Impact of loss of BH3-only proteins on the development and treatment of MLL-fusion gene-driven AML in mice

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Inhibition of the apoptosis pathway controlled by opposing members of the Bcl-2 protein family plays a central role in cancer development and resistance to therapy. To investigate how pro-apoptotic Bcl-2 homology domain 3 (BH3)-only proteins impact on acute myeloid leukemia (AML), we generated mixed lineage leukemia (MLL)-AF9 and MLL-ENL AMLs from BH3-only gene knockout mice. Disease development was not accelerated by loss of Bim, Puma, Noxa, Bmf, or combinations thereof; hence these BH3-only proteins are apparently ineffectual as tumor suppressors in this model. We tested the sensitivity of MLL-AF9 AMLs of each genotype in vitro to standard chemotherapeutic drugs and to the proteasome inhibitor bortezomib, with or without the BH3 mimetic ABT-737. Loss of Puma and/or Noxa increased resistance to cytarabine, daunorubicin and etoposide, while loss of Bim protected against cytarabine and loss of Bim had no impact. ABT-737 increased sensitivity to the genotoxic drugs but was not dependent on any BH3-only protein tested. The AML lines were very sensitive to bortezomib and loss of Noxa conveyed significant resistance. In vivo, several MLL-AF9 AMLs responded well to daunorubicin and this response was highly dependent on Puma and Noxa but not Bim. Combination therapy with ABT-737 provided little added benefit at the daunorubicin dose trialed. Bortezomib also extended survival of AML-bearing mice, albeit less than daunorubicin. In summary, our genetic studies reveal the importance of Puma and Noxa for the action of genotoxics currently used to treat MLL-driven AML and suggest that, while addition of ABT-737-like BH3 mimetics might enhance their efficacy, new Noxa-like BH3 mimetics targeting Mcl-1 might have greater potential.

Cell Death and Disease (2016) 7, e2351; doi:10.1038/cddis.2016.258; published online 1 September 2016

Acute myeloid leukemia (AML) is a devastating disease primarily affecting children and older people. Although genetically diverse,1–9 most AMLs are oligoclonal at presentation, with perhaps only two to four driver mutations.1,3–6 Chromosomal translocations are common (~50% of cases) and those involving the mixed lineage leukemia (MLL) gene, the mammalian homolog of Drosophila trithorax gene located on chromosome 11 band q23, occur in ~10% of acute leukemias, including AML, acute lymphoblastic leukemia and leukemias of mixed or indeterminate lineage.6 MLL translocations are associated with poor prognosis.6,7 MLL encodes a large multi-domain protein that activates transcription through its C-terminal histone H3 lysine 4 (H3K4) methyl transferase domain. MLL translocations create a fusion gene containing the 5′ portion of MLL and the 3′ portion of the partner gene.8 The DNA-binding MLL portion of the resulting fusion protein binds MLL target genes, including Hox genes, and the partner moiety enforces constitutive expression through interaction with a higher order transcriptional elongation complex.6,7,9

Nearly 80 different MLL fusion partners have been identified in AML,7 two of the most common being AF9 and ENL. Transgenic mice expressing MLL-AF9 and MLL-ENL under the control of the endogenous mll promoter are highly prone to AML, although the long latency indicates a requirement for additional genetic event(s) before the emergence of fully malignant cells.10–12

Major improvements in AML therapy have remained elusive. Current ‘standard of care’ involves an initial phase of intense chemotherapy (remission induction therapy) followed by additional chemotherapy cycles and/or allogeneic stem cell transplantation. Most commonly, induction therapy involves administration of cytarabine with an anthracycline, usually daunorubicin or idarubicin, with etoposide sometimes also included. Because all these drugs act on DNA synthesis, they preferentially affect rapidly dividing cells. Cytarabine (cytosine arabinoside) is phosphorylated intracellularly and incorporated into DNA during S-phase, resulting in chain termination of the elongating nascent DNA chain.13 Anthracyclines and etoposide inhibit topoisomerase II, thereby increasing the frequency of double strand DNA breaks.14 Multiple additional activities have been ascribed to anthracyclines,15 including inhibition of DNA and RNA synthesis as a result of intercalation between base pairs and generation of damaging reactive oxygen species (ROS).16

By provoking DNA damage, ROS and other intracellular stresses, cytotoxic drugs kill cells (at least in part) by inducing

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Abbreviations: BH3, Bcl-2 homology domain 3; MLL, Mixed lineage leukemia; MLL-ENL, Fusion gene combining MLL with MLLT1; MLL-AF9, Fusion gene combining MLL with MLLT3; AML, Acute myeloid leukemia; KO, Knock out; WT, Wild type; GFP, Green fluorescent protein; IRES, Internal ribosomal entry site; SCM, Stem cell medium; IMDM, Iscove’s modified Dulbecco’s medium; FBS, Fetal bovine serum; ROS, reactive oxygen species

Received 06.7.16; accepted 25.7.16; Edited by G Raschella
the intrinsic (also known as the mitochondrial or stress-induced) apoptosis pathway, which is regulated by pro- and anti-apoptotic members of the Bcl-2 family (for reviews see refs 17–19). Bcl-2 and its closest relatives (Bcl-xl, Mcl-1, A1/BFL1, Bcl-w and, in humans, possibly also Bcl-B) promote cell survival by inhibiting apoptosis, whereas structurally similar relatives Bax and Bak (and possibly also Bok) instead promote apoptosis, as do the so-called ‘BH3-only proteins’ (Bim, Puma, Noxa, Bad, Bmf, Bik and Hrk), which have only one of the four Bcl-2 homology (BH) domains. In healthy cells, the pro-survival proteins hold Bax and Bak in check. Stress signals—such as DNA damage or oncogene expression—up-regulate expression of Bcl-2 homology domain 3 (BH3)-only proteins, which bind tightly to the hydrophobic surface groove of pro-survival Bcl-2-like proteins, thereby neutralizing their capacity to inhibit activated Bax and Bak. The most potent BH3-only proteins, Bim, Puma and tBid, can bind all pro-survival proteins whereas others show more selective binding:20,21 Noxa, for example, is specific for Mcl-1 and A1/BFL1, whereas Bad is specific for Bcl-2, Bcl-xl and Bcl-w. Certain BH3-only

Figure 1  Generation of murine AMLs. (a) E13.5 fetal liver cells from WT or BH3-only gene KO Ly5.2 C57BL/6 mice were infected with MLL-AF9/GFP, MLL-ENL/GFP or GFP MSCV retrovirus (Materials and methods section) and injected into sub-lethally irradiated (7.5 Gy) C57BL/6-Ly5.1 mice (2 × 10⁶ cells/mouse). (b,c) Kaplan–Meier survival analysis of mice transplanted with fetal liver cells of the indicated genotype after infection with MLL-AF9 virus; number of recipient mice is indicated in brackets. Mice were monitored regularly and euthanized humanely when showing signs of AML-induced stress. (d) Spleen weight and (e) WBC count of individual mice at autopsy. Data points represent individual mice with mean and S.E.M. indicated. P-values were determined by unpaired t-test with Welsh’s correction for differences in variance. *P < 0.05, **P < 0.01. Comparable data for mice transplanted with MLL-ENL virus-exposed cells is shown in Supplementary Figure S1
proteins can also bind transiently to the surface groove of Bax and Bak, inducing them to undergo conformational change and form homodimers on the outer mitochondrial membrane.\textsuperscript{22,23} The homodimers then aggregate to form homo-oligomeric pores,\textsuperscript{24} through which cytochrome c egresses into the cytoplasm to initiate the cascade of caspase activation responsible for dismantling the cell.

A new class of small molecule that mimics BH3-only proteins is generating much clinical interest. BH3 mimetics bind to pro-survival Bcl-2-like proteins in a manner similar to BH3-only proteins, releasing previously sequestered BH3-only proteins to activate Bax and Bak.\textsuperscript{25,26} Cancer cells have greater susceptibility to BH3 mimetic drugs than normal cells, in part because they often have higher levels of pro-survival proteins and hence higher ‘stores’ of BH3-only proteins.\textsuperscript{26,27} ABT-737, the first bonafide BH3 mimic to be developed, binds Bcl-2, Bcl-x\textsubscript{L} and Bcl-w but not Mcl-1 or A1,\textsuperscript{28,29} as does its orally bio-available derivative ABT-263 (Navitoclax).\textsuperscript{30} whereas the more recent ABT-199 (Venetoclax) is specific for Bcl-2 (ref. 31) and A-1155463 is specific for Bcl-xL.\textsuperscript{32} These BH3 mimetics have shown promising efficacy in a variety of preclinical models (eg refs 31,33) and ABT-263 and ABT-199 are now in advanced clinical trials for chronic lymphocytic leukemia and certain other malignancies.\textsuperscript{34–37} High levels of Mcl-1 (or A1/BFL1) are likely to cause resistance\textsuperscript{26,29,38} and BH3 mimetics specific for Mcl-1 are under development.

Using retroviruses expressing MLL-fusion genes,\textsuperscript{39–41} we are generating murine AMLs with a variety of apoptotic lesions. Here we report genetic studies designed to clarify whether BH3-only proteins are generating murine AMLs with a variety of apoptotic lesions. Results

Generation of murine AMLs lacking specific BH3-only proteins. MLL-AF9 and MLL-ENL driven AMLs were generated using retroviral transduction of fetal liver cells from wild type (WT) or BH3-only gene knock out (KO) mice and hematopoietic reconstitution of sub-lethally irradiated mice,\textsuperscript{39–41} as described in Figure 1a. The reconstituted mice are designated hereafter according to the virus and the genotype of the fetal liver cells (e.g., WT/green fluorescent protein (GFP) indicates mice reconstituted with WT fetal liver cells exposed to control GFP virus and noxa\textsuperscript{−/−}/MLL-AF9 indicates mice reconstituted with noxa\textsuperscript{−/−} fetal liver cells exposed to MLL-AF9 GFP virus).

Irrespective of the genotype of the donor stem/progenitor cells, AML developed in all mice reconstituted with MLL-AF9 or MLL-ENL virus-infected cells, most needing to be euthanized within a period of 30–65 days (Figures 1b and c and Supplementary Figure S1a). The sick mice had an enlarged spleen and elevated blood leukocytes (Figures 1d and e, Supplementary Figures S1b and c and Supplementary Table S1), as well as thrombocytopenia and anemia (Supplementary Figure S2). In contrast, GFP mice of all genotypes remained healthy until culled, usually after 120 days.

Loss of Bim, Puma, Noxa, Bmf or combinations thereof, made no significant difference to the kinetics of morbidity (Figures 1b and c and Supplementary Figure S1a) or degree of splenomegaly (Figure 1d,Supplementary Figure S1b), although circulating leukocytes were significantly elevated in sick bim\textsuperscript{−/−}/MLL-AF9 and bim\textsuperscript{−/−}/MLL-ENL mice compared with their WT/MLL-AF9 and WT/MLL-ENL counterparts, principally due to a greater increase in myeloid cells (Mac1\textsuperscript{+}/Gr1\textsuperscript{−} cells; see Supplementary Table S1). Curiously, blood leukocytes were not as elevated in sick bmf\textsuperscript{−/−}/MLL-AF9 mice as in sick WT MLL-AF9 mice but were comparable in bmf\textsuperscript{−/−}/MLL-ENL mice (Figure 1e and Supplementary Figure S1c).

Sick mice were autopsied and tissues subjected to histological and flow cytometric analysis (Supplementary Figure S3,Supplementary Table S1 and data not shown). As reported by others,\textsuperscript{39–41} the bone marrow and blood were replete with differentiated myeloid (Mac1\textsuperscript{+}/Gr1\textsuperscript{−}/) cells. Normal splenic architecture was effaced by these abundant malignant myeloid cells, which also infiltrated the liver, kidney and other tissues. WT and BH3-only gene KO MLL-AF9 and MLL-ENL AMLs had comparable pathology (data not shown).

Expression of apoptosis regulators. To further characterize the MLL-AF9 AMLs, we ascertained the expression pattern of key apoptosis regulators by western blot and quantitative PCR analysis of bone marrow cells from sick primary mice (4–10 per genotype; Figure 2 and Supplementary Figure S4). In addition, western blots were performed on spleen cells (Supplementary Figure S5).

Pro-survival Bcl-2 family proteins Bcl-2, Bcl-x\textsubscript{L}, Mcl-1 and A1 were detected in all AML genotypes analyzed at variable levels. No suitable antibody was available for Bcl-w protein but A1 were detected in all AML genotypes examined at variable levels. BH3-only proteins Bim, Puma, Bmf and Bad were readily detected in all genotypes. BH3-only proteins Bim, Puma, Bmf and Bad were detectable in all AMLs examined. Bim protein was detectable in some bim\textsuperscript{−/−}, puma\textsuperscript{−/−}, bmf\textsuperscript{−/−} and puma\textsuperscript{−/−} bim\textsuperscript{−/−} AMLs but not in any of the WT AMLs, although noxa transcripts were detectable in the WT AMLs.

In general, the AMLs appeared to have higher expression of Bcl-2 family members than Mac1\textsuperscript{+}/Gr1\textsuperscript{−} cells isolated by flow cytometry from normal bone marrow (not shown).

Expression of p53 or p19Arf protein in the absence of an apoptotic stimulus is indicative of mutation or loss of p53 respectively.\textsuperscript{41} However, none of 27 AMLs tested expressed detectable levels of either of these proteins (Figure 2a), suggesting that mutations affecting the p53 pathway were rare, as is also the situation in human AML.\textsuperscript{2}

Drug sensitivity of WT and BH3-only gene KO AMLs. To ascertain the in vitro drug sensitivity of the AMLs, short-term cell lines were established from multiple primary tumors of each genotype by culturing bone marrow or spleen cells from sick mice (Materials and methods section). The drugs tested
included the genotoxic agents cytarabine (cytosine arabinoside), daunorubicin and etoposide as well as ABT-737 and the proteasome inhibitor bortezomib. Drug doses were guided by published data and preliminary dose response tests.

Figure 3a shows the kinetics of cell death obtained for WT/MLL-AF9 AMLs (n = 4) exposed to each of these drugs. Killing with ABT-737 was more rapid than with the other agents, particularly cytarabine, but then plateaued. For subsequent studies, a 16 h time point was chosen and the cytarabine...
concentration was increased to 600 ng/ml to increase its efficacy.

To assess which BH3-only proteins might be critical for individual drug regimens, we compared the viability of the various BH3-only gene KO/MLL-AF9 AMLs with those of WT/MLL-AF9 AMLs treated in parallel. Three or more independent lines were tested for each genotype, except the puma−/− noxa−/− lines for which only two were available. Figure 3b shows the results for treatment with ABT-737 alone and Figure 3c–f for treatment with cytarabine, etoposide, daunorubicin or bortezomib, either alone (gray) or in combination with ABT-737 (black). An asterisk above a column indicates significantly greater resistance for specific BH3-only gene KO AMLs than WT AMLs and a hash indicates significantly better response of the indicated genotype to the combination therapy than to the single drug.

Neither Bim, Puma, Noxa or Bmf was essential for ABT-737 cytotoxic activity, since none of the corresponding gene KO
AMLs displayed significant resistance to this BH3 mimetic, and neither did any of the double BH3-only gene KO AML lines tested (Figure 3b).

Loss of Puma and/or Noxa increased resistance to the DNA damaging agents cytarabine, etoposide and daunorubicin. Loss of Bim increased resistance to cytarabine but not etoposide or daunorubicin and loss of Bmf had no significant impact for any of the drugs (Figures 3c–e).

Encouragingly, WT AMLs were highly sensitive to treatment with bortezomib, as were $\text{bim}^{-/-}$, $\text{puma}^{-/-}$ and $\text{bmf}^{-/-}$ AMLs (Figure 3f). Only loss of Noxa conferred significant resistance enhanced the sensitivity of $\text{puma}^{-/-}$ AMLs following bortezomib treatment (Figure 4b) and various BH3-only gene KO AMLs to each of these drugs and of $\text{bim}^{-/-}$ AMLs to cytarabine. Furthermore, despite their resistance to bortezomib as a single agent, $\text{noxa}^{-/-}/\text{MLL-AF9}$ AMLs appeared sensitive to the combination.

In an effort to better understand the responses triggered by the various drugs, three WT/MLL-AF9 cell lines (#1300, #1414 and #1411) and one $\text{bmf}^{-/-}/\text{MLL-AF9}$ line (#1415) were tested in the presence of the pan-caspase inhibitor QVD-OPH and analyzed after 6 h by western blot (Figure 4a and Supplementary Figure S6). Exposure to bortezomib markedly increased the levels of Noxa protein in two lines (#1414 and #1415) but not the others. Puma levels modestly increased in response to etoposide and Bim increased with ABT-737. Bcl-2 and Mcl-1 protein levels remained relatively constant in the face of all agents.

Comparable qPCR studies were performed 3 h after treatment for three WT/MLL-AF9 (Figure 4b) and various BH3-only gene KO/MLL-AF9 lines (Supplementary Figure S7 and data not shown). Noxa transcripts increased markedly in the WT AMLs following bortezomib treatment (Figure 4b) and also in the $\text{bim}^{-/-}$ AMLs (Supplementary Figure S7) but only modestly in the $\text{puma}^{-/-}$ AMLs. Puma RNA was elevated in WT AMLs in response to bortezomib (Figure 4b) and in Bim-deficient AMLs in response to most agents, particularly cytarabine, bortezomib and the various drug combinations. Smaller, more variable increases in $\text{bim}$, $\text{a1}$ and $\text{mcl-1}$ RNAs were noted in response to most agents but there was no significant change in $\text{bad}$, $\text{bid}$, $\text{bmf}$, $\text{bcl-2}$, $\text{bcl-w}$, $\text{bcl-xL}$ RNAs, for either WT or BH3-only gene KO AMLs.

**In vivo treatment of MLL-AF9 AMLs.** We next embarked on in vivo trials comparing the efficacy of traditional and novel therapeutic regimens for treating AML-bearing mice. Figure 5 presents results obtained for daunorubicin, either alone or in combination with ABT-737 (see Supplementary Figure S8 for results obtained for individual AMLs). Healthy mice were injected with $0.5 \times 10^6$ bone marrow cells from sick secondary MLL-AF9 mice (3 recipients per AML per treatment arm) and treatment was started 3 days later. Mice received 5 mg/kg body weight daunorubicin intravenously on days 1, 4 and 9 and either ABT-737 (75 mg/kg body weight) or vehicle intraperitoneally on days 1–5 and 8–12.

For the WT/MLL-AF9 AMLs (Figure 5a), controls receiving saline and vehicle (black) all died within 20 days and those treated with ABT-737 alone (blue) fared no better. In contrast, daunorubicin (red) significantly extended lifespan; of the 15 mice transplanted with five different WT/MLL-AF9 AMLs, almost 50% survived for more than 30 days, and two were still alive at the end of the experiment (100 days; Figure 5a).

Treatment with ABT-737 as well as daunorubicin did not significantly increase survival over daunorubicin alone (compare aqua and red lines), although more mice achieved long-term survival (5/15 versus 2/15). No overt correlation was evident between the degree of responsiveness of individual AMLs (Supplementary Figure S8) and their pattern of expression of pro-survival Bcl-2 proteins or BH3-only proteins (Figure 2 and Supplementary Figure S5). RNASeq analysis is being undertaken to account for the differences in responsiveness.

Of note, while MLL-AF9 AMLs lacking Bim (Figure 5b) responded similarly to WT/MLL-AF9 AMLs, those lacking either Puma (Figure 5c) or Noxa (Figure 5d), were very resistant to daunorubicin, even when it was combined with ABT-737. Thus, Puma and Noxa are critical for the cytotoxic action of daunorubicin.

In view of the high sensitivity of the AML lines to bortezomib in vitro, we were keen to test its efficacy in vivo. Unfortunately, tests performed on healthy C57BL/6 mice indicated relatively poor tolerance of the drug, evidenced by severe weight loss. AML-bearing mice were given the maximum tolerated dose (0.75 mg/kg) and any mice euthanized early due to weight loss (usually before 7 days) were censored. Figure 6 summarizes the outcome for mice developing AML following transplantation with three different WT/MLL-AF9 AMLs. Treatment with bortezomib provided a significant extension of life although, at this dose, efficacy was not as great as with daunorubicin, and combination therapy with ABT-737 provided no additional benefit.

**Discussion**

In this study, we tested the impact of loss of individual BH3-only proteins on the development and treatment of AMLs driven by MLL-fusion genes. The AMLs were generated by transplanting WT or BH3-only gene KO fetal liver cells infected with MLL-AF9 or MLL-ENL virus into sub-lethally irradiated recipient mice. Of note, loss of Bim, Puma, Noxa, Bmf, or combinations thereof, made no significant difference to the kinetics of morbidity (Figure 1 and Supplementary Figure S1), all recipients developing florid AML within 30–65 days. These results differ markedly from those obtained for lymphomagenesis in Eμ-myc mice, where disease was accelerated by loss of Bim, Puma or Bmf, although not Noxa. Thus, in contrast to Myc-driven lymphomagenesis, none of the BH3-only proteins tested appear to serve as a critical tumor suppressor for the development of MLL-driven AML. This is suggestive of redundant roles. However, the faster morbidity of the retroviral AML model (median 6 weeks) compared with the Eμ-myc lymphoma model (median 15 weeks) may have masked a tumor suppressor role.

To ascertain the sensitivity of the MLL fusion protein-driven AMLs to conventional and targeted cytotoxics, we first tested primary cell lines established by culturing bone marrow cells from sick reconstituted mice in medium supplemented with...
Expression of Bcl-2 family members following treatment. Expression of Bcl-2 family members in cultured primary WT/MLL-AF9 AMLs following treatment with cytotoxic agents. (a) Western blot analysis of protein expression in AMLs #1300 and #1414 after 6 h treatment with the indicated drugs. Numbers below bands indicate the intensity of each lane relative to the untreated control for each antibody. Quantitation was performed using Image Lab software. (b) qPCR analysis of RNA expression in AMLs #1300, #1411 and #1601 after 3 h treatment. ΔΔCT values normalized to HMBS control and made relative to cells treated with QVD only to determine drug-induced fold change. Data represents mean fold change ± S.E.M., with dashed line indicating a value of 1; note the different axis for noxa expression. Comparable PCR analyzes of BH3-only gene KO/MLL-AF9 AMLs are summarized in Supplementary Figure S7. AML cell lines maintained in culture in IMDM with 10% FCS and supplemental IL-3 (Materials and methods section) were treated with 600 ng/ml cytarabine [C], 25 nM bortezomib [B], 200 ng/ml daunorubicin [D], 300 ng/ml etoposide [E], either alone or in combination with 1 μg/ml ABT-737 [737] as indicated, in presence of the pan-caspase inhibitor QVD-OPH (25 μM)
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Figure 5  In vivo treatment of MLL-AF9 AMLs with daunorubicin in combination with ABT-737. Kaplan–Meier survival curves for mice transplanted with MLL-AF9 AMLs. 8–10-week-old Ly5.1 C57BL/6 mice (non-irradiated) were injected with 0.5 × 10⁶ bone marrow cells from sick secondary WT/MLL-AF9 or BH3-only gene KO/MLL-AF9 mice (three recipients per tumor per treatment arm) and treatment was started 3 days later: 5 mg/kg daunorubicin [D] intravenously on days 1, 4 and 9 and/or 75 mg/kg ABT-737 [737] intraperitoneally on days 1–5 and 8–12; controls received saline [S] and ABT-737 vehicle [V]. Transplanted mice were monitored daily for symptoms of AML and euthanized if morbidly ill or at the end of experiment (100 days post treatment start). A total of (a) 5 WT/MLL-AF9 AMLs (#1211, 1223, 1224, 1411 and 1414), (b) 4 bim⁻/⁻ MLL-AF9 AMLs (#1158, 1213, 1249 and 1250), (c) 4 pumα⁻/⁻ MLL-AF9 AMLs (#1218, 1225, 1226 and 1229) and (d) 5 noxa⁻/⁻ MLL-AF9 AMLs (#1306,1308, 1309, 1310 and 1311) were tested (see also Supplementary Figure S8 for individual tumor results). Statistical significance was determined by Log-rank (Mantle-Cox) test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Daunorubicin treatment alone, or in combination with ABT-737, significantly prolonged survival of WT/MLL-AF9 and bim⁻/⁻/MLL-AF9 AMLs compared with saline treatment (P<0.0001), pumα⁻/⁻/MLL-AF9 had significantly prolonged survival when treated with daunorubicin alone (P=0.0173) or with daunorubicin in combination with ABT-737 (P=0.0026), as did noxa⁻/⁻/MLL-AF9 when treated with daunorubicin alone (P=0.0008) or in combination with ABT-737 (P=0.0001). For all genotypes there was no significant difference (P>0.5) in survival between daunorubicin treatment and treatment with the combination of daunorubicin and ABT-737.

IL-3. Each of the standard chemotherapeutic drugs was relatively effective at killing WT/MLL-AF9 AMLs, etoposide and daunorubicin more so than cytarabine at the concentrations tested. As might be predicted for DNA damaging agents such as these, loss of either Puma or Noxa, both p53 targets, increased resistance. Loss of Bim increased resistance to cytarabine but not to etoposide or daunorubicin, whereas loss of Bmf had no significant impact. ABT-737 was not very effective as a single agent but significantly improved the response to the standard agents suggesting that it may improve clinical response or allow lower concentrations of traditional cytotoxics to be used.

Bortezomib was highly effective at killing WT/MLL-AF9 AMLs in vitro. Significantly, whereas pumα⁻/⁻, bim⁻/⁻ and bmf⁻/⁻ AMLs were as sensitive as WT/MLL-AF9 AMLs, Noxa-deficient AMLs displayed increased resistance. Noxa RNA levels increased markedly within 3 h in primary WT/MLL-AF9 lines exposed to bortezomib and high levels of Noxa protein were observed within 6 h in two bortezomib-treated lines (one WT and the other bmf⁻/⁻) but not in two other WT lines that were nevertheless highly sensitive to bortezomib (Figure 4a and Supplementary Figure S6). Differential kinetics of Noxa induction or breakdown may explain the varied results.

Several mechanisms have been implicated in the cytotoxicity induced by proteasome inhibitors, including stabilization of p53 and IκB, and induction of ER stress and the unfolded protein response (reviewed in refs 50,51), all of which trigger apoptosis through the Bcl-2 family-regulated pathway. The apparent lack of Puma-dependence implies that Noxa induction by bortezomib in MLL-AF9 AML cells occurs through a p53-independent mechanism. Noxa transcription can be upregulated by c-myc in tumor cells in response to bortezomib.52

Noxa strongly binds to and inhibits anti-apoptotic Mcl-1,20,21 thereby facilitating activation of Bak and Bax. Since Noxa promotes Mcl-1 degradation via the BH3-only E3 ubiquitin ligation Mule,53 Mcl-1 levels might be expected to rise in bortezomib-treated cells but this was not apparent for the AML lines (Figure 4a). Presumably bortezomib-mediated Noxa up-regulation titrates the level of Mcl-1 sufficiently to kill the cells.

We transplanted five independent WT/MLL-AF9 AMLs into immunodeficient mice to test their sensitivity in vivo to daunorubicin and bortezomib, both alone or in combination
Materials and Methods
Mice. The noxa−/−, pumara−/−, bim−/− (ref. 56) and bmf−/− (ref. 57) mice have been described previously; all were maintained on a C57BL/6 J (Ly5.2) background at the Walter and Eliza Hall Institute (WEHI). C57BL/6-Ly5.1 mice were originally obtained from Jackson Laboratories. Experimental protocols were approved by WEHI's Animal Ethics Committee.

AML generation. MSCV retroviruses encoding internal ribosomal entry site (IRES)/GFP, MLL-AF9/IRES/GFP26 or MLL-ENL/IRES/GFP DNA28 were prepared by transfection of Phoenix cells. For infection, cells were prepared from three cryopreserved members of each lineage of normal or leukemic aorta-gonad-mesonephros (AGM) region, reconstituted into lethally irradiated (5 Gy) Ly5.1 recipients (200 μl/mouse). Bone marrow-derived cells of sick recipients (T1) were cryopreserved. For treatment studies, non-irradiated mice (8–10 week female C57BL/6 Ly5.1) were injected intravenously with 103 T1 cells.

In vitro drug treatment. Primary AMLs (T0) were expanded by transplanting 2 × 106 spleen cells via tail vein injection into C57BL/6 Ly5.1 mice. Bone marrow cells of sick recipients (T1) were cryopreserved. For treatment studies, non-irradiated mice (8–10 week female C57BL/6 Ly5.1) were injected intravenously with 0.5 × 106 bone marrow-derived T1 tumor cells and drug regimens commenced 3 days later: on days 1, 4, and 5, 75 mg/kg body weight ABT-737 (dissolved in 10% dimethyl sulfoxide and 90% vehicle) or an equal volume of 100 μl saline was injected intravenously; on days 1–5 and 8–12, 75 mg/kg body weight bortezomib or an equal volume (100 μl) of saline was injected intravenously followed by a flush of saline (700 μl) using a butterfly catheter; on days 1, 4, 8 and 11, 0.75 mg/kg body weight daunorubicin or 5 mg/m2 body weight bortezomib, either alone or in combination with 1 mg/m2 ABT-737. Following treatment, cells were washed once with balanced salt solution (150 mM NaCl, 3.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 7.4 mM HEPES-NaOH, 1.2 mM KH2PO4 and 0.8 mM K2HPO4) containing 5% FBS and resuspended in the same medium with Annexin V-Alexa Fluor 647 (kindly provided by Daniel Gray, WEHI) and 4 μg/ml propidium iodide. Cell viability was determined on an LSR I flow cytometer using FlowJo software.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements. We thank our institute colleagues A Roberts, A Strasser and JM Adams for useful discussions and review of the manuscript; K Hughes, G Siciliano, J Corbin and J McManus for excellent technical assistance; and the

Cell Death and Disease
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