

Published in final edited form as:

Leukemia. 2013 October ; 27(10): . doi:10.1038/leu.2013.151.

Bcl-xL anti-apoptotic network is dispensable for development and maintenance of CML but is required for disease progression where it represents a new therapeutic target

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Abstract

The dismal outcome of blast crisis chronic myelogenous leukemia (CML-BC) patients underscores the need for a better understanding of the mechanisms responsible for the development of drug-resistance. Altered expression of the anti-apoptotic Bcl-xL has been correlated with BCR-ABL leukemogenesis; however, its involvement in the pathogenesis and evolution of CML has not been formally demonstrated yet. Thus, we generated an inducible mouse model in which simultaneous expression of p210-BCR-ABL1 and deletion of *bcl-x* occurs within hematopoietic stem and progenitor cells. Absence of Bcl-xL did not affect development of the chronic phase-like myeloproliferative disease, but none of the deficient mice progressed to an advanced phenotype, suggesting the importance of Bcl-xL in survival of progressing early progenitor cells. Indeed, pharmacologic antagonism of Bcl-xL, with ABT-263, combined with PP242-induced activation of BAD markedly augmented apoptosis of CML-BC cell lines and primary CD34⁺ progenitors but not those from healthy donors, regardless of drug-resistance induced by bone marrow stromal cell-generated signals. Moreover, studies in which BAD or Bcl-xL expression was molecularly altered strongly support their involvement in ABT-263/PP242-induced apoptosis of CML-BC progenitors. Thus, suppression of the antiapoptotic potential of Bcl-xL together with BAD activation represents an effective pharmacologic approach for patients undergoing blastic transformation.

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Conflict-of-interest disclosure: The authors declare no competing financial interests related to this work.

INTRODUCTION

Despite successful implementation of imatinib and second generation tyrosine kinase inhibitors (TKI) as first line therapies for chronic myelogenous leukemia (CML) in chronic phase (CML-CP), the majority of CML-BC and Philadelphia-positive (Ph⁺) B-cell acute lymphoblastic leukemia (B-ALL) patients do not show long-term responses to TKIs or any other therapeutic option¹⁻⁶. The molecular mechanisms responsible for blastic transformation and drug-resistance in CML-BC are still unclear but likely involve both BCR-ABL1 kinase-dependent and -independent mechanisms⁴. Presence of BCR-ABL1 mutations can only in part explain the development of TKI-resistance⁷; in fact, both cell autonomous (e.g. enhanced Src and LYN kinase activity)⁸ and microenvironment-induced signals^{9, 10} contribute to development of drug-resistance and increased survival of CD34⁺ CML-BC progenitors⁴. The latter seems to depend, at least in part, on increased levels and/or activity of antiapoptotic Bcl-2¹¹, Bcl-xL^{9, 12, 13}, and Mcl-1^{9, 14, 15}. While Mcl-1, but not Bcl-2, is essential for survival of normal and Ph⁺ leukemic stem cell (LSC) populations¹⁶⁻¹⁹, the role of Bcl-xL in their maintenance *in vivo* is still unknown. Although loss of Bcl-xL by itself or its pharmacologic antagonism in combination with that of Bcl-2 in B-ALL mouse models did not dramatically improve survival²⁰⁻²², exposure of TKI-resistant CML-BC stem and progenitor cells to the Bcl-xL/Bcl-2 antagonist ABT-737 induced apoptosis by partially restoring sensitivity to imatinib²³. However, therapeutic CML-BC strategies involving pharmacologic antagonism of Bcl-xL could be further refined and potentiated not only by associating a Bcl-xL/Bcl-2 antagonist with TKIs, as BCR-ABL1 kinase mutation-independent relapse is the common outcome for TKI-treated CML-BC patients²⁴, but also by combining the orally bioavailable formulation of ABT-737 (i.e. ABT-263) that reportedly has a clinically-manageable toxicity profile²⁵, with other non toxic drugs capable of further modulating apoptosis. Since the BCR-ABL1-regulated²⁶⁻²⁸ pro-apoptotic factor BAD is the primary binding partner of Bcl-xL²⁵, and it undergoes phosphorylation (inhibition) upon cytokine- or oncogene-induced activation of Akt and mTORC1/2 signaling²⁹, pharmacologic restoration of BAD activity combined with suppression of Bcl-xL activity might fully restore TKI sensitivity or, *per se*, strongly initiate apoptosis of CML-BC progenitors even when BCR-ABL kinase-independent signals (e.g. microenvironmental-induced^{9, 10}) control survival of CML-BC progenitors. Thus, it is highly plausible that dual inhibitors of the BCR-ABL1-activated^{30, 31} and PI3K-Akt-dependent mTORC complexes^{1/2}²⁹ (e.g. OSI-027³² and PP242³³) that reportedly limit proliferation and colony forming ability of mononuclear cells (MNCs) from CML-BC patients^{34, 35}, have the strong ability to activate BAD and likely potentiate the effects of Bcl-xL/Bcl-2 antagonism in CML-BC.

Here we show deletion of the *bcl-x* gene in the BCR-ABL1⁺ LSC-enriched cell compartment neither altered stem cell frequency nor improved mice survival albeit none of the *bcl-x* deficient mice underwent disease progression and developed a lymphoid CML-BC-like leukemia phenotype³⁶; suggesting that Bcl-xL may be important for the survival of BCR-ABL1⁺ progenitors undergoing progression. Additionally, we found that PP242 has the ability to activate BAD and potentiate the effects of ABT-263-mediated antagonism of Bcl-xL. Combination of ABT-263 with PP242 efficiently and selectively induced apoptosis in BCR-ABL1⁺ cell lines and primary CML-BC progenitors, but not CD34⁺ progenitors from healthy donors, and overcame TKI-resistance induced by signals generated by stromal cells. Furthermore, shRNA studies confirmed efficacy of this strategy depends, at least in part, on PP242-induced BAD activation. Likewise, genetic manipulation of the BCR-ABL1/Bcl-xL/BAD interplay through shRNA-mediated impairment of the BCR-ABL1-regulated heterogeneous ribonuclear protein A1 (hnRNP A1)³⁷ resulted in lower levels of Bcl-xL expression and BCR-ABL1 kinase activity, and increased sensitivity of CD34⁺ CML-BC

progenitors to the pro-apoptotic activity of PP242, suggesting the efficacy of ABT-263 in these studies results from its ability to inhibit Bcl-xL, and not Bcl2. Furthermore, antagonism of Bcl-xL while activating BAD may represent an efficient pharmacologic approach to augment TKI-based therapeutic protocols for CML patients with advanced and drug-insensitive stages of the disease.

METHODS

Generation and analysis of the Bcl-xL-deficient BCR-ABL⁺ transgenic mice

Inducible SCL^tTA-BCR-ABL1-cre-bcl-x fl/fl mice were generated through cross breeding of SCL^tTA³⁶, pTRE-BCR-ABL1³⁸, and tet-O-cre³⁹ lines, with mice carrying loxP sites flanking exons 1 and 2 of the *bcl-x* gene⁴⁰. Breeding was done while administering tetracycline in drinking water³⁸; PCR-mediated genotyping was performed as described³⁸ with gene specific primers (Table 1). Efficiency of recombination within *bcl-x* was assessed by 3-primer (A, B and C) PCR⁴⁰ on DNA isolated from bone marrow (BM) and splenic MNCs. Following recombination, primers A and C generate the 280 base pair product (bp). In the presence of a non-recombined allele, primers A and C do not amplify and the 300 bp product from primers A and B is observed. Induction of BCR-ABL1 (p210) transgene and *cre recombinase* was achieved by tetracycline withdrawal. Mice were induced at 6 to 8 weeks of age and studies were performed with approval of the Medical College of Wisconsin's IACUC.

Culture of cell lines and primary cells, colony forming, and long term culture-initiating cell (LTC-IC) assays

The CML-BC cell lines 32D-BCR-ABL1 (6.15 clone), LAMA84 (kindly provided by Dr. A. Reid, Imperial College, London UK) and K562 were maintained in culture in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS and 2 mM L-glutamine. For maintenance, cellular fractionation, and drug treatments, 32Dcl3 and derived lines were cultured in the presence of 10% (v/v) WEHI conditioned medium as source of IL-3. For experiments requiring the use of conditioned medium (CM) from the telomerase-immortalized (TERT⁺) human mesenchymal stem cell lines (hTERT⁺ stromal line⁴¹; kindly provided by Dr. D. Campana, NUS, Singapore), LAMA84 cells were maintained in 100% CM 18 hours preceding and during drug treatments (24 hr.).

Frozen CD34⁺ Normal Bone Marrow (NBM) cells from different healthy donors were obtained from Cincinnati Children's Hospital and The Ohio State University (OSU). Studies with human CML specimens included those obtained from The Ohio State University Leukemia Tissue Bank; the Division of Hematology, Maisonneuve-Rosemont Hospital, Montréal QC and from the Department of Hematology, Aarhus University, Denmark, and were carried out with approval from The OSU Institutional Review Board. The percentage of Ph⁺ cells analyzed by FISH ranged from 91% to 100%. The CD34⁺ fraction was isolated by magnetic cell sorting (MACS, Miltenyi Biotec, Auburn, CA) and cultured in IMDM containing 30% FBS, 2 mM L-glutamine and supplemented with recombinant human cytokines (StemSpan CC100; Stem Cell Technologies, Vancouver, BC). Mouse lineage-negative/Sca-1⁺/c-Kit⁺ (LSK) cells were isolated from femur and/or spleen of induced and non-induced (WT) animals as described³⁶. All *in vitro* studies using primary mouse cells were done with the OSU IACUC's approval. Colony forming (CFC) and replating assays, determination of LTC-IC frequency, and lentiviral production and transduction were performed as described in Supplemental Methods.

Isolation of stem/progenitor cell-enriched fractions and flow cytometry-based assays

Total and lineage-depleted mouse BM cells were isolated as described³⁶. FACS-mediated analysis of hematopoietic markers was performed with combinations of the following antibodies: anti-Gr-1 PE, anti-Mac-1 FITC, anti-B220 APC, anti-CD19 PeCy7, anti-Ter119 PeCy7, and anti-c-kit APC AF750 (eBioscience, San Diego, CA), anti-CD71 Biotin and, anti-Sca-1 PeCy7 (BD Biosciences). CML specimens were subjected to CD34 positive-selection, and the hematopoietic stem cell-enriched fraction (CD34⁺/CD38⁻) along with common myeloid progenitors (CMP, CD34⁺/CD38⁺/CD123⁺/CD45R⁻) and granulocyte monocyte progenitors (GMPs, CD34⁺/CD38⁺/CD123⁺/CD45R⁺) were separated following staining with anti-CD34 AF647 (4H11) and anti-CD38 PeCy7 (HIT2) (eBioscience), anti-CD123 PE (9F5) (BD Pharmingen), and anti-CD45R PE Texas Red (Invitrogen) antibodies and cell sorting (Aria, Becton Dickinson, Franklin Lakes, NJ). Determination of the percentage of apoptotic cells in untreated and after 3 (cell lines) and 6 (primary cells) days of drug treatment were assessed by Annexin V PE staining (BD Biosciences) and Sytox Blue Live/Dead Stain (Invitrogen). All analyses were performed on a tri-laser fluorescent-activated cell sorter (FACS) (LSRII, Becton Dickinson). Cells were thereafter used for RNA isolation, Real Time PCR and Western blot analyses as described in detail in Supplemental Methods.

Reagents (Chemical Inhibitors and Plasmids)

Culture medium containing cell lines and primary cells seeded at a density of 10⁵ and 10⁶ per milliliter, respectively, were exposed to inhibitors at the doses indicated in the results section. Cell lines were treated for 72 hours, except for LAMA84 cells which were treated for 24 hours due to sensitivity to all treatments. The drugs used include Imatinib (Novartis), LY294002 (Cayman Chemical, Ann Arbor, MI), Rapamycin (Sigma, St Louis, MO), ABT-263 (ChemieTek, Indianapolis, IN), PP242 (Chemdea, Ridgewood, NJ), and U0126 (Promega).

The pLL3.7-hnRNPA1(shRNA) construct was obtained by cloning the annealed oligonucleotides 5'-tAGCAAGAGATGGCTAGTGCtcaagagaGCACTAGCCATCTCTTGCTtttttgaac-3' into the HpaI and NotI sites of the pLL3.7 lentiviral plasmid. Bases specific for hnRNP A1 shRNA are capitalized. The BAD shRNA-containing lentiviral construct was generated as described⁴².

Statistical analysis

Data are expressed as means ± SEM and were compared using the Student *t* and/or Fisher exact tests. *P* values < 0.05 are considered significant.

RESULTS

The survival factor Bcl-xL is dispensable for development of CML *in vivo*

BCR-ABL1-dependent induction of Bcl-xL expression, albeit not required for the emergence of Ph⁺-ALL in animals²², seems to be important, at least *in vitro*, for survival of CML-BC cell lines^{12, 13}. High levels of BCR-ABL1 expression similar to those found in CML-BC blasts⁴³ resulted in the imatinib-sensitive induction of survival factors Mcl-1 and Bcl-xL, but not Bcl-2, and in increased expression and activity of their post-transcriptional modulators^{37, 43, 44} (e.g. hnRNP A1) and upstream regulators of cell survival (e.g. Akt) (Fig. 1A, top left). Accordingly, Akt-regulated activity of pro-apoptotic BAD was restored upon kinase inhibition of BCR-ABL1, as indicated by the appearance of the non-phosphorylated (active⁴⁵) BAD in the mitochondrial (M) fraction of imatinib-treated 32D-BCR-ABL1 cells (Fig. 1A, bottom left). To assess whether expression of Bcl-xL has a role

in CML-development, maintenance and/or progression *in vivo*, we crossed SCLtTA-BCR-ABL1 (dTg) mice, which upon induction of BCR-ABL1 develop a CML-like myeloproliferative disorder (MPD) that progresses into a lymphoid blast crisis (L-BC)-like disease in 30% of mice³⁶, with inducible *bcl-x*-deficient animals²² to generate the SCLtTA-BCR-ABL1-cre-Bcl-x fl/fl (dTg/KO) mouse line (Fig. 1B, top). SCL-driven expression of BCR-ABL1 increased protein levels of Bcl-xL and that of its post-transcriptional modulator hnRNP A1³⁷ in MNC and stem cell-enriched (LSK) cell fractions, respectively, isolated from spleens of 8 and/or 12 week-induced dTg mice, (Fig. 1A, top and bottom right). Note that MNCs and LSKs from non-induced littermates (wild type; WT) were used as controls. However, the almost complete loss of Bcl-xL mRNA (~75% reduction) and protein (~90% reduction) expression in BM and/or splenic LSKs (Fig. 1B, bottom left) and MNCs (Fig. 1B, bottom right), respectively, neither altered the frequency of BCR-ABL1⁺ LSK cells (Fig. 1C) nor prevented the development of a CML-like MPD as indicated by increased presence of Gr-1⁺/Mac-1⁺ myeloid cells³⁶ in PB of 8, 12 and 16 week-induced dTg/KO animals (Fig. 2A, left and Suppl. Fig 1A). dTg/KO mice developed splenomegaly (Suppl. Fig 1B, left) and did not demonstrate significantly different overall survival ($p=0.14$) (Figure 1D), suggesting that the anti-apoptotic potential of Bcl-xL might be dispensable for both the maintenance of human Ph⁺ stem cell compartment and development of CML. In fact, succumbed dTg/KO mice had a phenotype mostly superimposable with that of the original SCLtTA-BCR-ABL1 mouse model³⁶. In addition to splenomegaly and high percentages of Gr-1⁺/Mac-1⁺ cells in PB, BM and spleen (Suppl. Fig. 1A), they also presented pale brittle bones (not shown), and massive infiltration of myeloid cells into spleen, liver and kidney (Suppl. Fig 1B, right). Likewise, deletion of Bcl-x did not alter the frequency of erythroid (Ter119⁺/CD71⁺) and lymphoid B- (B220⁺/CD19⁺) cells (Suppl. Fig. 1A). Consistent with the existence of a BCR-ABL1-induced and hnRNP A1-mediated posttranscriptional control of Bcl-xL expression³⁷, we found almost identical levels of *bcl-x* mRNA in WT and dTG LSK cells (Fig. 1B bottom left) whereas higher Bcl-xL protein (Fig. 1A and 1B bottom right) and hnRNP A1 levels (Fig. 1A bottom right) were detected in MNC and/or LSK cells from dTg animals.

Bcl-xL expression is required for CML disease progression *in vivo*

To determine whether Bcl-xL plays a role in CML blastic transformation, a cohort of 8-12 week-induced dTg (n=8) and dTg/KO (n=12) animals presenting with marked neutrophilia, as evidenced by the percentage of Gr-1⁺/Mac-1⁺ cells almost twice that of non-induced littermates [%Gr-1⁺/Mac-1⁺: 24.05±3.0 (dTg); 34.9±3.8 (dTg/KO); and 13.6±1.7 (non-induced control mice; n=3)], were monitored for signs of disease progression³⁶. A significantly increased number of B220⁺/CD19⁺ cells in PB (Fig. 2A, left) and the appearance of a B220^{dim}/CD19⁺ (Fig. 2A, right) population of lymphoblasts in the spleen was observed in 3 out of 8 dTg but not in the dTg/KO mice (n=12) between 8 and 12 weeks post BCR-ABL1 induction, indicating that loss of Bcl-xL impairs the transformation of a CML-CP-like disorder into a L-BC-like acute leukemia³⁶ ($p = 0.05$). Consequently, dTg mice with the transformed L-BC-like disease but not dTg/KO animals presented B220⁺/BP-1⁺ lymphoblasts in PB, lymph nodes, and BM as well (not shown). BM examination of dTg/KO animals demonstrated nearly complete gene recombination in purified populations of both myeloid (Gr-1⁺/Mac-1⁺) and lymphoid (B220⁺/CD19⁺) cells (Fig. 2B).

Inhibition of Bcl-xL triggers apoptosis of BCR-ABL1⁺ myeloid progenitors and is potentiated by reactivation of BAD

Previous studies report that it is the anti-apoptotic activity of Bcl-xL, but not Bcl-2, which reconstitutes most, albeit not entirely, the leukemogenic potential^{4, 12, 46} of BCR-ABL1 in CML-BC-progenitors. To assess whether Bcl-xL can be used as a therapeutic target in CML-BC, 32D-BCR-ABL1 and LAMA84, which are models of blast crisis, were used to assess sensitivity of these cells to the Bcl-xL/Bcl-2 antagonist ABT-263. In three

independent experiments, flow cytometric analysis of Annexin V- and Sytox Blue-stained cells revealed that treatment with a single dose of ABT-263 (1 μ M) induced a ~50% decrease in cell survival compared to vehicle-treated cells (Fig. 3A, left). Furthermore, ABT-263 (1 μ M) did not alter the percentage of dTg (n=4) LSK-derived colony forming cells (~10% inhibition) and their replating efficiency (Fig. 3A, middle). Similarly, the LTC-IC frequency of Lin⁻ BM cells from 8 week-induced dTg mice (n=3) remained almost identical in untreated and ABT-263-treated cells (~15% reduction) (Fig. 3A, right), suggesting that loss of Bcl-xL does not influence the self-renewal and survival of BCR-ABL1-transformed hematopoietic stem cells.

Thus, because of the important role played by BAD in BCR-ABL1-driven leukemogenesis²⁶⁻²⁹ and in the regulation of Bcl-xL activity²⁵, we evaluated whether pharmacologic activation of BAD achieved through interference with the PI3K/Akt/mTORC1/2²⁹ or MEK1/MAPK⁴⁷ signaling enhances ABT-263-induced apoptosis of BCR-ABL1⁺ cells. 32D-BCR-ABL1 cells were treated for 18 hours with the archetypical PI3-Kinase inhibitor LY294002 (20 μ M), mTORC1 inhibitor Rapamycin (0.1 μ M), mTORC1/2 inhibitor PP242 (0.1 μ M), or the MAP-Kinase inhibitor U0126 (25 μ M) and levels of phosphorylated (pBAD) and non-phosphorylated BAD as well as that of other survival signaling molecules (e.g. Akt, Mcl-1, Bcl-xL, Bcl-2 and c-Myc) were determined. Western blot analysis performed on subcellular fractionated (Fig. 3B, left) and total protein lysates (Fig. 3B, right) show that PP242, LY294002 and Rapamycin induced BAD activation as indicated by the readily detectable non-phosphorylated BAD in whole cell (WC) and mitochondrial (M) lysates (Fig. 3B, left). By contrast, inhibition of MEK1 by U0126 did not induce BAD activation (Fig. 3B), consistent with persistence of Akt- and p70 S6 kinase-dependent BAD phosphorylation on serine 136²⁹. As expected, BAD was heavily phosphorylated/inactive in vehicle treated (untreated) (Fig. 3B, left) 32D-BCR-ABL1 cells. Likewise, levels of Mcl-1 and that of c-Myc were significantly reduced by treatment with LY294002, PP242 or Rapamycin, and PP242 or Rapamycin, respectively (Fig. 3B, right), while expression of Bcl-xL and Bcl-2 were not influenced by suppression of PI-3K/Akt/mTORC1/2-mediated signals (Fig. 3B, right).

Activation of BAD in PP242-treated 32D-BCR-ABL1 and LAMA84 cells did not alter survival (Fig 3A); however, 90-95% were apoptotic (Annexin V⁺) after exposure of both BCR-ABL1⁺ lines to single treatment with a combination of 1 μ M ABT-263 and 0.2 μ M PP242 (n=3) (Fig. 3A, left). Although previous work reported a modest decrease (MTT-based assay) in proliferation/survival in PP242-treated BCR-ABL1⁺ cell lines³⁵, PP242 failed to induce apoptosis of both LAMA84 and 32D-BCR-ABL cells when used at lower concentrations (0.2 μ M) (Fig. 3A, top), most likely due to high Bcl-xL levels. The potentiating effect of this TORC1/2 inhibitor on the pro-apoptotic activity of ABT-263 in cell line models of blast crisis may depend on its ability to activate BAD which in turn, antagonizes the anti-apoptotic function of Bcl-xL²⁵. We tested this hypothesis by genetic manipulation of BAD expression with shRNA which showed that ~50% BAD knock-down in K562 cells (Fig. 3C, left) is sufficient to prevent PP242 from augmenting the pro-apoptotic effects of ABT-263 (Fig. 3C, right). Furthermore, Annexin V-based apoptosis assays revealed that 32D-BCR-ABL1 cells are two times more sensitive than 32Dcl3 cells to combined pharmacologic inhibition of Bcl-xL with ABT-263 (0.025-2 μ M) and activation of BAD by PP242 (0.005-0.4 μ M) with EC₅₀ values of 0.48 μ M ABT-263/0.1 μ M PP242 for 32D-BCR-ABL1, and 1.0 μ M ABT-263/0.2 μ M PP242 for 32Dcl3 cells (Fig. 3D).

The combination of ABT-263 with PP242 triggers apoptosis of CML-BC but not CML-CP or normal CD34⁺ progenitor cells, and overcomes microenvironment-induced TKI resistance

Methylcellulose-based clonogenic assays revealed that the combination of ABT-263 (0.1 μ M) and PP242 (0.05 μ M), used at one-tenth and one-fourth of the concentrations given to

cell lines, dramatically decreases size (not shown) and number (~90% inhibition) of cytokine-driven myeloid colony forming cells from CD34⁺ BM CML-BC, but not CML-CP (~15% reduction) progenitors (n=3) (Fig. 4A). Marked (~85-95%) apoptosis (Annexin V⁺) was induced by the same drug combination in CD34⁺ progenitors isolated from BM of CML-BC (n=6) (Fig. 4B, black bars) but not healthy (n=3) donors in which a 6-day exposure to both drugs resulted in a 40% reduction in viability (Fig. 4B, white bars). A significant but modest (~50% reduction) impairment of CD34⁺ CML-BC (n=3) colony formation was observed when these drugs were used separately (Fig. 4A). This correlated with a 30% decrease in viability of ABT-263- but not PP242-treated CD34⁺ BM cells from CML-BC (n=6) and healthy (n=3) individuals (Fig. 4B).

Previous studies show that the BM stroma protects CML-BC (cell lines⁹ and primary cells¹⁰) from TKI-induced cell death. To determine whether PP242 and ABT-263 treatment overcomes microenvironmentally-induced drug resistance, LAMA84 cells were cultured for 42 hrs. in conditioned medium from the TERT⁺ human mesenchymal stem cell line, while exposed for 24 hr. to either 1 μM imatinib or the combination 0.1 μM ABT-236 and 0.2 μM PP242. Flow cytometric analysis of Annexin V/Sytox Blue-stained cells showed near complete cell death in LAMA84 cells treated for 24 hr. with ABT-263 and PP242 regardless of the presence of BM stroma CM (Fig. 4C). As expected, TKI (e.g. imatinib)-induced apoptosis was strongly inhibited by culturing LAMA84 cells in hTERT⁺ stromal cell CM, suggesting that suppression of Bcl-xL/Bcl-2 and TORC1/2 pathways efficiently overcomes extrinsic BM stromal signals conferring resistance of Ph⁺ leukemic progenitors to TKI-induced apoptosis.

Impaired Bcl-xL expression by hnRNP A1 shRNAs mimics the pro-apoptotic effects of ABT-263 and potentiates efficacy of PP242

hnRNP A1 expression was found to gradually increase, in a BCR-ABL kinase-dependent manner, in paired CML-CP and -BC BM MNC samples³⁷. We found that levels of hnRNP A1 are specifically elevated in the CML-BC cell populations which, as reported, show innate or acquired TKI-resistance⁴⁸, have the ability to self-renew⁴⁹ and, most likely, represent the origin of the progressing cell clone⁴. In fact, hnRNP A1 expression was increased in CD34⁺/CD38⁻ (HSC) and GMP cell fractions of CML-BC (n=3) when compared to the CML-BC CMP (Fig. 5A, left and middle) and to HSC and GMP cells fractions from BM of healthy (n=3) and CML-CP (n=4) individuals (Fig. 5A). Expression of hnRNP A1 was ~3 times higher in chronic phase (n=4) and almost 10 times higher in a blast crisis patient (Fig. 5A, right).

Since hnRNP A1 is a positive post-transcriptional modulator of Bcl-xL expression³⁷, we modulated levels of hnRNP A1 with shRNA to better understand the molecular mechanisms by which the Bcl-xL/Bcl-2 antagonist ABT-263 exerts its pro-apoptotic activity in BCR-ABL⁺ CML-BC progenitors. Knock-down of hnRNP A1 in primary CD34⁺ CML-BC BM cells (n=3) resulted in downregulation of Bcl-xL but not Bcl-2 (Fig. 5B, left), and mimicked the effect of ABT-263 when hnRNP A1 shRNA-expressing CD34⁺ CML-BC BM cells (n=3) were exposed to 0.1 μM PP242 (Fig. 5B, right). Annexin V staining revealed shRNA-mediated decreased levels of hnRNP A1 impaired survival of CD34⁺ CML-BC progenitors by ~60% 6 days after GFP-selection compared to vector-transduced progenitors (Fig. 5B). Viability of shRNA infected cells was further reduced (*p* 0.05) upon addition of 0.1 μM PP242 (~20% survival), suggesting that expression of Bcl-xL rather than Bcl-2 is important for survival of CML-BC progenitors, and that ABT-263 exerts its proapoptotic activity in CML-BC cells through inhibition of Bcl-xL. As expected, shRNA-mediated suppression of hnRNP A1 expression (~65% inhibition) resulted in downregulation of the hnRNP A1-target SET, thereby leading to PP2A reactivation^{4, 50}, and, consequently, downregulation of BCR-

ABL1 and its downstream effectors (e.g. hnRNP E2 and K)^{43, 44} in CD34⁺ CML-BC progenitors (Fig. 5B).

DISCUSSION

The dismal outcome of patients with CML-BC treated with either TKIs or other experimental drugs reflects our lack of a clear understanding of which BCR-ABL kinase-dependent and/or -independent pathways are significantly contributing to disease progression^{2, 4}. Among these, several regulators of apoptosis (e.g. Bcl-xL) have been proposed to be important for survival of CML-BC progenitors⁵¹; however, whether their contribution is critical for disease progression *in vivo* is still unclear. By using a mouse model of CML blastic transformation³⁶, we showed that the anti-apoptotic factor Bcl-xL is dispensable for development and maintenance of a CML-CP-like disease in mice but required for transformation into an L-BC-like disorder (Fig. 1, 2 and S1). Development of leukemia in the absence of *bcl-x* expression *in vivo* was unexpected because of both the dependence of Bcl-xL expression on BCR-ABL1 kinase activity, and the numerous *in vitro* studies suggesting a role for Bcl-xL in BCR-ABL1 kinase-dependent and -independent survival of CML-BC cells and their resistance to pro-apoptotic stimuli^{9, 12, 13}. We also showed that genetic and pharmacologic (ABT-263) loss of Bcl-xL expression and/or activity did not alter BCR-ABL1⁺ stem cell (LSK) number, survival and self-renewal activities while preventing *in vivo* expansion of more committed progenitors which, like the CML-BC GMPs^{4, 49}, represent a secondary CML cell population demonstrating increased BCR-ABL1 expression, survival/proliferation advantage, increased genomic instability and, likely, self-renewal. However, while the L-BC-like disease maintains BCR-ABL1 kinase-dependence in dTg mice, relapse and BCR-ABL kinase-independence are two phenomena commonly observed in TKI-treated CML-BC patients^{36, 38}. Furthermore, despite the proposed role for Bcl-2 in disease progression^{46, 52}, expression studies done in CML patients indicate that disease progression does not directly correlate with Bcl-2 levels⁵³, suggesting that Bcl-xL, and possibly its negative regulator BAD, may play an important role in both CML-BC development and BCR-ABL1-independent TKI resistance, which is likely induced by microenvironment-generated signals rather than depending on the presence of leukemic cell clone(s) harboring BCR-ABL1 mutations^{9, 10}.

In support of a significant biological role played by both Bcl-xL and BAD in CML-BC and not CML-CP, we showed that low concentrations of the orally-available Bcl-2/Bcl-xL inhibitor ABT-263 (100 nM) exerts a strong and selective cytotoxicity towards CD34⁺ CML-BC but not CP or normal progenitors (Fig. 3 and 4) when used in combination with suboptimal concentrations of drugs (e.g. 50 nM PP242) which lead to BAD activation (Fig. 3). Indeed, treatment of both BCR-ABL1⁺ cell lines and CD34⁺ CML-BC progenitors with combined low doses of ABT-263 and PP242 reduced viability by ~90% without having any significant effect on CD34⁺ hematopoietic cells from healthy individuals. The anti-leukemic effect of a combined Bcl-xL/Bcl-2 antagonist (i.e., ABT-737 or ABT-263) and PP242 treatment has been previously investigated in cell line models of Burkitt's lymphoma (0.5 μ M ABT-737/1.25 μ M PP242) and acute T-cell leukemia (T-ALL) (0.01-1 μ M ABT-263/0.01-1 μ M PP242)^{54, 55}. However, while the ABT-263/PP242 combination strongly resulted in apoptosis of primary CML-BC cells and cell lines, these drugs had only a modest killing (30% induction of apoptosis) in Burkitt's lymphoma and a very limited synergistic effect in T-ALL cell lines^{54, 55}, suggesting that the Bcl-xL/BAD interplay specifically plays a crucial role in survival of CML-BC but not all leukemic progenitors. Note that alone, neither ABT-263 nor PP242 had a significant effect on survival of CML-BC progenitors when used at 0.1 μ M and 0.050 μ M concentrations, respectively (Fig. 4), although it has been shown that higher doses of PP242 decreased clonogenic potential of CML-BC cells³⁵, most likely through its inhibitory effect on mTORC1/2-Akt1-regulated Mcl-1 expression (Fig. 3).

Consistent with our data obtained with 100 nM ABT-263 in both leukemic and normal CD34+ progenitors, it has been reported²³ that suppression of Bcl-xL/Bcl-2 activities by 100 nM ABT-737 accounts only for 20-30% of apoptosis. Furthermore, low or no sensitivity to the ABT-737/ABT-263 compounds, even if used at concentrations as high as 10 μ M, has been reported for Ph+ cell lines and primary CML stem/progenitor cells^{23, 25, 56}. The limitation of this drug as a single therapeutic agent in CML-BC is supported by evidence indicating resistance to its pro-apoptotic activity is induced in malignancies (e.g., CML-BC^{9, 12, 13}) where Bcl-xL and/or Mcl-1 are overexpressed^{23, 57}.

Given that microenvironment-induced TKI resistance has also been in part associated with the ability of extracellular BM soluble factors to increase Mcl-1, Bcl-xL, survivin, and mTORC1/2 levels in leukemic progenitors^{9, 58}, and that downregulation of Mcl-1 restores sensitivity of leukemic cells to ABT-737^{59, 60}, it is likely that a combined ABT-263/PP242 would be more effective than the single agent approaches. Indeed, we not only provided evidence indicating that PP242 is capable of reducing Mcl-1 levels but we also showed that ABT-263/PP242 treatment efficiently (90% induction) promoted apoptosis of CML-BC cells even in the presence of external factors (hTERT⁺ stromal cell CM) capable of inducing TKI resistance (Fig. 3 and 4). Mechanistically, shRNA-mediated suppression of BAD or hnRNP A1 that, in turn, leads to Bcl-xL but not Bcl-2 downregulation, allowed us to determine that inhibition of Bcl-xL and restoration of BAD activity mostly accounts for the apoptosis induced in CD34⁺ CML-BC progenitors by the Bcl-xL/Bcl2 antagonist ABT-263 and mTORC1/2 inhibitor PP242, respectively (Fig. 5). However, it is likely that PP242-induced inhibition of the mTORC1/2- and Akt-mediated survival signals also plays a key role in the apoptotic response of leukemic progenitors to the ABT-263/PP242 combination (Fig. 6).. Additionally, the strong apoptotic effect of the ABT-263/PP242 combination might also depend on interference with other BCR-ABL1 kinase-dependent and -independent survival signals. In fact, co-treatment of ABT-737 with imatinib induced not only a ~50% and ~25% apoptosis in CML-BC^{23, 56} and normal progenitors²³, respectively, but also restored TKI sensitivity of CD34⁺/CFSE^{MAX} CML-BC and CD34⁺/CD38⁻ CML-CP stem cell-enriched populations^{23, 56}, suggesting that BCR-ABL1-dependent and -independent survival pathways are simultaneously affected.

In conclusion, although we cannot determine whether the combination of ABT-263 with PP242 would be more effective than TKIs in CML-BC therapy, our *in vitro* data strongly suggest that pharmacologic inhibition of Bcl-xL together with activation of its negative regulator BAD has a greater and more profound deleterious effect (90% induction of apoptosis in the presence of external cytokines) on survival of CML-BC progenitors regardless of the drug-resistance induced in these cells by BM-generated signals^{9, 10}.

Finally, it remains unknown whether TKIs with ABT-737/ABT-263 treatment would be sufficient to induce a sustained molecular remission, or the ~50% surviving drug-resistant CML-BC progenitors would account for relapse. Of clinical interest, our data support the application of an alternative therapeutic protocol in which patients would be initially debulked with TKIs to reduce leukemic blast burden and, thereafter, treated with ABT-263 and PP242 to eliminate residual TKI-resistant CML-BC clones.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

D.P. is a Scholar of The Leukemia and Lymphoma Society. This work was supported in part by NIH grants CA095512 and CA163800 (D.P.), CA16058 (OSU-CCC); Fonds de Recherche Quebec Sante– TheCell (D.C.R.);

Lauri Strauss Leukemia and BloodCenter Research Foundations (C.S.H.); the Danish Medical Research Council, the Danish Cancer Society and the Karen Elise Jensen Foundation (P.H) grants. J.G.H. was supported in part by NIH training grant HL-07209. We thank L. Hennighausen (NIH, Bethesda, MD) for providing Bcl-x f/f mice; H. Albertz and C. Reinbold (FACS Core Facility, Blood Research Institute, Milwaukee, WI) for technical assistance; J. Perrin (OSU Medical Center, Columbus OH) for helping in procuring CML specimens, and S. Lee (OSU Medical Center, Columbus OH) for editorial assistance.

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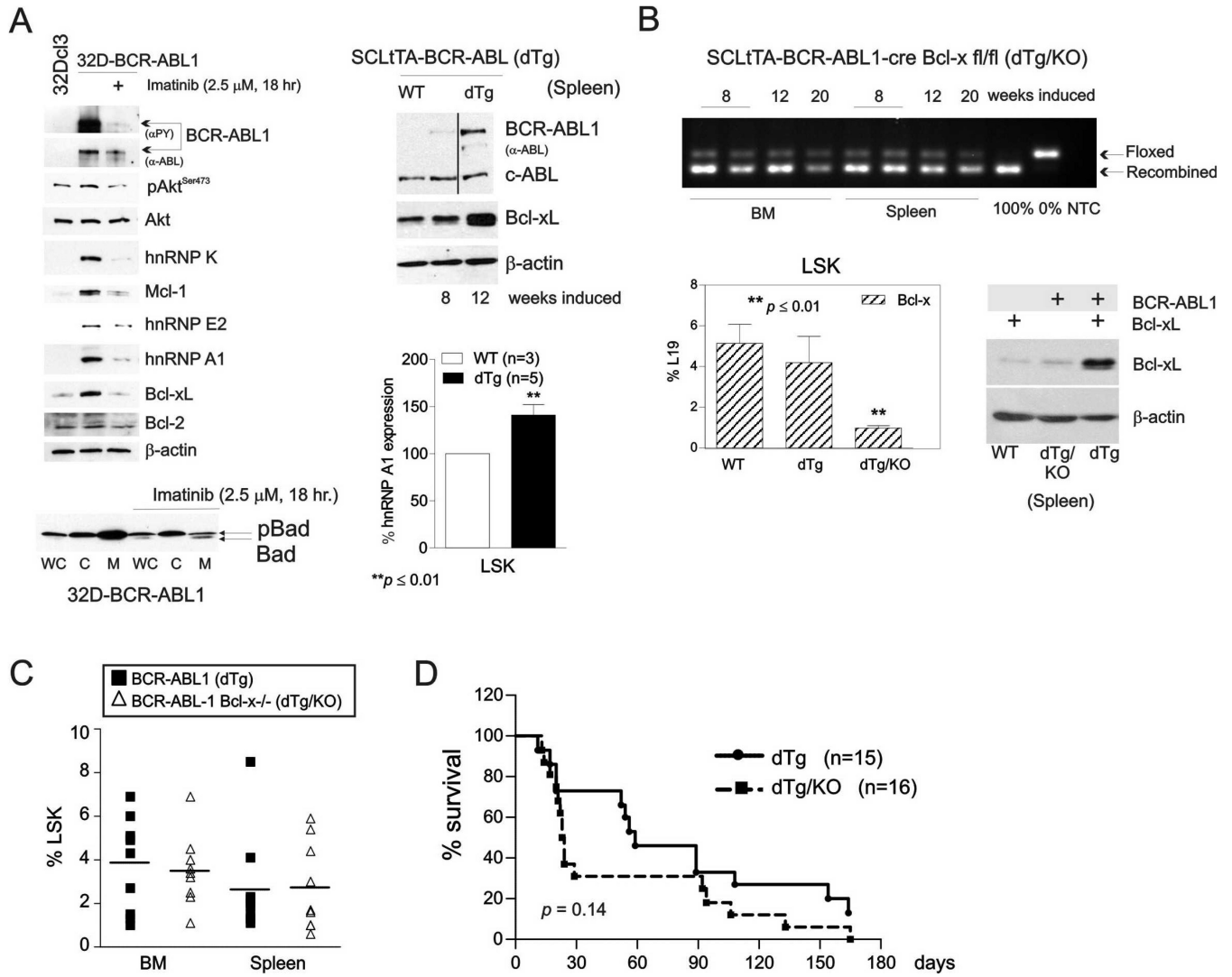


Figure 1. Expression of Bcl-xL is dispensable for BCR-ABL1-driven myeloproliferative disease (MPD) in vivo

(A) *Top left*: Western blots show levels and/or activity of Mcl-1, Bcl-xL, Bcl-2, hnRNP K, hnRNP E2, hnRNP A1, total (α-ABL) and active (αPY) BCR-ABL1, and active Akt (pAkt^{Ser473}) in parental, untreated and imatinib-treated BCR-ABL1-expressing myeloid progenitor 32Dcl3 cells. *Top right*: Bcl-xL and BCR-ABL1 protein expression in mononuclear cells (MNCs) from spleens of wild type (non-induced: WT) (lane 1) and leukemic SCLtTA-BCR-ABL1 (dTg) (lanes 2 and 3) mice. *Bottom left*: BAD expression in whole cell lysates (WC), cytoplasmic (C), and mitochondrial (M) subcellular fractions of untreated and imatinib-treated 32D-BCR-ABL1 cells. *Bottom right*: Flow cytometry analysis of hnRNP A1 expression in lineage-negative/Sca-1⁺/c-Kit⁺ (LSK) cells from the bone marrow of WT and 8-week induced dTg mice. (B) *Top*: PCR shows levels of recombination of Bcl-x floxed alleles in the bone marrow (BM) and spleen of 8, 12, and 20 week-induced SCLtTA-BCR-ABL1-cre Bcl-x fl/fl (dTg/KO) mice. Controls demonstrating complete recombination, absence of recombination, and the absence of a template (NTC) are shown in the last three lanes. *Bottom left*: bcl-x mRNA levels determined by Real-Time PCR in LSK cells from BM of WT and leukemic dTg and dTg/KO mice is reported as percentages of L19 expression. Results are given as mean ± Standard Error of the Mean

(SEM); $n = 3$. *Bottom right*: Levels of Bcl-xL protein in MNCs from spleen lysates of WT, leukemic dTg/KO, and dTg mice. **(C)** *Left*: Frequency by flow cytometry of lineage-negative/Sca-1⁺/c-Kit⁺ (LSK) cells in BM and spleen of dTg and dTg/KO mice. **(D)** Kaplan-Meier plot shows survival times of dTg and dTg/KO leukemic mice. Asterisks (*) indicate statistical significance.

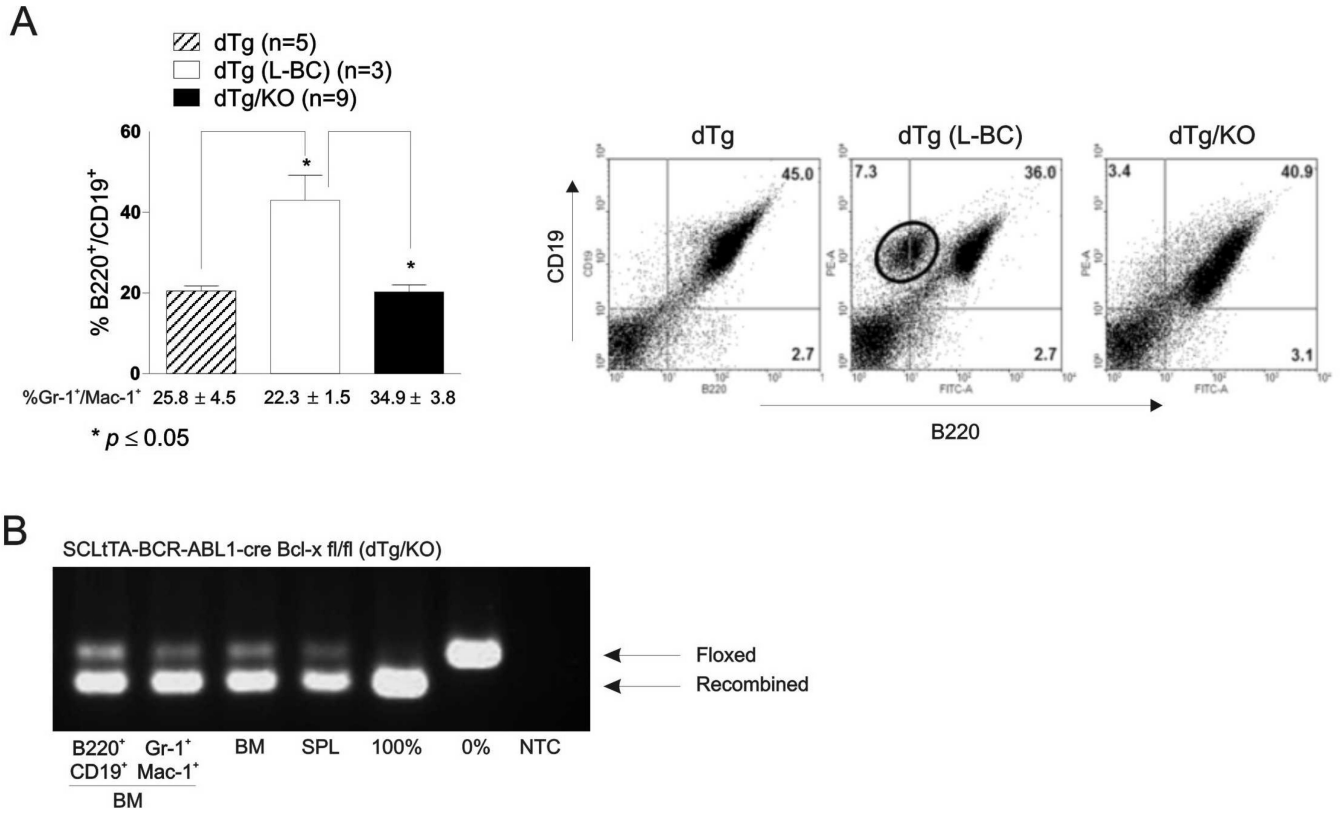


Figure 2. Bcl-x deficient BCR-ABL1 expressing mice do not progress to lymphoid blast crisis
(A) Left: Flow cytometric analysis shows percentage (mean ± SEM) of B220⁺/CD19⁺ and Gr-1⁺/Mac-1⁺ cells in the peripheral blood of leukemic dTg, dTg which progressed to lymphoid blast crisis (L-BC), and dTg/KO mice. **Right:** representative FACS panels of B220/CD19 populations in the spleen of 12 week-induced: dTg (n=5) (left panel), dTg (L-BC; n=3) (middle panel), and dTg/KO (n=12) (far right panel) mice. **(B)** PCR demonstrates levels of recombination in B220⁺/CD19⁺ (lane 1) and Gr-1⁺/Mac-1⁺ (lane 2) cells from the marrow, as well as whole bone marrow (BM) (lane 3) and spleen (SPL) (lane 4) of dTg mice 12 weeks after induction. Lane 5 and 6 are controls demonstrating complete and absence of recombination. Results shown are representative of three different mice.

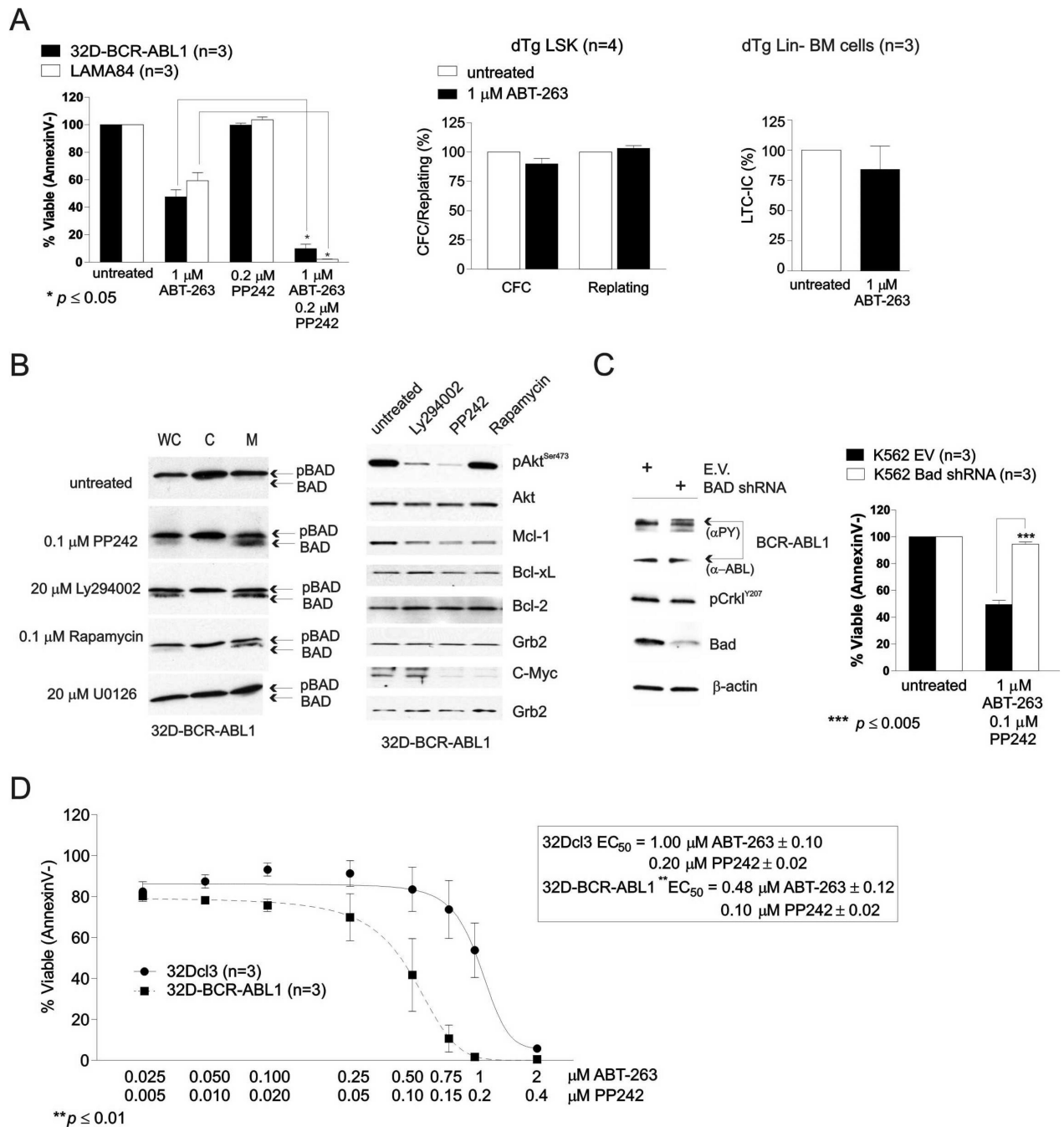


Figure 3. Inhibition of Bcl-xL triggers apoptosis of BCR-ABL1⁺ myeloid progenitors and is potentiated by reactivation of BAD

(A) *Left*: Plot shows effect of ABT-263 and PP242 used alone or in combination on survival (expressed as the mean \pm SEM of the percentage of AnnexinV⁻ cells; n=3) of untreated and drug-treated 32D-BCR-ABL1 (black bars) and LAMA84 (white bars) cells; *middle*: Methylcellulose-based clonogenic potential and replating efficiency of untreated and ABT-263-treated LSK cells from spleens of dTg mice; *right*: effect of ABT-263 treatment on the long-term culture-initiating cell frequency in lineage-negative (Lin⁻) cells from BM of 8 week-induced dTg mice. (B) Levels of the active (BAD) and inactive (pBAD) BAD (left panels) and of Akt, Mcl-1, Bcl-xL, Bcl-2, and c-Myc in whole cell (WC) and

cytoplasmic (C) and mitochondrial (M) subcellular fractions (left panels) and total lysates (right panels) of 32D-BCR-ABL1 cells untreated or treated with the indicated chemical inhibitors of known survival/proliferation pathways. **(C) Right:** Survival analysis shows that shRNA-mediated BAD downregulation suppresses the potentiating killing activity of PP242 in ABT-263-treated K562 cells. *Left:* Western blot shows downregulation of BAD by shRNA. Levels of total (-ABL) and active (PY) BCR-ABL1, pCrkl^{Y207}, and in empty vector (EV) and BAD shRNA-expressing K562 cells are shown as controls. **(D):** Annexin V-staining shows effect of increasing doses of ABT-263/PP242 combination on survival of parental (solid line) and BCR-ABL1-expressing 32Dcl3 cells (dashed line). *Inset* shows EC₅₀ values for 32Dcl3 and 32D-BCR-ABL1 cells. All data are representative of at least three independent experiments.

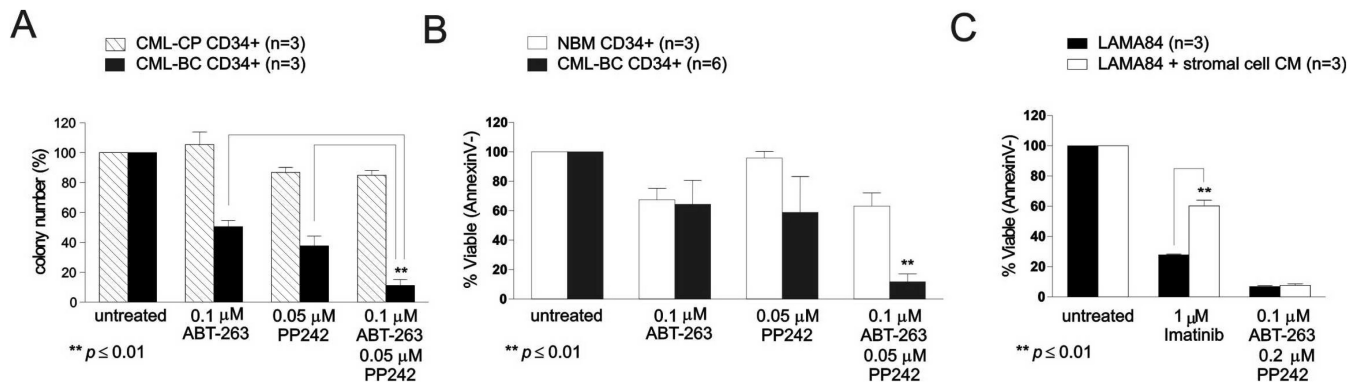
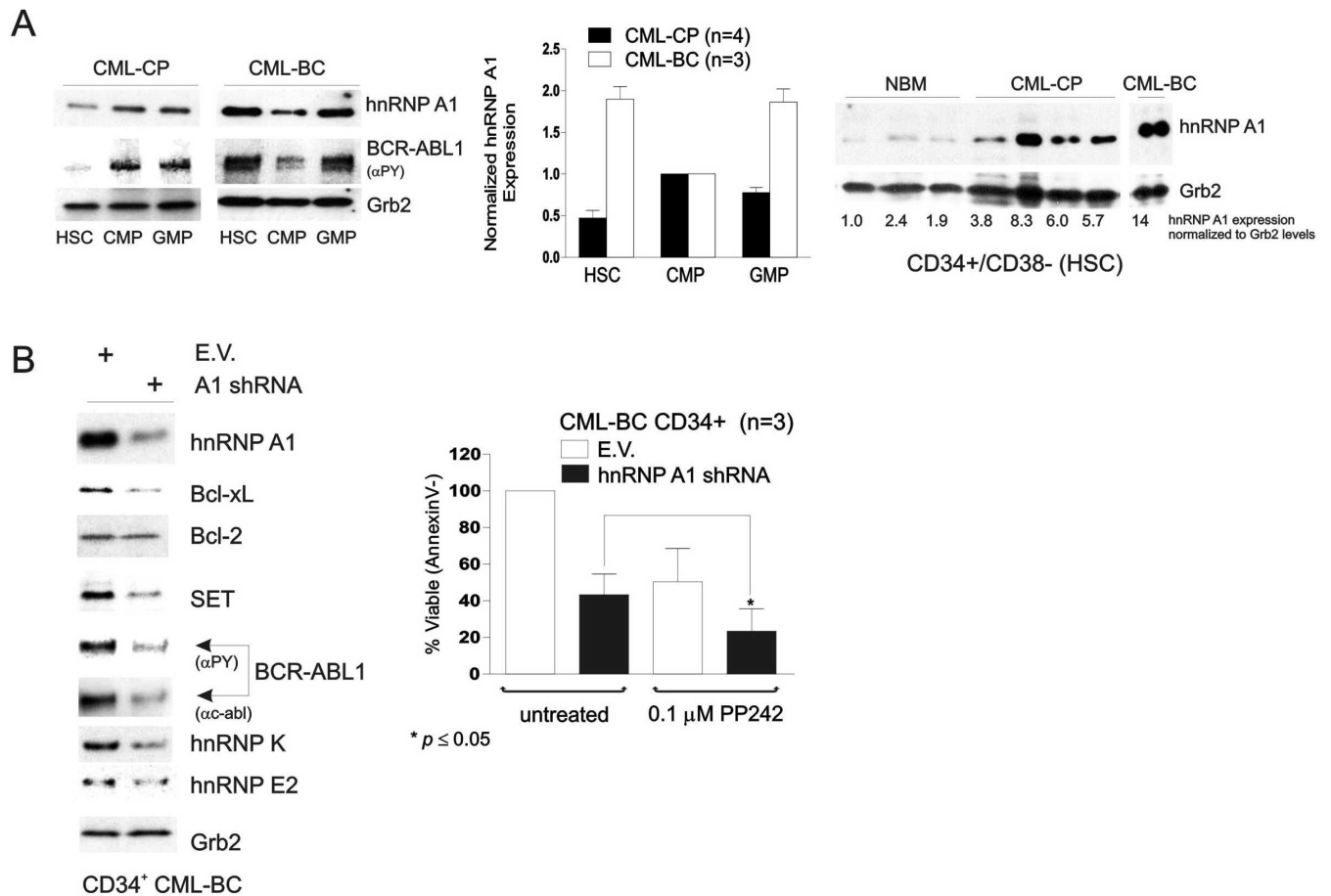


Figure 4. The combination ABT-263 and PP242 triggers apoptosis of leukemic but not normal CD34⁺ progenitor cells, and overcomes microenvironment-induced TKI resistance (A) Clonogenic and/or (B) survival analyses (mean ± SEM, $p < 0.01$) of untreated (DMSO) and drug (ABT-263 and/or PP242)-treated CD34⁺ BM progenitors from CML-CP, CML-BC patients and/or healthy individuals (NBM: normal bone marrow). (C) Survival analysis (mean ± SEM; n=3) shows effect of extrinsic drug-resistance signals provided by hTERT⁺ stromal cell-conditioned medium (CM) on the pro-apoptotic activity of Imatinib and ABT263/PP242 in Ph⁺ LAMA84 cells.



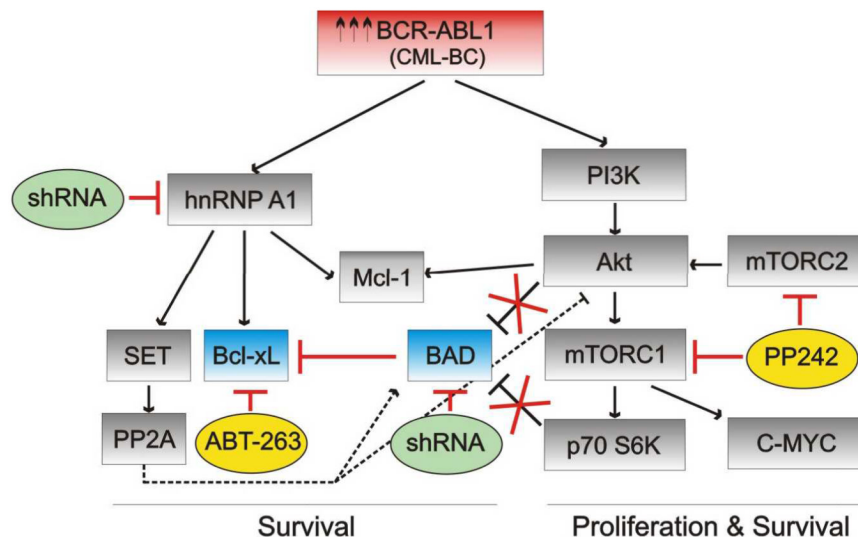


Figure 6. Effect of altered Bcl-xL and BAD expression and activity on BCR-ABL1 signaling in CML-BC

Schematic representation of the survival/proliferation pathways induced by high levels of BCR/ABL-kinase activity and likely affected by altered Bcl-xL and BAD expression/activity (blue) upon exposure of CML-BC cells to ABT-263 or PP242 (yellow), or after shRNA-mediated downregulation (green) of BAD or hnRNP A1 in CML-BC. Dashed lines indicate pathways normally inactive in CML-BC. Red lines indicate pathways influenced by drug-treatment or shRNA expression.

Table 1

Sequences of primers used for genotyping, real-time, and 3-primer PCR

Primers used for genotyping
Bcl-x f forward 5'-GTCCTGGCCCTGTCACTTA-3'
Bcl-x f reverse 5'-CCCTTCCCACCTCACTTCCT-3'
BCR/ABL forward 5'-GAGCGTGCAGAGTGGAGGGAGAACA-3'
BCR/ABL reverse 5'-GGTACCAGGAGTGTTCCTCCAGACTG-3'
Cre forward 5'-ACCTGAAGATGTTCCGCGATTATCT-3'
Cre reverse 5'-ACCGTCAGTACGTGAGATATCT-3'
fTA forward 5'-GCTAGGTGTAGAGCAGCCTAC-3'
fTA reverse 5'-GGCGCATACTATCAGTAGTA-3'
Primers used for real-time PCR
Bcl-x forward 5'-ACTTTTGTGGATCTCTACGGGAAC-3'
Bcl-x reverse 5'-CTGAAGAGTGAGCCCAGCAG-3'
Bcl-2 forward 5'-CTTCGCAGAGATGTCCAGTCAG-3'
Bcl-2 reverse 5'-GAACTCAAAGAAGGCCACAATCC-3'
Mcl-1 forward 5'-TTGTAAGGACGAAACGGGACTG-3'
Mcl-1 reverse 5'-TATGCCAGACCAGCCCCTAC-3'
BCR/ABL forward 5'-CGTCCACTCAGCCACTGGAT-3'
BCR/ABL reverse 5'-GGCTTCACTCAGACCCTGAGG-3'
L19 forward 5'-TCTGGTTGGATCCCAATGAGA-3'
L19 reverse 5'-GTCACAGGCTTGCGGATGAT-3'
3-primer PCR (recombination)
Primer A 5'-CGGTTGCCTAGCAACGGGGC-3'
Primer B 5'-CTCCACAGTGGAGGACCTCG-3'
Primer C 5'-TCAGAAGCCGAATATCCCC-3'