Supplemental materials

Supplemental experimental procedures

Tissue culture, retroviral infections, cell death induction and apoptosis assays

Cell lines (FDC-P1: mouse myelomonocytic, MCF-7: breast epithelial, HeLa CCL-2: human cervical carcinoma, Eμ-*myc* mouse B lymphoma, Phoenix Ecotropic packaging cells) and mouse embryonic fibroblasts (MEFs) were all cultured in Dulbecco's Modified Eagles (DME) medium supplemented with 10% fetal calf serum (FCS), and in some cases also with 250 μM L-asparagine, 50 μM 2-mercaptoethanol or 1,000 U/mL IL-3 (for FDC-P1 cells). MEFs used in some experiments (Fig. 1) were a gift from Dr S. Korsmeyer (Cheng et al., 2001); other C57BL/6-derived MEFs have been described (Willis et al., 2005). Bax/Bak DKO cells re-expressing HA-tagged Bax or Bak and FDC-P1 cells expressing FLAG-tagged Bcl-2 and Bcl-x_L have been described (Huang et al., 1997a; Willis et al., 2005).

Cells expressing Noxa, Noxa3E, Bad, Bcl-2, Bcl-x_L, Mcl-1 or A1 were generated by retrovirally infecting the cells with pMIG or pMIH retroviruses (Chen et al., 2005). Retroviral constructs were transiently transfected into Phoenix Ecotropic packaging cells and viral supernatants were used to infect cells as described (Chen et al., 2005). To allow infection of human cells, these cells were first transfected with an expression plasmid encoding mouse ecotropic retroviral receptor (gift of J. Silke) by nucleofection (Amaxa). Twenty-four hours later, the cells were infected with retroviruses (Noxa, Noxa 3E or RNAi constructs) that had been packaged in Phoenix cells, and the antibiotic-resistant (hygromycin or puromycin) cells were then expanded.

Cell death was induced by retroviral infection with constructs expressing BH3-only proteins (Chen et al., 2005); by continuous exposure for the indicated times to HA14-1 (Calbiochem), BH3I-1 (Chembridge), Compound 6 (gift of K. Watson), Antimycin A (Sigma), Chelerythrine chloride (Calbiochem), Gossypol (Sigma), ABT-737 (Abbott Laboratories), Cytosine Arabinoside (Ara-C; Pharmacia), Etoposide (Pharmacia), Seliciclib (R-roscosvitine/CYC202; Sigma) or Cycloheximide (Sigma); by 10 Gy γ-irradiation (provided by a ^{60}Co source); or by IL-3 deprivation of FDC-P1 cells (Vaux et al., 1988). Cell viability was quantified by flow cytometric analysis of cells excluding 5 μg/mL PI (Sigma) using a FACScan® (BD). At each time point, the assay was performed in triplicate on multiple independent clones of each genotype and the experiments repeated at least 3 times. In some experiments, the cells

were cultured in the presence of a broad-spectrum caspase inhibitor, 50 μM zVAD.fmk (Bachem).

Long-term survival (colony) assays were performed by plating equal numbers of retrovirally infected cells and scoring for GFP^{+ve} clones 6 d later (Chen et al., 2005). Equal numbers of cells in replicate wells were exposed to ABT-737 (1 μM), HA14-1 (50 μM) or Antimycin A (50 μM); the media and drug were replenished after 72 h and scored for macroscopically visible colonies on day 7. The results are presented as a percentage of the number of colonies formed in the presence of drug relative to that in its absence.

Immunoblotting

Cell lysates were prepared in lysis buffer (20 mM Tris-pH 7.4, 135 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol) containing 1% Triton-X-100 (TX-100), supplemented with protease inhibitors (Roche or Sigma). Proteins were resolved by SDS:PAGE (Novex gels; Invitrogen), transferred onto nitrocellulose membranes and detected by immunoblotting using rat monoclonal anti-Bim (3C5; Alexis), -Apaf-1 (18H2, Alexis); hamster monoclonal anti-mouse Bcl-2 (3F11; BD); mouse monoclonal anti-HA (HA.11; CRP), -Bcl-2 (Bcl-2-100; (Pezzella et al., 1990); -Bax (2D2 and 5B7; Sigma), -Bak (Ab-1; Calbiochem), -HSP 70 (N6; gift of Drs. W. Welch and R. Anderson); rabbit polyclonal anti-Bak (B5929; Sigma), -mouse Mcl-1 (Rockland), -human Mcl-1 (554103; BD). Secondary antibodies included HRP-conjugated anti-rat IgG or anti-hamster IgG (SouthernBiotech), anti-mouse or anti-rabbit IgG (Chemicon); IRDye800-conjugated (Rockland) or AlexaFluor 680-conjugated (Molecular Probes) anti-rat, anti-mouse or anti-rabbit IgG antibodies. The proteins were detected by enhanced chemiluminescence (ECL; GE Healthcare) or using the Odyssey (Li-Cor) imaging system.

Affinity measurements and solution competition assays

Affinity measurements using recombinant proteins (human except for mouse Mcl-1 and A1) were performed at room temperature on a Biacore 3000 biosensor as described (Chen et al., 2005); full-length human Bax was produced as described (Suzuki et al., 2000). As well as peptides previously described (Chen et al., 2005; Wilson-Annan et al., 2003), we used mouse NoxaBH3B (PADLKDECAQLRRIGDKVNLRQKLLN) and its E74F mutant (PADLKDFCAQLRRIGDKVNLRQKLLN; mutated residue underlined). Isothermal titration

calorimetry (ITC) was performed using a VP-ITC microcalorimeter (Microcal). Experiments were performed in 20 mM Tris pH 8.0, 150 mM NaCl, 1% DMSO, 1% Tween 20 at 25°C. Titrations consisted of 24 x 12 μL injections of peptide or compound at 40 μM into 1.34 mL of protein (5 μM). The data were analyzed to fit to a one-site model using the Microcal software.

Flow cytometric analysis

Transgene expression was confirmed by flow cytometric analysis, as previously described (Huang et al., 1997b), or by immunoblotting of cell lysates. Cytochrome *c* release was assayed as described (Waterhouse et al., 2004), using the mouse monoclonal anti-cytochrome *c* antibody 6H2.B4 (BD), and Bax activation was assessed using the mouse monoclonal anti-Bax clone 3 (BD) as described (Willis et al., 2005). The samples were analyzed using a FACScan® (BD).

***In vitro* cytochrome *c* release assays**

Cells were pelleted and lysed in 0.05% digitonin containing lysis buffer (20 mM Hepes pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, supplemented with Complete Protease Inhibitor Cocktail from Roche). The crude lysates, containing mitochondria, were incubated with 0 or 5 μM ABT-737 for 1 h at 30°C, then pelleted at 13,000 rpm at 4°C for 5 min. The supernatant was retained as the soluble (S) fraction while the pellet (P), which contains intact mitochondria, was solubilized in RIPA buffer.

Supplemental references

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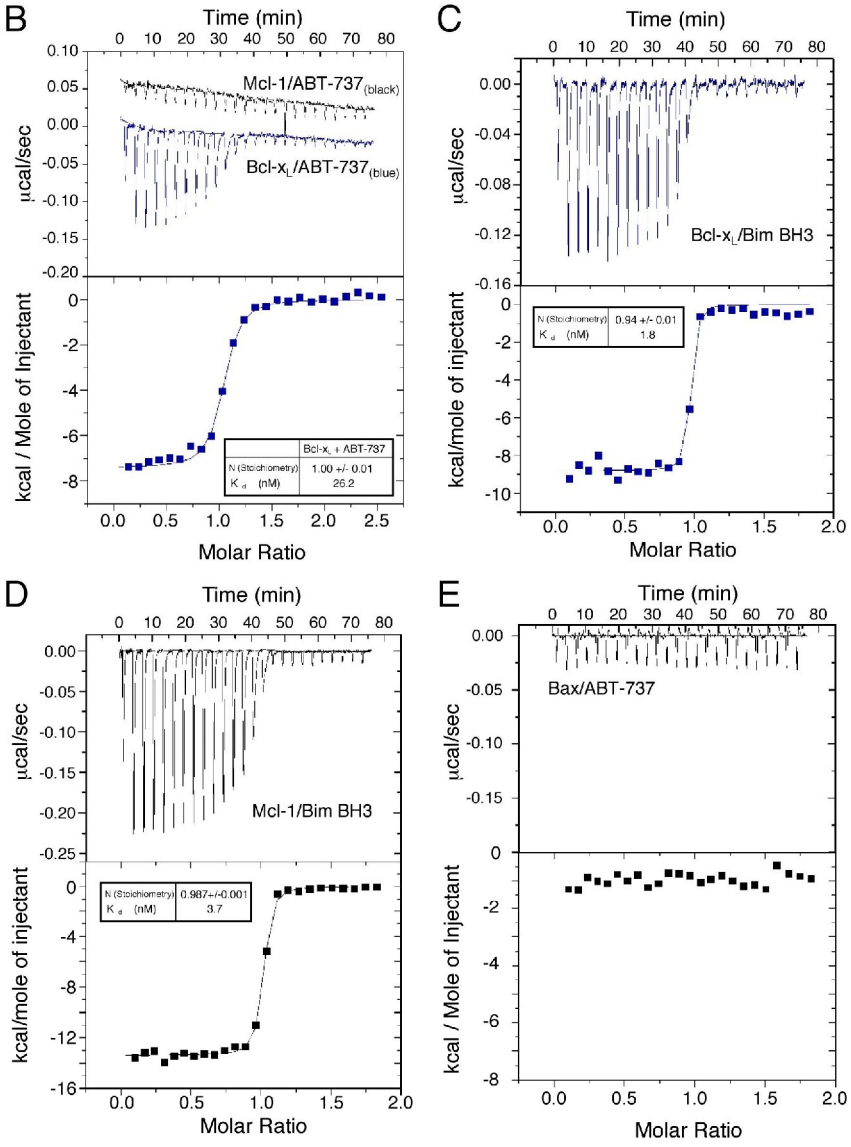
Supplemental figures

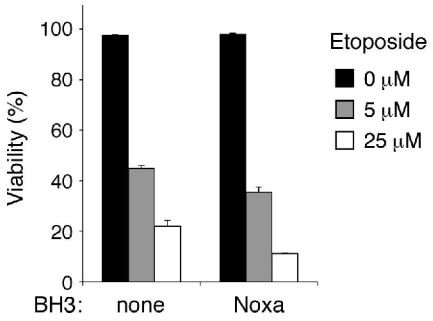
VanDelft / Wei Fig. S1

A

	IC_{50} (nM)	
	ABT-737	Bad
Bcl-2 ΔC22	3.5	16
Bcl-w ΔC29	9.6	30
Bcl-x_L ΔC24	5.7	5.3
Mcl-1 ΔC23	>2,000	>100,000
A1 ΔC20	>2,000	15,000



VanDelft / Wei Fig.S2



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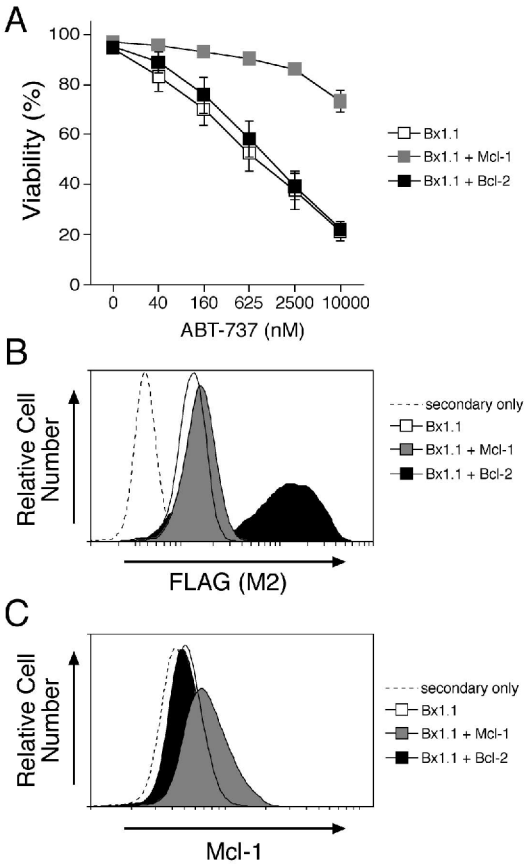

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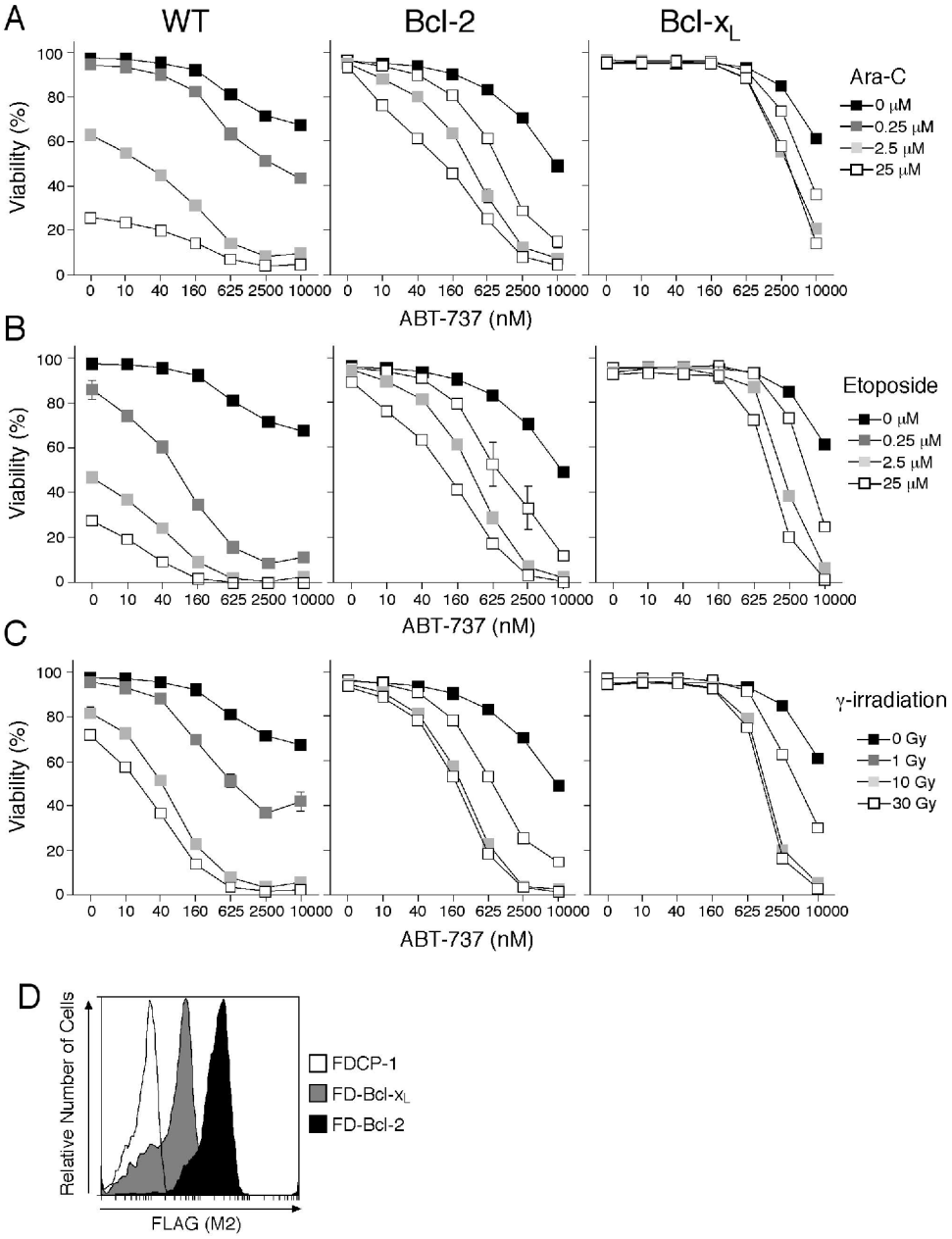

Supplemental figure legends

Figure S1. ABT-737 binding to Bcl-2 family proteins

A: The relative affinities (IC_{50} in nM) of a BadBH3 peptide (as reported previously (Chen et al., 2005) and ABT-737 for mammalian pro-survival proteins. The affinities were determined in solution competition assays using pro-survival proteins that had been C-terminally truncated to facilitate their production in bacteria (Chen et al., 2005; Day et al., 2005; Hinds et al., 2003).

B: Tight stoichiometric (1:1) binding is observed between ABT-737 and Bcl-x_L (blue), but none between ABT-737 and Mcl-1 (black), determined by isothermal titration calorimetry (ITC). The upper panels display the raw titration data. Each trough represents the heat absorbed during a single injection of ligand. The raw data for binding to Bcl-x_L (blue) and Mcl-1 (black; offset by 0.05 cal mol^{-1}) is displayed (upper panels). Each point on the curves in the lower panels represents the integration of the area of the inflection produced by each injection.

C, D: Tight binding of 26-mer Bim BH3 to Bcl-x_L (**C**) and Mcl-1 (**D**).

E: No binding is detected between ABT-737 and Bax.

The insets show the kinetics and stoichiometry of binding as determined by ITC.

Figure S2. Viability of MEFs stably expressing Noxa

The viability (determined by PI uptake) of wild-type parental MEFs (left) or a sub-line stably expressing Noxa (right) left untreated, or 24 h after treatment with Etoposide (5 or 25 μM). Data represent means $\pm$ SD from 3 independent experiments.

Figure S3. Overexpression of Mcl-1, but not Bcl-2, confers resistance to ABT-737

A: The parental factor-dependent myeloid (FDM) cells (clone Bx1.1) (Ekert et al., 2004), or derivatives stably expressing FLAG-tagged Mcl-1 or Bcl-2, were treated with ABT-737 (0-10 μM) and their viability determined 24 h later. Data represent means $\pm$ SD from 3 independent experiments.

B, **C**: Overexpression of Mcl-1 or Bcl-2 in FDM cells. The flow cytometric analysis shows FDM (clone Bx1.1) cells stably expressing FLAG-tagged Mcl-1 (stippled) or Bcl-2 (black), which had been fixed, permeabilized and stained with (B) the mouse monoclonal anti-FLAG M2 antibody to allow direct comparison of Bcl-2 or Mcl-1 expression (detected using a FITC-conjugated goat anti-mouse antibody) or (C) the rat monoclonal anti-Mcl-1 antibody to allow comparison of

Mcl-1 overexpression relative to endogenous levels (detected using a FITC-conjugated goat anti-rat antibody). Unfilled histograms: staining of parental FDM cells.

Figure S4. Sensitization by ABT-737 to cytotoxic agents

A-C: The viability of parental FDC-P1 cells or derivative lines overexpressing Bcl-2 or Bcl-x_L cells was determined 24 h after exposure to cytosine arabinoside (**A**, Ara-C; 0-25 μM), etoposide (**B**, 0-25 μM) or γ-irradiation (**C**, 0-30 Gy), in the absence or presence of ABT-737 (up to 10 μM).

D: Overexpression of Bcl-2 or Bcl-x_L in FDC-P1 cells. Flow cytometric analysis of representative FDC-P1 clones stably expressing FLAG-tagged Bcl-2 (black) or Bcl-x_L (stippled) which had been fixed, permeabilized and stained with the mouse monoclonal anti-FLAG M2 antibody, to allow direct comparison of Bcl-2 or Bcl-x_L expression (detected using a FITC-conjugated goat anti-mouse antibody). Unfilled histogram: staining of parental FDC-P1 cells.

Data in **A**-**C** is from representative experiments.