3.3 to 10 strong antagonism, and $>$10 very strong antagonism. The following drugs were used: dexamethasone (DEX), vincristine (VCR), etoposide (ETO), Nutlin-3 (Sigma-Aldrich), L-asp (Leunase; Aventis, Lane Cove, NSW, Australia), topotecan (TPT; Hycamtin, GlaxoSmithKline Australia, Pty. Ltd., Boronia, VIC, Australia), fenretinide (4-HPR; Avanti Polar Lipids, AL), and ABT-737 (kindly provided by Abbott Laboratories, Abbott Park, IL).

Apoptosis Assays. Mitochondrial transmembrane potential (MTP, $\psi\Delta$) was measured using the MitoProbe JC-1 assay kit for flow cytometry (Molecular Probes, Eugene, OR). In brief, xenograft cells were cocultured on a confluent layer of MS-5 stromal cells overnight, as described above, before treatment with 100 nM ABT-737 for up to 48 h. Cells were harvested and stained with JC-1 and anti-human CD45 antibody or PI. The percentage of cells with loss of MTP or viability was measured using a FACSCalibur flow cytometer.

Caspase activity was measured using the *para*-nitroaniline (pNA) Caspase-3 Colorimetric Assay (R&D Systems, Minneapolis, MN). Xenograft cells were treated with 100 nM ABT-737 for up to 48 h. In some experiments, cells were treated for 16 h with 75 μM z-VAD-fmk, a pan-caspase inhibitor (R&D Systems) before ABT-737 exposure. Cells were harvested and their viability assessed using 0.2% trypan blue exclusion. Cells were lysed according to the manufacturer's instructions and protein concentration was quantified using the bicinchoninic acid assay (Pierce, Rockford, IL). The enzymatic reaction for caspase activity was performed according to the manufacturer's instructions and was expressed relative to vehicle-treated controls with reference to a pNA standard curve.

Plasma membrane externalization of phosphatidylserine (PS) was visualized by Annexin-V-fluorescein isothiocyanate (BD Biosciences Pharmingen, San Diego, CA) binding using standard flow cytometric methods. Cells were gated as early apoptotic ($Annexin\text{-}V^+/PI^-$) or late apoptotic/necrotic ($Annexin\text{-}V^+/PI^+$).

Protein Analysis Methods. Methods for the preparation of whole-cell extracts, determination of protein concentrations, and analysis of cellular proteins by immunoblotting have been described in detail elsewhere (Bachmann et al., 2007). Polyclonal or monoclonal antibodies specific for the following proteins were used: Bcl-2, Bcl-X_L, Bak, Bax (BD Biosciences Pharmingen); Bcl-w (clone 16H12; Millipore, Billerica, MA); Mcl-1 (clone RC13) and p53 (clone DO-1) (Santa Cruz Biotechnology, Santa Cruz, CA); Puma, Bim, Actin (Sigma-Aldrich); Noxa (clone 114C307.1; Imgenex, San Diego, CA) and A1 (clone 51B2). Secondary antibodies used were horseradish peroxidase conjugates of either anti-mouse, -rabbit, or -rat IgG (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

For immunoprecipitation, lysates (200 μg of protein per reaction) were incubated with 3 μg of anti-hamster Bcl-2 antibody (BD Biosciences) at 4°C with rotation for a minimum of 5 h, followed by the addition of 50 μl of 50% (v/v) protein A Sepharose 4 Fast Flow beads (GE Healthcare) and kept at 4°C with rotation overnight. The beads were washed four to five times with 1 ml of lysis buffer and pelleted (12,000*g*, 5 min). Bound proteins were eluted by heating at 70°C for 10 min in SDS loading buffer. Eluates were fractionated by SDS-polyacrylamide gel electrophoresis (Invitrogen), transferred to nitrocellulose membranes (Millipore), and immunoblotted as described above.

TABLE 1
In vitro responses of leukemia cell lines and ex vivo and in vivo responses of pediatric ALL xenografts to ABT-737

Designation	Model Type	Leukemia Subtype	Cytogenetics of Original Patient Biopsy	Ex Vivo/In Vitro (IC_{50})	In Vivo (25 mg/kg)		In Vivo (60 mg/kg)	
					LGD	*P* vs. Control	LGD	*P* vs. Control
ALL-2	Xenograft	c-ALL	46, XX, NAD	9.8 nM	2.7	0.4045	15.1	0.0059
ALL-3	Xenograft	Pre-B	46, XX, del(11;q23)	3.4 nM	27.4	<0.0001	30.7	<0.0001
ALL-4	Xenograft	Ph^+-ALL	9q34(abl x2),22q11 (bcr x2)(abl con bcr x1)/9q34(abl x2),22q11(bcr x2)	16 nM	0.2	0.8216	12.0	0.0006
ALL-7	Xenograft	Biphen	46, XY, t(17;19)(q21;q13)	1.3 nM	20.6	<0.0001	23.7	0.0001
ALL-10	Xenograft	c-ALL	47, XY, +mar(12)/46, XY (13)	1.0 nM	12.9	<0.0001	24.8	<0.0001
ALL-11	Xenograft	c-ALL	46, XX, del(12p13), del(13q12) (8)/46, XX	45 nM	6.7	0.0257	29.4	0.0006
ALL-17	Xenograft	c-ALL	N.D.	3.7 nM	13.9	0.0002	14.3	0.0734
ALL-18	Xenograft	c-ALL	N.D.	3.0 nM	N.D.	N.D.	N.D.	N.D.
ALL-19	Xenograft	c-ALL	46, XY, NAD	5.5 nM	0.2	0.9175	14.5	0.0001
HL-60	Cell Line	AML	N.D.	>10 μM	N.D.	N.D.	N.D.	N.D.
K562	Cell Line	CML	N.D.	>10 μM	N.D.	N.D.	N.D.	N.D.
Nalm6	Cell Line	Pre-B	N.D.	>10 μM	N.D.	N.D.	N.D.	N.D.
Jurkat	Cell Line	T-ALL	N.D.	5 μM	N.D.	N.D.	N.D.	N.D.
Molt4	Cell Line	T-ALL	N.D.	918 nM	N.D.	N.D.	N.D.	N.D.
REH	Cell Line	Pre-B	N.D.	677 nM	N.D.	N.D.	N.D.	N.D.
CEM	Cell Line	T-ALL	N.D.	303 nM	N.D.	N.D.	N.D.	N.D.
Hal-01	Cell Line	Pro-B	N.D.	192 nM	N.D.	N.D.	N.D.	N.D.

AML, acute myelogenous leukemia; Biphen-ALL, biphenotypic ALL; CML, chronic myelogenous leukemia; c-ALL, common ($CD10^+$) pre-B ALL; NAD, no abnormality detected; N.D., not done; Ph^+-ALL, Philadelphia chromosome-positive ALL; Pre-B, B-cell precursor ALL.

Results were visualized by autoradiography, and signals were quantified by filmless autoradiographic analysis using a VersaDoc 5000 Imaging System. Data were analyzed using QuantityOne software (version 4.0; Bio-Rad Laboratories, Hercules, CA). A positive control cell lysate included in each gel was used to normalize between blots. Pearson correlations were used to compare protein levels with in vitro sensitivity of the cell lines, and in vivo sensitivity of xenografts, to ABT-737.

In vivo efficacy of ABT-737 against pediatric ALL xenografts. Procedures by which we established xenografts from pediatric ALL biopsy specimens in NOD/SCID mice and assessed their in vivo drug sensitivity have been described in detail elsewhere (Liem et al., 2004). In brief, groups of eight NOD/SCID mice were inoculated with 3 to 5×10^6 human leukemia cells previously harvested from the spleens of engrafted mice. Engraftment and response to drug treatment was assessed by flow cytometric quantification of the proportion of human CD45-positive (huCD45$^+$) cells versus total murine CD45$^+$ and huCD45$^+$ cells in the murine peripheral blood (%huCD45$^+$ cells). When the %huCD45$^+$ cells reached 1%, mice were randomized to receive drug or vehicle control treatments. All drug administration was intraperitoneal and consisted of TPT, L-asp, or ABT-737 on Monday through Friday for 4 weeks; ETO (Pfizer, West Ryde, NSW, Australia) Monday to Friday every 2 weeks, VCR every 7 days for 4 weeks. The %huCD45$^+$ cells were monitored during and after the course of treatment. Mouse event-free survival (EFS) was calculated as the number of days from randomization (-6 days from treatment initiation) until the %huCD45$^+$ cells reached 25%. Mouse EFS was graphically represented by Kaplan-Meier analysis (Kaplan, 1958) and survival curves were compared by logrank test. For comparisons between xenografts and drug treatments, the median EFS for control mice was subtracted from the median EFS for drug-treated mice to generate a leukemia growth delay (LGD). Mice were also monitored closely for signs of drug-related toxicity (weight loss, lethargy, ruffled fur, etc.) and euthanized at the first indication of morbidity. Mice were excluded from the group if they developed spontaneous murine thymic lymphomas. All experimental studies had prior approval from the Animal Care and Ethics Committee of the University of New South Wales.

Results

ALL Cell Lines and Xenografts Exhibit Variable Sensitivity to ABT-737 In Vitro and In Vivo. We first compared the in vitro cytotoxic effects of ABT-737 against a panel of eight leukemia cell lines and ex vivo cultures from nine ALL xenografts. The cell-line panel exhibited heterogeneous sensitivity to ABT-737; IC_{50} values ranged from 192 nM (the pre-B cell line Hal-01) to >10 μM (Nalm-6, K562, and HL-60; Fig. 1A and Table 1). To validate the results obtained using the MTT cytotoxicity assay, the viability of two cell lines (Nalm-6 and Jurkat) exposed to increasing concentrations of ABT-737 was assessed using the trypan blue exclusion assay. The results were comparable with those reported in Table 1, with IC_{50} values of >10 and 3.6 μM for Nalm-6 and Jurkat cell lines, respectively (data not shown). There was no apparent lineage-specific relationship with ABT-737 sensitivity; a range of IC_{50} values were observed over the cell lines tested. In contrast to the cell lines, all nine ALL xenografts were acutely sensitive to ABT-737 ex vivo (Fig. 1A), IC_{50} values ranging from 1 to 45 nM (Table 1). The median IC_{50} of the xenograft panel (3.7 nM) was 810-fold less than that of the panel of cell lines (3.0 μM; Table 1).

ABT-737 has demonstrated single agent in vivo activity against various human solid tumor xenograft models and murine malignancies (Oltersdorf et al., 2005; Trudel et al., 2007). We therefore next assessed the in vivo efficacy of ABT-737 against a panel of eight human BCP-ALL xenografts derived from patients who exhibited diverse clinical outcomes and which manifest as a systemic disease in NOD/SCID mice. At a dose of 25 mg/kg [approximately 25% of the maximal tolerated dose (MTD)] ABT-737 significantly delayed the progression of five of eight xenografts (ALL-3, -7, -10, -11, and -17) by 7 to 27 days compared with control animals (Fig. 1, B and C, and Table 1). At a higher dose (60 mg/kg), ABT-737 delayed the progression of all xenografts by 12 to 31 days (Table 1). The median and ranges of EFS values of treated and control mice for all xenografts are shown in Supplemental Table 1. The response to ABT-737 was dose-dependent (Fig. 1D), albeit marginally, for ALL-3, -7, and -17.

Because we have shown above that ALL xenograft cells are sensitive to ABT-737 both ex vivo and in vivo, we tested whether ex vivo culture conditions that mimic the bone marrow microenvironment (low oxygen or stromal coculture) affected sensitivity to ABT-737. These conditions have previously been shown to alter sensitivity of leukemia cells to chemotherapeutic drugs (Yang et al., 2006). Although low oxygen decreased the sensitivity of Molt-4 cells to 4-HPR, it had no effect on the sensitivity of ALL-3 or ALL-18 to ABT-737 (Fig. 2A). Furthermore, the sensitivity of ALL-3 to DEX was substantially decreased by stromal coculture, although stromal coculture had no effect on the sensitivity of ALL-3 and ALL-18 to ABT-737 (Fig. 2B). These results may explain in part why the exquisite ex vivo sensitivity of these cells is also reflected in vivo.

Consistent with their acute sensitivity to ABT-737, ex vivo cultured ALL xenograft cells underwent rapid loss of MTP and viability (Fig. 2C) and activation of effector caspases-3/7 upon exposure to ABT-737 (Fig. 2D, left). Pre-exposure of ALL-3 cells to z-VAD-fmk prevented caspase activation, substantially inhibited ABT-737-induced PS externalization, and delayed the loss of cell viability (Fig. 2D, right), confirming the importance of the intrinsic apoptotic pathway in ABT-737-induced death of ALL cells.

Bim Is an Important Determinant of ABT-737 Sensitivity. Previous studies have shown that high Bcl-2 or low Mcl-1 expression levels correlate with increased in vitro sensitivity of cancer cell lines to ABT-737 (van Delft et al., 2006; Lin et al., 2007; Tahir et al., 2007). Consistent with these findings, Mcl-1 (but not Bcl-2) protein expression levels significantly ($P = 0.017$; $R^2 = 0.639$) correlated with the in vitro ABT-737 sensitivity of the panel of eight leukemia cell lines (Fig. 3A, left). Strangely enough, high levels of Noxa ($P = 0.048$, $R^2 = 0.505$) and Bim ($P = 0.013$, $R^2 = 0.673$) protein expression also correlated with in vitro resistance of these cell lines. For original immunoblots, see Supplemental Fig. 1A. Of critical clinical importance is to identify biomarkers that predict in vivo sensitivity of cancer cells to novel chemotherapeutic drugs. In this regard, a panel of eight xenografts established from direct explants of ALL biopsy material represents a useful experimental model. In contrast to the panel of cell lines, no relationship was apparent between the expression levels of antiapoptotic Bcl-2 protein family members, including Mcl-1, and the in vivo sensitivity of ALL xenografts to ABT-737 (Fig. 3A, right). It is noteworthy that only high Bim expression significantly correlated with in vivo sensitivity to ABT-737 ($P = 0.007$; $R^2 = 0.619$ at 25 mg/kg, $P = 0.1385$; $R^2 = 0.253$ at 60 mg/kg; $P = 0.025$, $R^2 = 0.489$

versus the sum of LGDs at 25 and 60 mg/kg doses). For original immunoblots, see Supplemental Fig. 1B. In addition, the amount of Bim protein that was associated ($P = 0.02$, $R^2 = 0.62$) or not associated ($P = 0.048$, $R^2 = 0.51$) with Bcl-2 significantly correlated with in vivo ABT-737 sensitivity at 25 mg/kg (Fig. 3B). To further explore the role of Bim in ABT-737

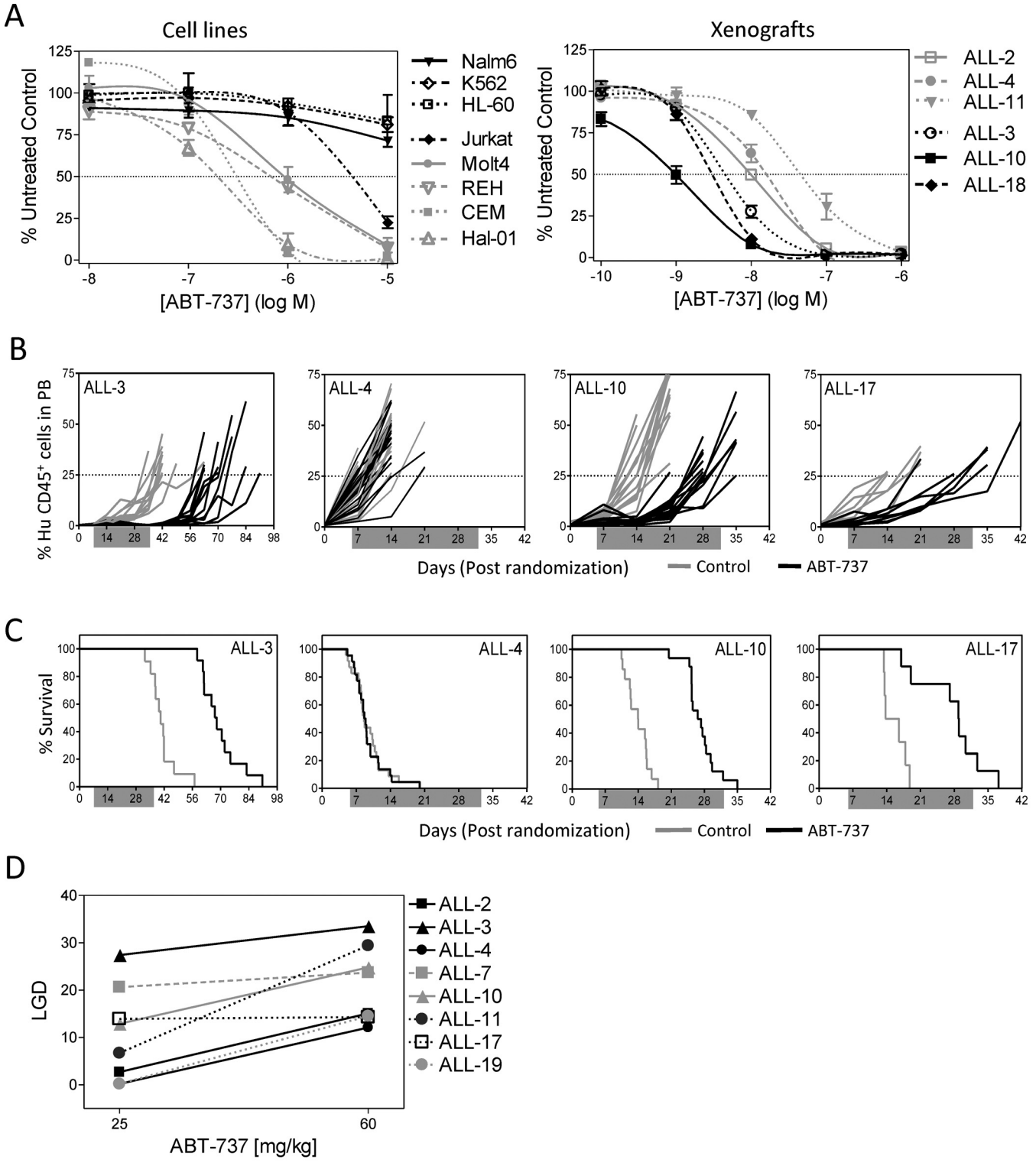

Fig. 1. In vitro and in vivo sensitivity of leukemia cell lines and ALL xenografts to ABT-737. A, the sensitivity of leukemia cell lines in continuous culture (left) or ALL xenograft cells retrieved from cryostorage (right) to ABT-737 was assessed by MTT assay after a 48-h drug exposure. Data represents the mean ± S.E.M. of three separate experiments. B, the responses of four representative xenografts treated with ABT-737 (25 mg/kg, Mon–Fri × 4 weeks; black) or vehicle control (gray) are illustrated as a percentage of human CD45$^+$ cells in the peripheral blood (PB) of individual mice over time or the proportion remaining event-free (C). Shaded areas correspond to the duration of treatment. D, the leukemia growth delay (LGD) of eight xenografts in response to ABT-737 at two doses, 25 and 60 mg/kg; see Table 1 and Supplemental Table 1 for all values.

sensitivity of normal lymphocytes, ex vivo cultured pro- and pre-B lymphocytes from Bim(−/−) mice were found to be relatively resistant to ABT-737 compared with those from wild-type Puma(−/−) mice (Fig. 3C) and xenografts.

In an effort to understand the diversity in ABT-737 responses between leukemia cell lines and xenograft cells, we directly compared protein expression levels of Bcl-2 family members, which were all normalized to a common control cell

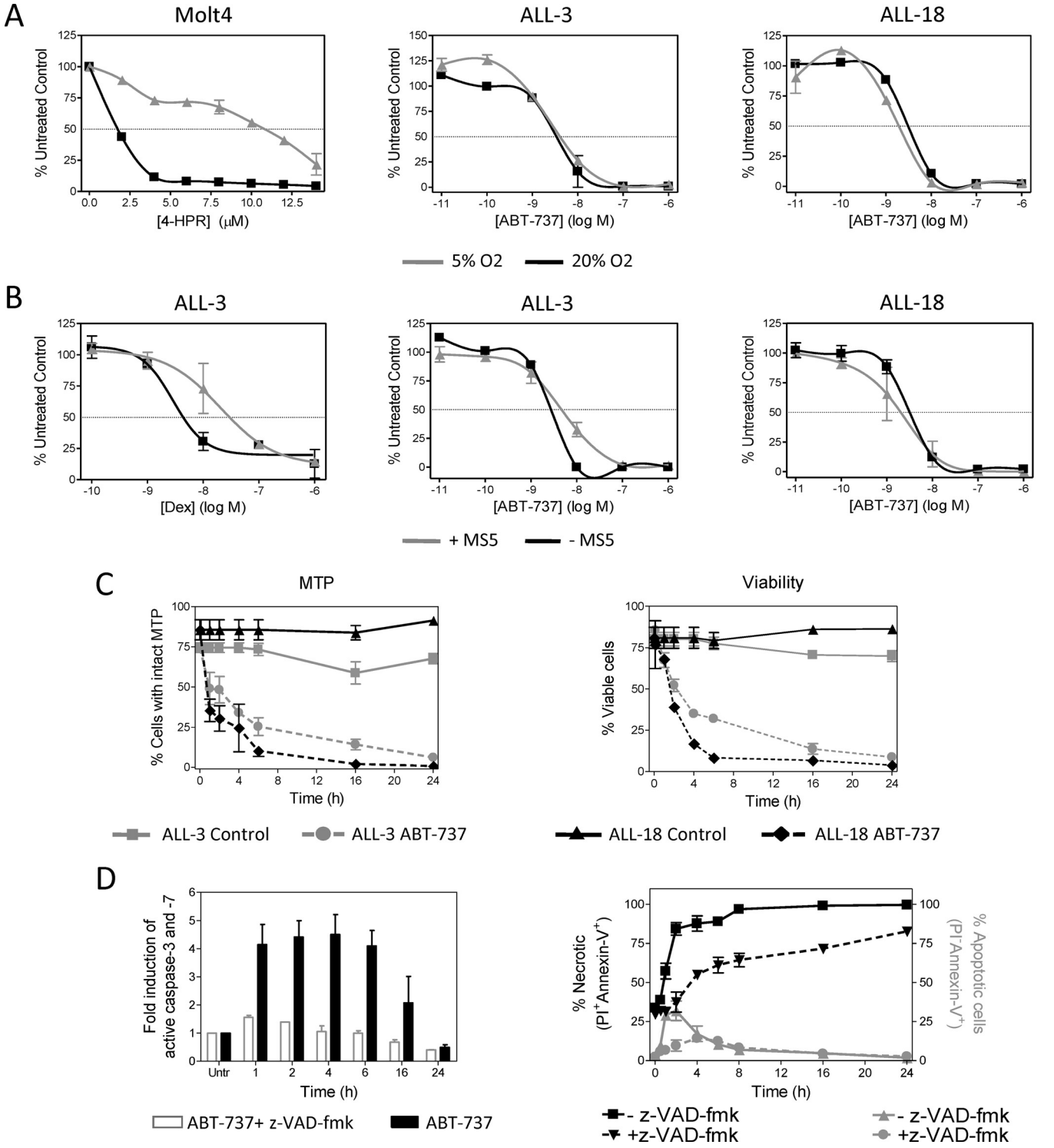

Fig. 2. In vitro effects of ABT-737 in ALL xenograft cells. A, the effect of low oxygen (5%) conditions on Molt-4 cells treated with 4-HPR (left) and ALL-3 or ALL-18 treated with ABT-737 (middle and right, respectively). B, effect of MS5 support layer on ALL-3 treated with DEX (left) and ALL-3 or ALL-18 treated with ABT-737 (middle and right, respectively). Experiments in A and B were assessed by MTT cytotoxicity assay. C, the loss of MTP, measured by JC-1 staining (left), and cell viability, measured by PI staining (right), was determined after exposure to 100 nM ABT-737 in ALL-3 and -18. D, left, caspase activity, measured by DEVD-pNA cleavage in ALL-3 treated with ABT-737 over 24 h with or without pre-exposure to z-VAD-fmk. D, right, PS externalization and cell viability measured by Annexin V and PI staining in ALL-3 cells treated with ABT-737 over 24 h with or without pre-exposure to z-VAD-fmk. Data represent the mean ± S.E.M. of a minimum of two independent experiments.

lysate that was included in each gel. Although leukemia cell lines expressed significantly higher levels of Bcl-w and Bcl-X_L and higher levels of Mcl-1 that approached significance, xenograft cells expressed higher levels of Bcl-2 (Fig. 3D, top row). Moreover, the three cell lines that were most sensitive to ABT-737 (REH, CEM, and Hal-01) expressed levels of Mcl-1 that were comparable with those in xenograft cells (Fig. 3D). In terms of pro-apoptotic proteins, the cell lines expressed significantly higher levels of Puma, Bim, and Bak, but lower levels of Bax, than xenograft cells (Fig. 3D, bottom row). Except for Bcl-2, relative expression levels of Bcl-2 family members were less variable across the panel of nine xenografts compared with the eight leukemia cell lines.

Overall, these results indicate a role for Bim in the in vitro and in vivo sensitivity of normal and malignant preB lymphocytes to ABT-737. They also highlight fundamental differences in expression of Bcl-2 family proteins between autonomously dividing cell lines and ALL xenografts established from direct explants, which may partly explain the divergence in their sensitivity to ABT-737.

Synergistic Interactions between ABT-737 and Chemotherapeutic Drugs against Pediatric ALL. ABT-737 augments the activity of established drugs against cancer cell lines (Chen et al., 2007; Tahir et al., 2007), including the in vivo efficacy of a three-drug regimen (VCR, DEX, and L-asp) against pediatric ALL xenografts (Kang et al., 2007). We reasoned that it would be possible to use this xenograft model to rationally design effective combination regimens between ABT-737 and drugs known to be active in the treatment of pediatric ALL, which could be rapidly translated to the clinic. To develop this paradigm, we selected an aggressive xenograft derived from a child at early relapse (ALL-19), which

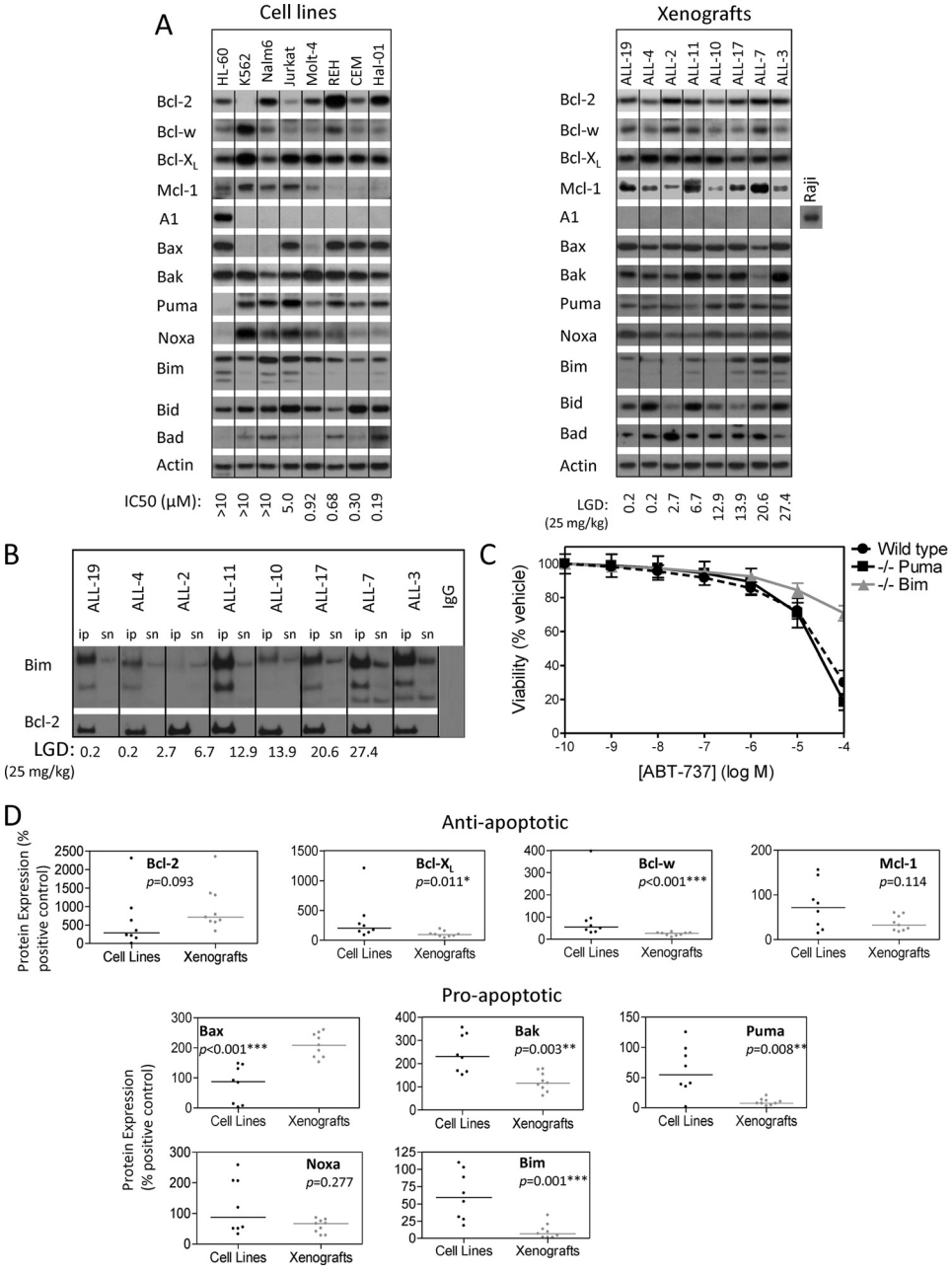

Fig. 3. Relationship between levels of Bcl-2 family protein expression and sensitivity of leukemia cell lines, ALL xenografts, and murine pro/preB lymphocytes to ABT-737. A, basal protein expression of Bcl-2 family members was analyzed by immunoblotting in a panel of eight leukemia cell lines arranged in order of increasing in vitro sensitivity to ABT-737 (left), and eight xenografts arranged in order of increasing in vivo sensitivity to ABT-737 at 25 mg/kg (right). B, immunoprecipitation (ip) of Bcl-2, showing bound Bim in ip fraction and unbound in supernatant (sn), of eight xenografts arranged in order of increasing in vivo sensitivity to ABT-737 at 25 mg/kg. C, in vitro sensitivity of pro- and pre-B lymphocytes isolated from Bim(−/−), Puma(−/−), and wild-type mice treated with increased concentrations of ABT-737. D, the basal levels of anti- (top) and proapoptotic (bottom) proteins in ALL xenografts compared with leukemia cell lines. Each data point represents a single xenograft and is the mean of three separate experiments. *, $p < 0.1$; **, $p < 0.01$; ***, $p \leq 0.001$. Data presented in A and B are representative of a minimum of two separate experiments, whereas data points in C represent the mean ± S.E.M. of three separate experiments.

was previously shown to exhibit relative resistance to VCR and DEX in vivo (Liem et al., 2004). Using fixed-ratio combination ex vivo cytotoxicity assays, ABT-737 exerted strong synergy (average CI ≤0.3) with L-asp (Fig. 4A, left, and Supplemental Table 2), and synergy (average CI ≤0.7) with TPT, VCR, and ETO (Fig. 4, B–D, left panels, and Supplemental Table 2). It is noteworthy that the ex vivo synergy between ABT-737 and these four established drugs was reflected in vivo (Fig. 4, middle and right panels, and Supplemental Table 3). Although ABT-737 at a dose of 25 mg/kg produced little or no delay in the progression of ALL-19, the combination with L-asp resulted in a delay that was >18 days greater than the sum of effects of the individual drugs. Likewise, ABT-737 enhanced the antileukemic efficacy of TPT, VCR, and ETO by >26 days, >16 days, and >4 days, respectively. Thus, ABT-737 broadly augments the efficacy of established chemotherapeutic drugs against pediatric ALL in vivo.

The most stringent definition of therapeutic synergy is "a therapeutic effect achieved with a tolerated regimen of a combination treatment that exceeds the optimal effect achieved at any tolerated dose of monotherapy associated with the same drugs used in the combination" (Rose and Wild, 2004). When ABT-737 was combined with L-asp or TPT, at the respective MTDs of each of the two-drug combinations, the effects were significantly greater than single-agent L-asp ($P = 0.0023$) or TPT ($P = 0.0001$) alone at their respective MTDs (Fig. 5A and Supplemental Table 4). In the case of the TPT/ABT-737 combination, the effects were significantly greater than ABT-737 alone at its MTD ($P = 0.0001$), whereas the L-asp/ABT-737 combination was equivalent to single-agent ABT-737 at its MTD (Fig. 5A and Supplemental

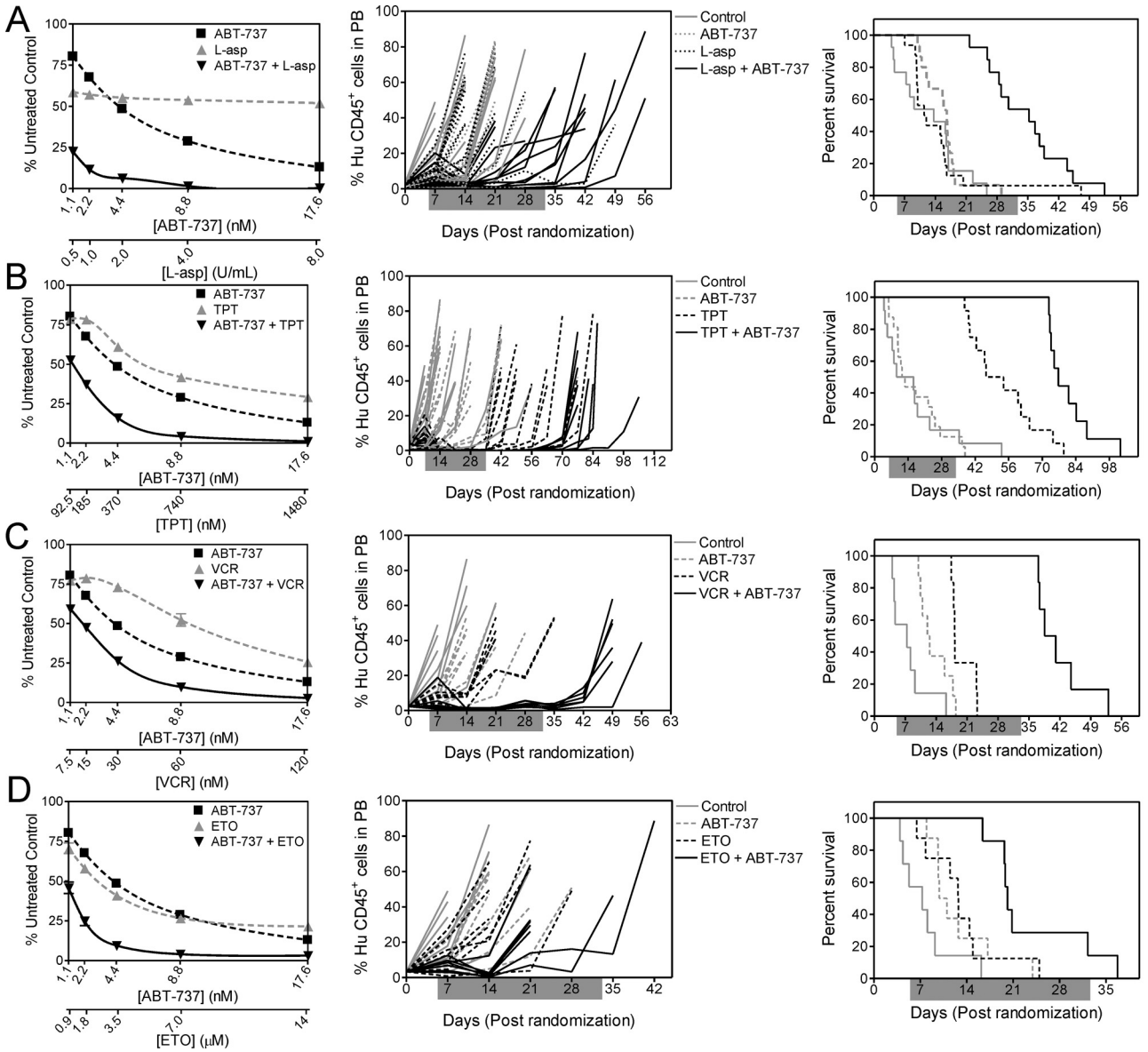

Fig. 4. ABT-737 exerts synergistic in vitro and in vivo antileukemic efficacy with a broad range of chemotherapeutic drugs known to be active against pediatric ALL. In vitro cytotoxicity assays (left) of fixed-ratio combinations of ABT-737 with L-asp (A), TPT (B), VCR (C), and ETO (D). The percentage of human $CD45^+$ cells in the PB of individual mice (middle panels) and Kaplan-Meier plots of mouse EFS (right) for ABT-737 (25 mg/kg) combined with L-asp (1000 IU/kg; A), TPT (1 mg/kg; B), VCR (0.15 mg/kg; C), and ETO (6 mg/kg; D). Shaded areas correspond to the duration of treatment. Data on the left represent the mean ± S.E.M. of a minimum of three independent experiments.

Table 4). These findings provide additional evidence for the rational combination of a Bcl-2 inhibitor with L-asp or TPT in the treatment of pediatric ALL.

To test the generality of our findings, fixed-ratio combination cytotoxicity assays were carried out on an additional five xenografts, and all showed synergy or strong synergy between ABT-737 and L-asp or TPT (Supplemental Figs. 2 and 3 and Supplemental Table 5).

Rationale for Combining ABT-737, TPT, and L-asp in the Treatment of ALL. Because we have shown above that ABT-737 exerts synergistic ex vivo and in vivo antileukemic effects when combined with either TPT or L-asp, we further explored the rationale to develop this three-drug combination. First, we examined the effects of these drugs on the levels of key apoptosis regulatory proteins in ex vivo-cultured xenograft cells. Consistent with its properties as a DNA-damaging agent, a concentration of TPT that is achievable in the plasma of patients with cancer (Zamboni et al., 1998) caused a transient increase in p53 expression in ALL-19 cells within 2 h of exposure but had no significant effects on the levels of the antiapoptotic proteins Mcl-1, Bcl-2, Bcl-w, or Bcl-X_L or pro-apoptotic Noxa, Puma, or Bim (Fig. 5B, left, and data not shown). In contrast, exposure of ALL-19 cells to L-asp caused a rapid and specific down-regulation of Mcl-1 compared with other Bcl-2 family proteins and only a delayed induction of p53 (Fig. 5B, right). This effect was confirmed in two additional xenografts (ALL-2 and ALL-17) after a 4-h exposure to either L-asp or TPT (Supplemental Fig. 4). These results suggest that TPT (via p53 activation), L-asp (Mcl-1 down-regulation), and ABT-737 (inhibition of Bcl-2/Bcl-X_L/Bcl-w) target nonoverlapping components of the intrinsic apoptosis pathway, which may result in synergistic cytotoxicity against ALL cells ex vivo and in vivo.

On this assumption, we tested the triple drug combination against ALL-19. The combination of TPT, L-asp, and ABT-737 was strongly synergistic ex vivo (average CI = 0.19; Fig. 5D, left, and Supplemental Table 2), whereas the combination of TPT with L-asp was moderately antagonistic (average CI = 1.3; Supplemental Table 2). It is noteworthy that the three-drug combination delayed the in vivo progression of ALL-19 by >50 days longer than expected if the effects of the three drugs were merely additive (Fig. 5C and Supplemental Table 6). In this experiment, ABT-737 and L-asp alone were ineffective in delaying the progression of ALL-19, TPT caused a significant delay (34.9 days; $P = 0.0002$ versus controls), whereas the triple combination resulted in a delay of 85.5 days. In the triple-combination group, only three of seven mice reached a leukemia-related event; deaths of the remaining mice were presumed to be age-related. It is noteworthy that the in vivo synergistic effect of the triple combination was much greater than either the double combination of ABT-737/L-asp or ABT-737/TPT.

To verify the generality of the in vivo synergy between TPT, L-asp, and ABT-737 an additional two chemoresistant xenografts were tested. In each case, the three-drug combination resulted in LGDs that were greater than the sum of the LGDs for each single agent (Supplemental Fig. 5 and Supplemental Table 7).

To further understand the mechanism by which TPT and ABT-737 cause synergistic cytotoxicity against ALL cells, we used the MDM2 antagonist Nutlin-3 to activate the p53 pathway in the absence of DNA damage. The synergistic effects of ABT-737/Nutlin-3 were almost identical to those of ABT-737/TPT (Fig. 5D, right), supporting the notion that p53 activation, rather than DNA damage per se, is the underlying mechanism.

Discussion

The principal findings of this study are that 1) Bim protein expression levels seem to be an important determinant of in vivo and ex vivo sensitivity of normal and malignant immature B lymphocytes to ABT-737; and 2) rationally combining ABT-737 with established chemotherapeutic drugs results in highly synergistic in vivo antileukemic effects.

The exquisite ex vivo sensitivity of the pediatric ALL xenografts used in this study seems more closely aligned with that of primary ALL cells than with continuously cultured cell lines (Del Gaizo Moore et al., 2008), supporting the relevance of using direct explants of biopsy material to establish xenografts in immune-deficient mice for preclinical drug testing. Moreover, the ex vivo and in vivo sensitivity of the pediatric ALL xenografts to ABT-737 seems to be due to several factors.

First, the panel of xenografts express higher Bcl-2 protein levels than the panel of autonomously growing cell lines used (Fig. 3D). Recent studies suggest that Bcl-2 dependence, rather than basal Bcl-2 expression levels, have a greater impact on the cellular response to inhibitors such as ABT-737 (Deng et al., 2007; Del Gaizo Moore et al., 2008). In the xenograft cells, in which most of the Bim protein is sequestered by Bcl-2 (Fig. 3B), treatment with ABT-737 will cause displacement of Bim, resulting in Bax/Bak activation and apoptosis. This model is consistent with both the direct and indirect pathways of Bax/Bak activation (Letai et al., 2002; Willis et al., 2007).

Second, our data also suggest that Bcl-2 dependence in the leukemia cell lines is less important in determining cell survival than in the xenograft and primary ALL cells. Therefore, it could be predicted that expression levels of pro-survival proteins not targeted by ABT-737 will be important determinants of sensitivity in cell lines. This is indeed the case, where Mcl-1 expression levels significantly correlated with ABT-737 sensitivity in the leukemia cell lines. Furthermore, the levels of Mcl-1 expression in the entire xenograft panel were comparable with those in the three cell lines that were most sensitive to ABT-737 (Fig. 3D). Thus, although high Mcl-1 expression does not correlate with in vivo ABT-737 resistance, the overall low level of expression in the ALL xenografts seems to contribute to their relative sensitivity.

Third, overall expression levels of Bcl-2 family members were less variable across the panel of xenografts compared with the cell lines. This suggests that the intrinsic apoptotic pathway is highly deregulated in the cell lines and that defects within the pathway are likely to occur at multiple levels. Moreover, leukemia cell lines are more prone to sustain inactivating mutations in Bax and p53 that are not reflective of the primary disease, which also may affect effective apoptosis-triggering mechanisms (Molenaar et al., 1998; Drexler et al., 2000). For example, three of the cell lines (K562, Nalm-6, Molt-4) seemed to express no Bax protein.

Fourth, we show that Bim (and importantly the amount of Bim associated with Bcl-2) significantly correlated with the in vivo sensitivity of the panel of xenografts to ABT-737.

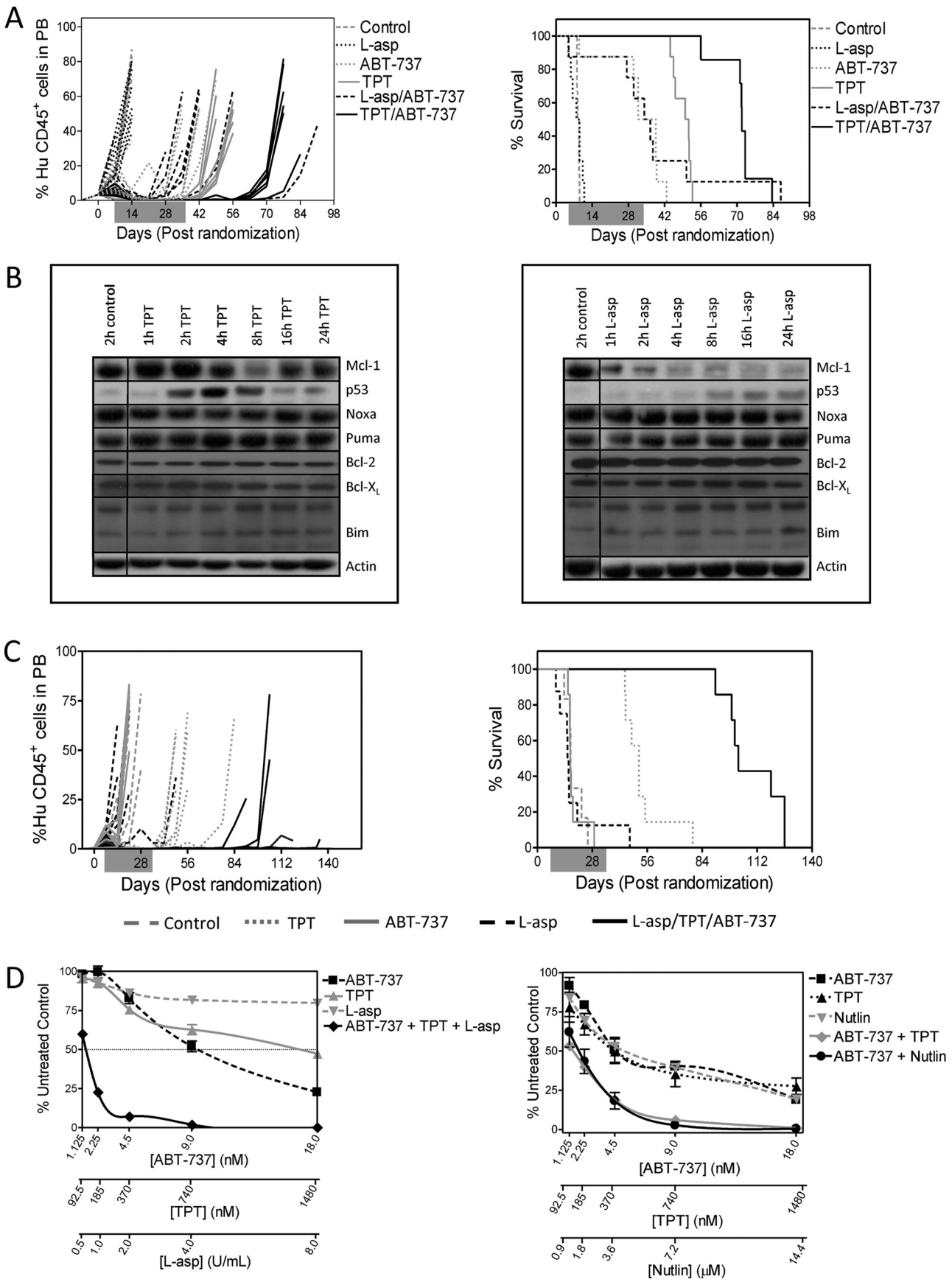

Fig. 5. Targeting the apoptotic machinery of pediatric ALL using the BH3-mimetic ABT-737. A, the responses of individual mice engrafted with ALL-19 to treatments at their respective MTDs are illustrated as a percentage of human CD45$^+$ cells in the PB of individual mice over time (left) or EFS (right). The MTD for each treatment was as follows: L-asp (2000 IU/kg), TPT (1.5 mg/kg), ABT-737 (100 mg/kg), or combinations of L-asp (1500

This correlation is in agreement with the in vitro ABT-737 sensitivity of a panel of human diffuse large B cell lymphomas (Deng et al., 2007), but in contrast with the in vitro sensitivity of the cell lines used in this study. The importance of Bim expression levels in relation to ABT-737 response was further strengthened by experiments demonstrating that Bim(−/−) lymphocytes were more resistant to ABT-737 than their wild-type and Puma(−/−) counterparts. Therefore, the principal mechanism of in vivo ABT-737 resistance in the xenograft panel seems to be reduced expression of a BH3-only protein, Bim, rather than defects in effector proteins (Bax/Bak) or increased expression of antiapoptotic proteins (e.g., Mcl-1) (Deng et al., 2007). However, whereas our results suggest an important role for Bim in the sensitivity of ALL xenograft cells to ABT-737, further studies using Bim knockdown are required to demonstrate a direct contribution.

In agreement with a previous study (Del Gaizo Moore et al., 2008), we have also shown that ABT-737 induces cell death via the mitochondrial pathway in ALL cells. In addition, it has previously been shown using cell lines that pretreatment with a pan-caspase inhibitor can wholly inhibit ABT-737-induced cell death (Del Gaizo Moore et al., 2008). In contrast, we show that in xenograft cells pan-caspase inhibition delays, but does not prevent, cell death. This provides evidence that ABT-737 is likely to induce ALL cell death even if caspase activation was blocked. Our results are consistent with a recent study, which demonstrated that, in addition to inducing apoptosis via the intrinsic apoptotic pathway, ABT-737 can induce cell death by promoting outer mitochondrial membrane rupture, a caspase independent process, in primary chronic lymphocytic leukemia cells (Vogler et al., 2009).

Although this study has shown that, even at a low dose, ABT-737 is relatively effective in vivo as a single agent against a heterogeneous panel of ALL xenografts, the clinical applicability of Bcl-2 inhibitors is most likely to involve combinations with established drugs (Oltersdorf et al., 2005; Kang et al., 2007; Trudel et al., 2007; Kuroda et al., 2008). In this study, we show that ABT-737 synergizes ex vivo and in vivo with a broad range of chemotherapeutic drugs (L-asp, TPT, VCR, and ETO) against an aggressive and chemoresistant xenograft. Using this method, we investigated the possibility of rationally designing novel drug combinations against refractory childhood ALL and strengthening the supporting evidence for the inclusion of this class of compound in patient therapy.

We provide evidence to suggest that L-asp and TPT act through different mechanisms to synergize with ABT-737. L-asp exposure resulted in rapid and specific down-regulation of Mcl-1 expression and produced a synergistic antileukemic effect when combined with ABT-737 ex vivo and in vivo. Although alternative methods have been used to down-regulate Mcl-1 and sensitize tumor cells to ABT-737 (van Delft et al., 2006), the specific effect of a drug routinely used in the treatment of pediatric ALL patients, in this case L-asp, on Mcl-1 has not previously been demonstrated despite its known effects on inhibiting protein synthesis. It is likely that the effect of L-asp on Mcl-1 is more pronounced compared with other Bcl-2 family members because of the relatively short half-life of Mcl-1 (Iglesias-Serret et al., 2003).

In contrast to the effects of L-asp on Mcl-1, TPT caused rapid up-regulation of p53 expression with no significant effects on Bcl-2 family protein expression. The proapoptotic Noxa and Puma were not up-regulated, which is surprising because they are transcriptionally up-regulated by p53 in response to DNA damage in other model systems (Villunger et al., 2003). Moreover, both Noxa and Puma were induced by cyclophosphamide in causing in vivo synergy with ABT-737 against aggressive Myc-driven lymphomas (Mason et al., 2008). Our results suggest that p53 mediates apoptosis by directly targeting mitochondria in ALL xenograft cells (Mihara et al., 2003). The synergistic effects of Nutlin-3 with ABT-737 were almost identical with those of TPT, suggesting that p53 activation per se, rather than DNA damage, was the underlying mechanism of synergy between TPT and ABT-737. However, additional studies using either p53-mutant or knockout cells are required to demonstrate a causal relationship in this regard. It is noteworthy that the synergistic effects between L-asp, TPT, and ABT-737 were replicated in five additional xenografts, confirming the generality of the interactions.

Based on the above evidence, we designed a three-drug regimen that, by targeting different components of the intrinsic apoptotic pathway, we reasoned should result in a strong synergistic effect (Supplemental Fig. 6). The triple combination was indeed highly synergistic both ex vivo and in vivo, and the in vivo results were confirmed in an additional two independent xenograft lines. The ability of ABT-737 to reverse L-asp resistance in vivo is likely to be of clinical relevance, because poor clinical outcome in pediatric ALL has been associated with L-asp resistance (Fine et al., 2005). Moreover, recent evidence suggests that TPT has some clinical activity against relapsed pediatric ALL (Hijiya et al., 2008). Therefore, the combination of L-asp/TPT and a Bcl-2 inhibitor (e.g., ABT-263) represents a promising combination for the treatment of relapsed/refractory ALL. At the least, our results provide strong preclinical evidence for the inclusion of a Bcl-2 inhibitor in novel combinations with established drugs in clinical trials against relapsed/refractory childhood ALL.

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IU/kg) + ABT-737 (25 mg/kg) and TPT (1 mg/kg) + ABT-737 (25 mg/kg). Shaded areas correspond to the duration of treatment. B, protein expression assessed by immunoblotting after the ex vivo exposure of ALL-19 cells to 150 nM TPT (left) or 7 U/ml L-asp (right). C, in vivo triple combination treatment with L-asp, TPT, and ABT-737 of mice engrafted with ALL-19 compared with single agent treatments represented as a percentage of human $CD45^+$ cells in PB (left) or EFS (right). D, comparison of the synergistic cytotoxicity exerted by TPT in combination with L-asp and ABT-737 (left) and Nutlin-3 (right) when combined with ABT-737 against ex vivo cultured ALL-19 cells assessed by fixed ratio cytotoxicity assays. Data in B and D are representative of a minimum of two separate experiments.

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Address correspondence to: Richard B. Lock, Leukemia Biology Program, CCIA, P.O. Box 81 High St, Randwick NSW 2031, Australia. E-mail: rlock@ccia.unsw.edu.au