Requirement for Anti-Apoptotic MCL-1 in the Survival of BCR-ABL B-Lineage Acute Lymphoblastic Leukemia

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Key Points:

2. In BCR-ABL\(^+\) B-ALL human and mouse cells, combining TKIs with small molecule inhibitors of BCL-2 can potentiate sensitivity to cell death.

Abstract

The response of Philadelphia chromosome (Ph\(^+\)) acute lymphoblastic leukemia (ALL) to treatment by BCR-ABL tyrosine kinase inhibitors (TKIs) has been disappointing, often resulting in short remissions typified by rapid outgrowth of drug-resistant clones. Therefore, new treatments are needed to improve outcomes for Ph\(^+\) ALL patients. In a mouse model of Ph\(^+\) B-lineage ALL (B-ALL), MCL-1 expression is dysregulated by the BCR-ABL oncofusion protein and TKI treatment results in loss of MCL-1 expression prior to the induction of apoptosis, suggesting that MCL-1 may be an essential pro-survival molecule. To test this hypothesis, we developed a mouse model in which conditional allele(s) of \(Mcl-1\) can be deleted either during leukemia transformation or later after the establishment of leukemia. We report that endogenous MCL-1’s anti-apoptotic activity promotes survival during BCR-ABL-transformation and in established BCR-ABL\(^+\) leukemia. This requirement for MCL-1 can be overcome by overexpression of other anti-apoptotic molecules. We further demonstrate that strategies to inhibit MCL-1 expression potentiate the pro-apoptotic action of BCL-2 inhibitors in both mouse and human BCR-ABL\(^+\) leukemia cell lines. Thus, strategies focused on antagonizing MCL-1 function and expression would be predicted to be effective therapeutic strategies.
Introduction

Therapeutic strategies cure more than 80% of children with acute lymphoblastic leukemia (ALL), but some patients require intensive treatment and develop complications due to side-effects\(^1\). Furthermore, for adults the ALL survival rate remains below 40%, in part due to the dominant role of the BCR-ABL oncogene in adult ALL cases\(^1,2\). In Philadelphia chromosome (Ph\(^+\)) leukemia, the characteristic t(9;22) chromosomal translocation fuses a breakpoint cluster region (BCR) derived from human chromosome 22 to a portion of the c-ABL proto-oncogene from chromosome 9, leading to the formation of the BCR-ABL fusion onco-proteins p210\(^{\text{BCR-ABL}}\) (p210) and p185\(^{\text{BCR-ABL}}\) (p185) that are typically detected in chronic myelogenous leukemia (CML) and Ph\(^+\) ALL cells respectively\(^3,4\). Both p185 and p210 encode constitutively-active tyrosine kinases essential for cell transformation\(^5\).

The treatment of BCR-ABL-expressing CML has been revolutionized by tyrosine kinase inhibitors (TKIs) of BCR-ABL like imatinib, which induces and maintains remission in the majority of CML cases without serious side-effects when administered as a single agent\(^6\). However, ~5% of CML patients per year develop resistance typically due to secondary mutations in the BCR-ABL oncogene\(^7\). In contrast, the response of Ph\(^+\) B-ALL to treatment by TKIs results in short remissions and is typified by rapid outgrowth of drug-resistant clones\(^8\). As a result, both pediatric and adult Ph\(^+\) ALL patients require maximally-intensive combination chemotherapy and stem cell transplants, but even these measures are often unable to achieve long-term survival\(^1\).

Apoptosis is necessary to regulate the proper development and maintenance of tissue homeostasis in metazoans by eliminating damaged or obsolete cells. However,
apoptotic dysregulation can lead to a variety of human pathologies including cancer. Intrinsic apoptosis is triggered when signals activate and/or induce BH3-only molecules, promoting BAX and BAK activation facilitating the release cytochrome *c* from the mitochondria that initiates apoptosome assembly and drives downstream caspase activation\(^9,10\). Genetic loss of both *Bax* and *Bak* prevents the induction of intrinsic apoptosis\(^9\). Anti-apoptotic family members such as BCL-2, BCL-X\(_L\), BCL-w, BFL-1, and MCL-1 antagonize this process and as a result are often overexpressed to prevent cell death in malignant cells\(^11\).

A mouse model of human Ph\(^+\) B-ALL has been established by transducing *Arf*−deficient (*Arf*\(^{-/-}\)) mouse bone marrow (BM) cells with either the p210 or p185 fusion proteins generating rapidly-growing BCR-ABL-expressing pre-B lineage cells that lose Interleukin-7 (IL-7)-dependence\(^12\). Cytokine signaling by IL-7 can overcome the effects of TKI treatment on the leukemic cells, while inhibition by a Jak inhibitor blocked IL-7’s protective effects\(^12\). Furthermore, leukemic cells derived from common gamma-chain deficient BM were more sensitive *in vivo* than wild-type leukemic cells to imatinib treatment\(^13\). Thus, cytokine signaling can antagonize the effects of TKI treatment.

To identify whether *Mcl-1* is a therapeutic target for treating Ph\(^+\) B-ALL, a mouse model of Ph\(^+\) B-ALL was developed in which *Mcl-1* could be genetically ablated either during transformation or later in BCR-ABL-transformed cells. We demonstrate that endogenous MCL-1 expression is required both during initiation and maintenance of BCR-ABL\(^+\) leukemia. Furthermore, we demonstrate that TKIs can potentiate the killing induced by a selective BCL-2 antagonistic small molecule (navitoclax) in mouse and human BCR-ABL\(^+\) leukemic cell lines.
Materials and Methods

**Mice.** *Mcl-1*-conditional; *Bim*-conditional; *Bax*-conditional Bak\(^{-/-}\); *Arf\(^{-/-}\); Rosa-ER\(^{Cre}\) \(^{T2}\) mice have been described previously\(^{14-17}\). For in vivo deletion, mice transplanted with p185\(^{+}\) Rosa-ER\(^{Cre}\) \(^{T2}\) leukemia received 5 doses of tamoxifen (1 µg/dose, Sigma, MO) emulsified in sunflower oil vehicle (Sigma) by gavage. All mice were bred in accordance with St. Jude Children’s Research Hospital (SJCRH) animal care and use committee.

**Plasmids, Expression Constructs, and Generation of Mutants.** *Mcl-1\(^{GRRL}\)* changes Glycine-243 to Arginine and at Arginine-244 to Leucine in mouse *Mcl-1* by site-directed mutagenesis. Mutagenic PCR primer sequences are available by request. Human *BCL-X\(_L\)* and *BCL-2* were from Dr. D. Green (SJCRH, Memphis, TN). BCR-ABL (p185) plasmid was from Dr. O. Witte (UCLA, CA). Stable cells were generated by retroviral transduction and puromycin selection (Sigma Aldrich, MO, 2 µg/ml final).

**Ecotropic Retroviral Production and Cell Transduction.** Retroviruses were produced as previously described\(^{18}\).

**Bone Marrow Transplantation.** BM was harvested and transduced for 3 hours with retrovirus and 8 µg/ml polybrene in the absence of cytokines, washed, and 1 million cells were injected in to lethally irradiated (1100 Rads) C57BL/6 recipients (Jackson Laboratory, ME) by tail-vein intravenous route. Peripheral blood was monitored for leukemia and recipients were observed for morbidity. Each transplant was performed on at least two initial bone marrow infections. For secondary transplants, leukemic BM was mixed 1:1 with control BM and transplanted into lethally-irradiated recipients (at least 10 recipients per initial leukemia).
**Immunohistochemistry.** Specimens were fixed in 10% formalin, paraffin embedded, and sectioned 4 µm thick. Sections were stained with hematoxylin and eosin (H&E), deparaffinized and immunohistochemistry for Pax5 detection was performed using goat anti-human Pax5 (SC1974; Santa Cruz, CA). Goat polymer (GHP 516; Bio Care Medical, PA) with deaminobenzidine detection system (TA125 HDX; ThermoShandon, CA) was used to visualize. Representative images from leukemic mice are presented.

**Cells and Cell Culture.** Mouse p185+ Arf−/− B-ALL and human Ph+ cell lines (TOM119, OP-120, BV17321) were grown in RPMI (Life Technologies, CA) with 20% fetal bovine serum, 55 µM 2-mercaptoethanol, 2 mM glutamine, penicillin, and streptomycin. When indicated recombinant IL-7 (20 ng/ml, Peprotech, NJ) was added to the culture media. Imatinib (Novartis, NJ), dasatinib (Bristol-Myers Squibb, NJ), and navitoclax (ABT-263, from Selleckchem, TX) solubilized in DMSO were added at indicated concentrations.

**Western Blotting, Co-Immunoprecipitation, and Antibodies.** Protein expression from cell lysates and immunoprecipitation was performed as previously described22. Each immunoblot is representative of at least 3 separate experiments performed independently. Antibodies used were: anti-MCL-1 (Rockland Immunochemical, PA), anti-BIM (BD Biosciences, CA), anti-BCL-XL (BD Biosciences), anti-BCL-2 (Clone 6C8), phospho-specific and total STAT5 antisera (Dr. J. Ihle, SJCRH), anti-phospho-specific and total ABL antisera (Cell Signaling, MA), anti-Cre (EMD Chemical, MA), anti-BCL-w (Cell Signaling), anti-BFL-1 (Pierce Biotechnology, IL), anti-PARP (Cell Signaling) and anti-Actin mouse monoclonal (Millipore, MA). Secondary antisera were anti-rabbit or anti-mouse horseradish peroxidase-conjugated (Jackson Immunochemical, ME).
**Cell Death Experiments.** Cell viability was determined by staining with Annexin-V-FITC and propidium iodide (BD Biosciences) and flow cytometry. For experiments on mouse cell lines, each experiment was performed on at least two independently-generated cells from *in vitro* transduced mouse BM. Three separate experiments were performed, each in triplicate.

**Analyses of Cre and Mcl-1 5’ LoxP Site Sequencing.** PCR amplified products of *Cre* cDNA (4 cell lines, 15 clones analyzed) or 5’ LoxP sites (3 lines, 12 clones) from isolated control (*Mcl-1<sup>wt</sup>*) or *Mcl-1<sup>ff</sup>* p185-transformed leukemic cell lines were cloned and sequenced.
Results

BCR-ABL Maintains MCL-1 Expression in pre-B cells

Treatment of p185 BCR-ABL-expressing (hereafter referred to as p185⁺) Arf⁻/⁻ cells with TKIs results in cell cycle arrest and apoptosis induction that can be attenuated by exogenous cytokines including IL-7₁²,₁³. To determine how inhibition of BCR-ABL-signaling affects the expression of members of the BCL-2 family, p185⁺ Arf⁻/⁻ cells were treated with TKIs (Fig. 1 A&B). Exposure to either TKI, which blocked STAT5 and BCR-ABL phosphorylation, decreased MCL-1 expression, but did not alter BCL-2 or BCL-X₇ expression (Fig. 1 A&B). The loss of MCL-1 was alleviated by adding exogenous IL-7 to the cultures, suggesting that signaling can bypass drug-dependent inhibition of BCR-ABL signaling and maintain MCL-1 expression (Fig. 1 A&B). While other cytokines could render leukemic cells resistant to TKIs, IL-7 was the most potent in preventing TKI-induced cell cycle arrest and apoptosis (personal communication from H. Singh and R.K. Guy, St. Jude Children’s Research Hospital).

To assess whether MCL-1 expression correlates with the response of p185⁺ Arf⁻/⁻ cells to TKI inhibition, we generated p185⁺ Arf⁻/⁻ cells from either wild-type (Mcl-1⁷⁺) or Mcl-1 heterozygous BM. Loss of one Mcl-1 allele decreased MCL-1 protein levels by ~50% (Fig. 1C). We transduced and selected for p185⁺ Arf⁻/⁻ cells that constitutively over-express MCL-1 protein (Fig. 1C). When p185⁺ Arf⁻/⁻ cells expressing different levels of MCL-1 were treated with TKIs, MCL-1 expression level correlated with the sensitivity of the p185⁺ Arf⁻/⁻ cells to TKI treatment (Fig. 1D&E). Consistent with its ability to induce MCL-1, IL-7 treatment reduced the cell-killing activity of both imatinib and dasatinib similar to the effects of MCL-1 over-expression and could enhance
protection of Mcl-1 heterozygous cell lines (Fig. 1D&E). Therefore, MCL-1 expression levels respond growth factor and BCR-ABL signaling and dictate TKI sensitivity.

**Requirement for MCL-1 during in vitro Leukemia Initiation**

To determine whether MCL-1 is an important survival molecule during the transformation of pre-B cells with BCR-ABL, a genetic approach was used to delete one or both Mcl-1 genomic alleles by transducing BM cells from Mcl-1<sup>f/f</sup>, Mcl-1<sup>f/wt</sup>, or Mcl-1<sup>wt</sup> mice with a p185-IRES-Cre vector (Fig. 2A). Simultaneous expression of p185 with Cre efficiently drove outgrowth of Mcl-1<sup>wt</sup> or Mcl-1<sup>f/wt</sup> p185-transformed cells; however, the outgrowth of p185<sup>+</sup> cells from Mcl-1<sup>f/f</sup> BM was delayed (Fig. 2A). Genomic analysis of the p185-transformed cells from the cultures confirmed that the Mcl-1 locus was deleted from p185<sup>+</sup> Mcl-1<sup>f/wt</sup> BM cultures, but there was no evidence of Mcl-1-deletion from p185<sup>+</sup> Mcl-1<sup>f/f</sup> BM cultures, indicating selection against Mcl-1-loss from cells during transformation (Fig. 2C).

In clinically aggressive Ph<sup>+</sup> B-ALL, the genetic deletion of the INK4-ARF locus occurs frequently<sup>23</sup>. Furthermore, it has been shown that in the murine model of BCR-ABL B-ALL, Arf-inactivation renders leukemic cells resistant to TKI treatment, enhances their survival in vivo, and facilitates the emergence of BCR-ABL kinase mutant, TKI-resistant clones<sup>12</sup>. To test the requirement for MCL-1 in the in vitro establishment of BCR-ABL-dependent cells, BM cells from Mcl-1<sup>f/f</sup> Arf<sup>−/−</sup>, Mcl-1<sup>f/wt</sup> Arf<sup>−/−</sup>, or Mcl-1<sup>wt</sup> Arf<sup>−/−</sup> mice were transduced with a p185-IRES-Cre vector. Transduction with p185 and Cre-recombinase drove outgrowth of Mcl-1<sup>wt</sup> or Mcl-1<sup>f/wt</sup> p185-transformed cells on an Arf<sup>−/−</sup> background (Fig. 2B). In contrast, delayed outgrowth of p185<sup>+</sup> cells from Mcl-1<sup>f/f</sup> Arf<sup>−/−</sup>
BM was observed (Fig. 2B), similar to that observed in \textit{Arf}^{wt} cultures (Fig. 2A). Genomic analysis of the p185\(^{+}\) cells from the cultures confirmed that deletion of the \textit{Mcl-1} locus was detectable from p185\(^{+}\) \textit{Mcl-1}^{\textit{f/wt}} \textit{Arf}\(^{-}\) BM cultures, but there was no \textit{Mcl-1}-deletion from p185\(^{+}\) \textit{Mcl-1}^{\textit{f/f}} \textit{Arf}\(^{-}\) BM cultures demonstrating a strong selection against \textit{Mcl-1}-loss also occurs even in the absence of \textit{Arf} (Fig. 2D). Analysis of protein expression confirmed that p185-IRES-Cre transduction in \textit{Mcl-1}^{\textit{f/wt}} \textit{Arf}\(^{-}\) BM causes a 50\% reduction in MCL-1 protein levels; whereas, MCL-1 protein expression remained unchanged in \textit{Mcl-1}^{\textit{f/f}} \textit{Arf}\(^{-}\) BM cells indicating a lack of \textit{Mcl-1} deletion (Fig. 2E). The p185-IRES-Cre-transduced \textit{Mcl-1}^{\textit{f/f}} \textit{Arf}\(^{-}\) cells in the cultures failed to express Cre protein despite possessing the \textit{Cre} cDNA, implying that these cells must have mutated \textit{Cre} to avoid deleting \textit{Mcl-1} (Fig. 2D&E). Sequencing of \textit{Cre} cDNA amplified from p185-IRES-Cre-transduced \textit{Mcl-1}^{\textit{f/f}} \textit{Arf}\(^{-}\) cells indicated that \textasciitilde 40\% of clones analyzed exhibited nonsense mutations in \textit{Cre} and that \textasciitilde 40\% of clones possessed missense mutations with some possessing both types (Sup. Fig. 1A). In contrast, sequencing of control (p185-IRES-Cre-transduced \textit{Mcl-1}^{\textit{wt}} \textit{Arf}\(^{-}\)) leukemic cells did not reveal any \textit{Cre} mutations illustrating the pressure against \textit{Mcl-1}-deletion in the p185-IRES-Cre-transduced \textit{Mcl-1}^{\textit{f/f}} \textit{Arf}\(^{-}\) cells (data not shown). These data indicate that in \textit{Arf}^{wt} or \textit{Arf}^{-/-} genetic backgrounds, MCL-1 expression is required for BCR-ABL-dependent pre-B cell transformation \textit{in vitro}.

**Requirement for MCL-1 during \textit{in vivo} Leukemia Initiation**

To test whether \textit{Mcl-1} is required for leukemia initiation \textit{in vivo}, BM from \textit{Mcl-1}^{\textit{f/f}} \textit{Arf}\(^{-}\), \textit{Mcl-1}^{\textit{f/wt}} \textit{Arf}\(^{-}\), or \textit{Mcl-1}^{\textit{wt}} \textit{Arf}\(^{-}\) mice was transduced with a p185-IRES-Cre
vector and transplanted into lethally-irradiated (1100 Rads) C57BL/6 recipient mice. Recipients receiving p185-IRES-Cre-transduced \( Mcl-I^{wt} \) \( Arf^+ \) BM cells rapidly succumbed to leukemia (Fig. 2F). The loss of even a single allele of \( Mcl-I \) (p185-IRES-Cre-transduced \( Mcl-I^{fwt} \) \( Arf^+ \) BM) significantly delayed the onset of the lethal leukemia (Fig. 2F). Transplanting p185-IRES-Cre-transduced \( Mcl-I^{f/f} \) \( Arf^+ \) BM cells, in which both \( Mcl-I \) alleles should be deleted, delayed leukemia onset even more than loss of a single \( Mcl-I \) allele; however, eventually all the recipients developed fatal leukemia (Fig. 2F).

While the \( Mcl-I \) background affected the time of leukemia latency, analysis of the moribund mice did not reveal any pathological differences in the leukemia as measured by leukocyte counts, liver mass, spleen size, and histopathology (Fig. 2G and Sup. Fig. 2). Genomic analysis indicated that the \( Mcl-I \) locus was deleted from cells recovered from moribund mice transplanted with p185-IRES-Cre-transduced \( Mcl-I^{fwt} \) \( Arf^+ \) BM cells. In contrast, there was no evidence of \( Mcl-I \)-deletion in the leukemic blasts isolated from mice transplanted with p185-IRES-Cre-transduced \( Mcl-I^{f/f} \) \( Arf^+ \) BM cells, indicating in vivo selection against \( Mcl-I \)-deletion during BCR-ABL-driven leukemogenesis (Fig. 2H). Protein analysis confirmed that leukemia from mice transplanted with p185-IRES-Cre-transduced \( Mcl-I^{fwt} \) \( Arf^+ \) BM expressed less MCL-1 protein than \( Mcl-I^{wt} \) leukemic cells, consistent with deletion of the conditional \( Mcl-I \) allele (Fig. 2I). However, MCL-1 protein was still detectable in the leukemic cells from mice transplanted with p185-IRES-Cre-transduced \( Mcl-I^{f/f} \) \( Arf^+ \) BM indicating a lack of deletion. This was due, at least in part, to the failure to express Cre protein due to nonsense mutations in \( Cre \) (Fig. 2I and Sup. Fig. 1B). Additionally, in other leukemic
samples we also detected missense mutations that resulted in mutant Cre protein (Sup. Fig. 1C). Thus, when Cre was co-expressed during the transformation with p185, there was a strong selection against Mcl-1 deletion during in vitro and in vivo leukemogenesis.

**Requirement for Anti-Apoptotic Function in Initiation of Leukemia In Vitro**

To assess whether MCL-1 loss induces the death of the cells, BM was isolated from Mcl-1<sup>fl</sup> Bax<sup>fl</sup> Bak<sup>-/-</sup> mice for p185-IRES-Cre transduction and the outgrowth of leukemic cells from the cultures followed (Fig. 3A). Control Mcl-1<sup>wt</sup> Bax<sup>fl</sup> Bak<sup>-/-</sup> BM cells rapidly expanded from culture consistent with a loss of both pro-apoptotic effectors BAX and BAK (Fig. 3A). Similarly, leukemia from the Mcl-1<sup>fl</sup> Bax<sup>fl</sup> Bak<sup>-/-</sup> transduced BM expanded from the cultures, but exhibited a delay when compared to Mcl-1<sup>wt</sup> Bax<sup>fl</sup> Bak<sup>-/-</sup> or Mcl-1<sup>wt</sup> cultures (Fig. 3A). In contrast, the outgrowth from Mcl-1<sup>fl</sup> cultures was dramatic delayed (Fig. 3A). Expression analyses indicated that unlike the p185<sup>+</sup> cells that grew from the Mcl-1<sup>fl</sup> cultures, which fail to delete MCL-1 or express Cre protein, the p185<sup>+</sup> cells from Mcl-1<sup>fl</sup> Bax<sup>fl</sup> Bak<sup>-/-</sup> cultures deleted MCL-1, BAX, and BAK and expressed the Cre-recombinase (Fig. 3B&D). Thus, removing the intrinsic death pathway, by Bax and Bak ablation, rescued the outgrowth of Mcl-1-deleted p185<sup>+</sup> cells in vitro implying that a consequence of MCL-1 loss is apoptosis induction.

Pro-apoptotic BIM participates in the induction of cell death in BCR-ABL<sup>+</sup> leukemia treated with TKIs<sup>24-26</sup>. To test whether Bim-loss could allow Mcl-1-deleted p185<sup>+</sup> cells to survive, Mcl-1<sup>fl</sup> Bim<sup>fl</sup> BM was transduced with p185-IRES-Cre and cultured. Surprisingly, while loss of Bim alone hastened the outgrowth of cultures when compared with controls, co-deletion of Mcl-1 and Bim delayed the outgrowth even more
than Mcl-1-deletion alone (Fig. 3A). Furthermore, the cells that grew out of the Mcl-1\(^{\text{f/f}}\) Bim\(^{\text{f/f}}\) cultures failed delete the genomic DNA for either Mcl-1 or Bim and maintained expression of both proteins (Fig. 3C&E). Similar to Mcl-1\(^{\text{f/f}}\) cultures, the Mcl-1\(^{\text{f/f}}\) Bim\(^{\text{f/f}}\) cells transduced with p185-IRES-Cre failed to express Cre-recombinase, again due to mutations in Cre (Fig. 3E and Sup. Fig. 1D). Thus, the loss of BIM expression was insufficient to allow the outgrowth of p185\(^{+}\) Mcl-1-deleted cells, indicating that additional pro-apoptotic molecules must induce death upon Mcl-1 deletion.

We mutated MCL-1’s BH3-domain binding groove by mutating Glycine-243 to Arginine and Arginine-244 to Leucine (referred to as MCL-1\(^{\text{GRRL}}\)). To validate that this mutant abolishes MCL-1’s anti-apoptotic function, MCL-1\(^{\text{GRRL}}\) was stably expressed to similar levels as MCL-1\(^{\text{wt}}\) in SV40-transformed Mcl-1\(^{\text{f/f}}\) Rosa-ER\(^{\text{Cre}}\) T2 MEFs that, when treated with tamoxifen, induced deletion of the endogenous Mcl-1 (Sup. Fig. 3A). Mcl-1-deletion rendered MEFs susceptible to staurosporine-induced death and ectopic expression of MCL-1\(^{\text{wt}}\) rendered them resistant (Sup. Fig. 3B). In contrast, stable expression of the MCL-1\(^{\text{GRRL}}\) mutant could not protect the MEFs from apoptosis indicating that the mutant is non-functional in antagonizing death (Sup Fig. 3B). MCL-1\(^{\text{wt}}\) co-immunoprecipitated with pro-apoptotic BIM; however, no interaction was detectable between BIM and MCL-1\(^{\text{GRRL}}\) protein (Sup Fig. 3C). These data demonstrate that the MCL-1\(^{\text{GRRL}}\) lacks the ability to sequester pro-apoptotic modulators, such as BIM, and therefore cannot prevent apoptosis.

To test whether MCL-1’s anti-apoptotic function is essential for leukemia in vitro initiation, Mcl-1\(^{\text{f/f}}\) Arf\(^{^{+}}\) pre-B cells were co-transduced with IRES-GFP vector expressing either vector alone, MCL-1\(^{\text{wt}}\), or MCL-1\(^{\text{GRRL}}\) along with p185-IRES-Cre to
simultaneously transform and delete endogenous \textit{Mcl-1}. Expression of \textit{Mcl-1}^{wt}, but not empty vector efficiently promoted the outgrowth of p185-IRES-Cre-transduced \textit{Mcl-1}^{\text{f/f}} \textit{Arf}^{-/-} cells from the cultures (Fig. 3F). In contrast, the expression of \textit{MCL-1}^{GRRL} was insufficient to promote the outgrowth of cells from the cultures indicating that \textit{MCL-1}'s anti-apoptotic function is required for promoting leukemic initiation \textit{in vitro} (Fig. 3F).

Since \textit{MCL-1}'s ability to inhibit cell death appears to be required for leukemic cell survival, we tested whether overexpression of other anti-apoptotic BCL-2 family members might suffice to protect the BM cells during \textit{in vitro} leukemic initiation. \textit{Mcl-1}^{\text{f/f}} \textit{Arf}^{-/-} BM were transduced with both p185-IRES-Cre and MIG-vector expressing \textit{Mcl-1}^{wt}, human \textit{BCL-2}, human \textit{BCL-XL} and then cultured in the absence of growth factors and monitored daily for the outgrowth of GFP+ cells. Expression of \textit{Mcl-1}^{wt}, but not empty vector promoted the outgrowth of p185-IRES-Cre-transduced \textit{Mcl-1}^{\text{f/f}} \textit{Arf}^{-/-} cells from the cultures (Fig. 3F). Additionally, expression of either BCL-2 or BCL-X \textit{L} supported the outgrowth of p185-IRES-Cre-transduced \textit{Mcl-1}^{\text{f/f}} \textit{Arf}^{-/-} cells indicating that ectopic expression of anti-apoptotic molecules rescued the survival of the cells lacking \textit{Mcl-1} (Fig. 3F). \textit{Mcl-1}-deletion was readily detectable by PCR from p185-IRES-Cre-transduced \textit{Mcl-1}^{\text{f/f}} \textit{Arf}^{-/-} cells expressing MCL-1, BCL-2, or BCL-X \textit{L} indicating that deletion in the cells ectopically-expressing anti-apoptotic molecules was tolerated (Fig. 3G). Likewise, analyses of the cultures after co-transduction demonstrated deletion of MCL-1 and corresponding expression of ectopic MCL-1, BCL-2, or BCL-X \textit{L} (Fig. 3H). These data indicated that concomitant expression of exogenous anti-apoptotic BCL-2 family members rescued the death induced by deletion of endogenous \textit{Mcl-1} during p185-IRES-Cre transformation.
**In Vitro Requirement for MCL-1 in Maintenance of Leukemia Survival**

BCR-ABL transformation results in the activation of many cellular signaling pathways that promote the proliferation and survival of leukemic cells\(^{27,28}\). To identify whether MCL-1 is also required for the continual survival of BCR-ABL-transformed cells, we established IL-7-dependent immortalized, but not transformed \(Mcl-1^{\text{ff}}\) pre-B cells generated from \(Mcl-1^{\text{ff}}\) mouse BM. These cells were transduced with p185-IRES-Luciferase to promote cytokine and stroma-independent growth (referred to hereafter as p185\(^+\) \(Mcl-1^{\text{ff}}\) B-ALL cells)\(^{12}\). To test whether MCL-1 is essential for promoting the survival of fully-transformed cells, either vector or \(Mcl-1^{\text{wt}}\) cDNA were stably expressed in the cells. The p185\(^+\) \(Mcl-1^{\text{ff}}\) B-ALL cells stably expressing either MSCV-vector or \(Mcl-1\) were then transduced with Cre-IRES-GFP (MIG-Cre) and the growth of GFP-expressing cells was followed. In vector-expressing cells, there was no outgrowth of GFP\(^+\) cells indicating that Cre-expression prevented the expansion of the p185\(^+\) \(Mcl-1^{\text{ff}}\) B-ALL cells (Fig. 4A). Cre-expression induced death in the cultures, but since the dead cells are GFP\(^-\) they could not be differentiated from non-MIG-Cre transduced cells (data not shown). Even when IL-7 was added to the cultures, Cre-expression prevented the outgrowth of p185\(^+\) \(Mcl-1^{\text{ff}}\) B-ALL cells indicating that exogenous growth factor additional was insufficient to overcome \(Mcl-1\)-deletion (Fig. 4A). In contrast, expression of MCL-1\(^{\text{wt}}\) in the p185\(^+\) \(Mcl-1^{\text{ff}}\) B-ALL cells promoted GFP\(^+\) Cre-expressing p185\(^+\) \(Mcl-1^{\text{ff}}\) B-ALL cells expansion demonstrating that ectopic MCL-1\(^{\text{wt}}\) expression could rescue the apoptosis induced by \(Mcl-1\)-deletion (Fig. 4A&B).
In Vitro Requirement for MCL-1’s Anti-Apoptotic Function in Leukemia Maintenance

To test whether MCL-1’s anti-apoptotic function is required for the survival of already existing transformed leukemic cells, Mcl-1\textsuperscript{wt}, Mcl-1\textsuperscript{GRRL} mutant cDNA or vector were stably expressed in p185\textsuperscript{+} Mcl-1\textsuperscript{f/f} Arf\textsuperscript{-/-} B-ALL cells. The cells were then transduced in parallel with MIG-Cre and the growth of GFP-expressing (Cre\textsuperscript{+}) cells was followed. MCL-1\textsuperscript{wt} expression promoted the efficient outgrowth of GFP\textsuperscript{+} cells from the cultures, but no GFP\textsuperscript{+} cells were expanded from cultures expressing empty vector (Fig. 4C). Similarly, expression of the non-functional MCL-1\textsuperscript{GRRL} mutant could not support the outgrowth of Mcl-1-deleted cells (Fig. 4C). These data indicate that BCR-ABL\textsuperscript{+} Arf\textsuperscript{-/-} B-ALL cells required MCL-1’s anti-apoptotic function.

Protein analyses demonstrated that endogenous MCL-1, BCL-2, and BCL-X\textsubscript{L} are expressed in the unmanipulated BCR-ABL transformed B-ALL cells, but the loss of MCL-1 was not tolerated in these cells indicating that the endogenous amounts of BCL-2 and BCL-X\textsubscript{L} are insufficient to maintain survival (Fig. 4D). To address whether overexpression of other anti-apoptotic BCL-2 family members could overcome Mcl-1 deletion in the BCR-ABL-transformed B-ALL cells, BCL-2 or BCL-X\textsubscript{L} were stably expressed in the p185\textsuperscript{+} Mcl-1\textsuperscript{f/f} Arf\textsuperscript{-/-} B-ALL cells and transduced with MIG-Cre and the outgrowth of GFP-expressing cells followed. Ectopic expression of human BCL-2 or BCL-X\textsubscript{L} promoted the survival and outgrowth of GFP\textsuperscript{+} cells from the cultures (Fig. 4C). Furthermore, protein analysis of the GFP\textsuperscript{+} cells after MIG-Cre transduction confirmed that these cells lacked MCL-1 expression when they expressed exogenous anti-apoptotic
cDNAs (Fig. 4D). Therefore, these data indicate that even transformed BCR-ABL⁺ B-ALL cells require endogenous MCL-1 to promote their survival and growth, but that the requirement for MCL-1 can be supplanted by overexpression of other anti-apoptotic BCL-2 family members.

In Vivo Requirement for Anti-Apoptotic MCL-1 Function in Leukemia Maintenance

We bred *Mcl-1* conditional mice to mice bearing a tamoxifen-activatable Cre (Rosa-ERCre<sup>T2</sup>) to delete *Mcl-1* in leukemic cells *in vivo* by tamoxifen administration. The CreER<sup>T2</sup> fusion protein is expressed regardless of the tamoxifen-treatment, but only localizes to the nucleus when tamoxifen is present. BM from *Mcl-1<sup>floxed</sup>* Rosa-ERCre<sup>T2</sup> or *Mcl-1<sup>wt</sup>* Rosa-ERCre<sup>T2</sup> (control) mice were infected with the p185-IRES-GFP virus and transplanted into lethally-irradiated recipients. After ~30 days, recipient mice exhibiting signs of leukemia were sacrificed and leukemic BM (>50% GFP⁺ cells) was mixed 1:1 with CD45.1⁺ wild-type BM and intravenously transplanted into lethally-irradiated recipients. Five days after the secondary transplant, recipients either received 5 doses of tamoxifen (1 µg/dose per day) or vehicle and were monitored for signs of leukemia progression and sacrificed when moribund. Both tamoxifen- and vehicle-treated mice harboring control leukemic cells rapidly developed a fatal leukemia indicating that tamoxifen-induced Cre activation did not alter leukemia progression (Fig. 5A). Strikingly, while the p185-transformed, *Mcl-1<sup>floxed</sup>* Rosa-ERCre<sup>T2</sup> vehicle-treated group all succumbed to leukemia (median survival 20 days); however, the progression of leukemia in the tamoxifen-treated group was significantly delayed (median survival 46 days) with
~40% tamoxifen-treated mice remaining leukemia-free for >200 days since leukemia transplant (Fig. 5A).

At sacrifice, moribund mice from both the tamoxifen-treated and control groups exhibited GFP+ cells in their peripheral blood and organs consistent with a p185+ leukemia (Fig. 5B and Sup. Fig. 4). The resulting leukemic (GFP+) cells from the tamoxifen-treated moribund mice still expressed MCL-1 despite tamoxifen treatment (Fig. 5C). To address how the leukemic cells had escaped deletion, the 5’ LoxP sites of the Mcl-1 conditional allele were amplified from leukemic cells isolated from moribund, tamoxifen-treated recipients bearing p185-transformed Mcl-1<sup>fl/fl</sup> Rosa-ER<sup>Cre</sup><sup>T2</sup> leukemic BM. Sequencing revealed deletions and mutations in the 5’ loxP Mcl-1 site in 80% of the surviving leukemic cells (Sup. Fig. 5A). Intriguingly, in some moribund tamoxifen-treated mice we detected the deletion of one Mcl-1 genomic allele, but also found mutations in the 5’ loxP site of the other (non-deleted) allele (Fig. 5D and Sup. Fig. 5B).

Histopathologically, the leukemia arising in the moribund mice were pathologically similar whether the mice received tamoxifen or vehicle treatment and uniformly expressed Pax5, a marker of B-lineage leukemia (Fig. 5E and Sup. Fig. 3). These data indicate that Mcl-1-deletion <i>in vivo</i> delayed leukemia progression and those tamoxifen-treated recipients that succumbed to disease exhibited selection against Mcl-1-deletion in the leukemic cells.
Potentiation of Tyrosine Kinase Inhibitor Activity by Small Molecule BCL-2 Inhibitors in Mouse BCR-ABL Leukemia

The small molecule inhibitor, navitoclax (ABT-263) specifically inhibits the anti-apoptotic function of BCL-2 and BCL-X\textsubscript{L} and exhibits efficacy in BCL-2-dependent cancers with limited toxicity\textsuperscript{29-31}. However, a common feature mediating tumor cell resistance to navitoclax and its progenitor compound (ABT-737) is the expression of other anti-apoptotic proteins, like MCL-1, which are not targeted by these compounds\textsuperscript{32-34}. As MCL-1 expression often correlates with tumor cell resistance to the cell-killing activity of navitoclax, we tested the death sensitivity of \textit{in vitro} generated p185\textsuperscript{+} \textit{Mcl-1\textsuperscript{wt} Arf\textsuperscript{-/-}} B-ALL cell lines in which endogenous \textit{Mcl-1} was deleted by Cre and replaced by other anti-apoptotic molecules. As expected, the BCR-ABL B-ALL cells expressing exogenous BCL-2 or BCL-X\textsubscript{L}, but lacking endogenous \textit{Mcl-1} expression, were more sensitive to navitoclax than p185\textsuperscript{+} \textit{Mcl-1\textsuperscript{wt} Arf\textsuperscript{+/+}} B-ALL cells (Fig. 6A). Furthermore, p185\textsuperscript{+} \textit{Mcl-1\textsuperscript{wt} Arf\textsuperscript{-/-}} B-ALL cells overexpressing MCL-1 were even more resistant to navitoclax than p185\textsuperscript{+} \textit{Mcl-1\textsuperscript{wt} Arf\textsuperscript{+/+}} B-ALL cells indicating that MCL-1’s expression level correlated with navitoclax sensitivity (Fig. 6A).

Navitoclax has been proposed to promote apoptosis by displacing pro-apoptotic BH3-only family members from anti-apoptotic molecules, thus facilitating pro-apoptotic effector (BAX and BAK) activation and cell death\textsuperscript{35,36}. To test this hypothesis, \textit{in vitro} generated p185\textsuperscript{+} \textit{Arf\textsuperscript{+/+} Mcl-1\textsuperscript{wt}} (referred to as BCR-ABL B-ALL cells) were treated with navitoclax and immunoprecipitated with anti-BIM antibodies to measure the BH3-only occupancy of anti-apoptotic BCL-2 family members, as BIM interacts with BCL-2, BCL-X\textsubscript{L}, and MCL-1\textsuperscript{37,38}. As the navitoclax dose increased the amount of BCL-2 and BCL-X\textsubscript{L}
immunoprecipitating with BIM decreased and there was a corresponding increase in MCL-1 protein associated with BIM (Fig. 6B). These data indicated that navitoclax displaced BIM from BCL-2 and BCL-X_L and suggests that the liberated BIM could then interact with MCL-1 (Fig. 6B). BIM is a relevant pro-apoptotic molecule as Bim-deficient p185-transformed leukemic cells, derived from Bim-conditional BM transduced with p185-IRES-Cre retrovirus, are partially resistant to TKI and navitoclax treatment consistent with previously published reports (Sup. Fig. 6)24-26,35. These data suggest that treatment with navitoclax may be less effective at killing BCR-ABL B-ALL cells because the leukemic cells may have sufficient MCL-1 to “buffer” the release of pro-apoptotic molecules, such as BIM, from BCL-2 and BCL-X_L.

Treatment of BCR-ABL B-ALL cells with TKIs decreased the amount of MCL-1 protein expressed and induced cell death, but did not modulate BCL-2, BCL-X_L, or BIM expression (Fig. 1A&B and Fig. 6C&D). However, TKI treatment did not disrupt the interaction between BCL-2, BCL-X_L, or MCL-1 with pro-apoptotic BIM as detected by co-immunoprecipitation (Fig. 6C&D). Since TKIs decreased MCL-1 protein expression, we hypothesized that we could potentiate the efficacy of navitoclax on BCR-ABL B-ALL cells by combining navitoclax with TKI treatment to decrease MCL-1 expression in the cells. We treated BCR-ABL B-ALL cells with low doses of imatinib (Fig. 6E) or dasatinib (Fig. 6F) that each induce ~10% apoptosis and co-administered increasing doses of navitoclax. As the navitoclax was increased we observed potentiation of the TKIs indicating a synergistic effect of combining navitoclax and TKI treatment (Fig. 6E&F). Growth factor signaling by IL-7 can partially overcome the death induced by TKI treatment; therefore, we tested whether IL-7 could overcome the potentiation of
combining TKI treatment and navitoclax. While exogenous IL-7 treatment reduced the effectiveness of TKI treatment and reduced the killing observed, a statistically significant effect of combining TKIs with navitoclax was observed (Fig. 6E&F). These data demonstrate combining TKIs and navitoclax can enhance activity to against BCR-ABL B-ALL cells and overcome the resistant effects of growth factor signaling.

**Potentiation of Tyrosine Kinase Inhibitor Activity by Small Molecule BCL-2 Inhibitors in Human BCR-ABL Leukemia Cell Lines**

To test whether similar potentiation of cell death can be observed in human Ph+ leukemia cell lines, we tested whether a similar relationship between BCR-ABL signaling and MCL-1 expression could be observed. In OP-1\textsuperscript{20} and TOM1\textsuperscript{19} (Ph+ B-ALL lines) and BV173\textsuperscript{21} (Ph+ CML blast crisis line) we observed that treatment with sub-optimal doses of either TKI decreased MCL-1 expression, but did not alter the expression of other anti-apoptotic BCL-2 family members (i.e. BCL-2, BCL-X\textsubscript{L}, or BFL-1) and only modestly decreased BCL-w in OP-1 cells (Fig. 7). Therefore, among anti-apoptotic BCL-2 family members only MCL-1 expression declines robustly in response to TKI treatment. We hypothesized that we may be able to potentiate the efficacy of navitoclax on the Ph+ cell line by combining with TKI treatment to decrease MCL-1 expression. Thus, we treated the human Ph+ cell lines with doses of TKIs that each induced ~10% apoptosis and co-administered increasing navitoclax doses (Fig. 7). As navitoclax was increased in the cultures we observed a potentiation of the TKIs to induce apoptosis (Fig. 7). These data indicate that like our observations in mouse BCR-ABL cell lines, combining TKIs and navitoclax in human Ph+ cell lines can also lead to enhanced
activity. While MCL-1 expression is most overtly decreased in response to TKI treatment, it is possible that combining TKI and navitoclax may effect targets other than MCL-1 leading to cell death. These data suggest that combining TKIs and navitoclax may be effective in treating human Ph+ leukemia.
Discussion

It has been reported that genetic deletion of \textit{Bcl2l1} (encoding the BCL-X anti-apoptotic protein) in a mouse model of BCR-ABL B-ALL did not adversely affect leukemogenesis and paradoxically resulted in the increased proliferation of the BCL-X-deficient B-ALL cells\textsuperscript{39}. In contrast, our experiments reveal that MCL-1 is the essential endogenous promoter of survival both during the initial transformation of B-lineage progenitors with BCR-ABL and also in p185-transformed leukemic cells. The requirement for MCL-1 is critical as both \textit{in vitro} and \textit{in vivo} leukemic cells exhibit strong selection pressure against \textit{Mcl-1}-deletion as mediated by mutagenesis of \textit{Cre} or mutation of one of the loxP sites flanking the \textit{Mcl-1} locus. The dependence on MCL-1 is striking as both B-lineage progenitors and leukemic cells simultaneously express other anti-apoptotic molecules; however, the endogenous expression of these molecules is insufficient to allow survival of the cells without MCL-1.

One potential rationale for the absolute dependence on MCL-1 is that it possesses unique functions that cannot be compensated for by other anti-apoptotic BCL-2 family members\textsuperscript{18}. However, while MCL-1 may have additional roles beyond preventing cell death, our data demonstrate that either loss of both pro-apoptotic effectors (BAX and BAK) or ectopic expression of human BCL-2 or human BCL-X\textsubscript{L} can rescue the survival and growth of \textit{Mcl-1}-deleted leukemic cells. Therefore, the primary function of MCL-1 in this mouse model of Ph\textsuperscript{+} B-ALL is to block apoptosis.

Our results are consistent with a model in which the “pool” of anti-apoptotic MCL-1 in cells is the critical regulator of cellular survival. Signaling, either through either cytokine receptors or by the BCR-ABL oncofusion promotes MCL-1 expression to
counter pro-apoptotic molecules in the cells. When signaling diminishes, either due to a lack of cytokines or after TKI treatment, MCL-1 levels decrease becoming insufficient to counter the pro-apoptotic molecules. Supporting this model, treatment of primary chronic lymphocytic leukemia with TKIs also reduces MCL-1 expression\cite{40}. In contrast, when exogenous BCL-2 and BCL-X\textsubscript{L} are overexpressed in the cells it expands the “pool” of anti-apoptotic modulators allowing the cells to counter pro-apoptotic expression despite a loss of MCL-1 function.

A dominant role for MCL-1 in promoting leukemic cell survival is also seen in mouse models of acute myelogenous leukemia (AML)\cite{41,42}. Strikingly, whether the AML cells were driven by c-Myc, MLL-ENL, MLL-AF9, Mixl1, AML1-ETO9a, or Hox19, the resultant leukemia were similarly dependent on MCL-1\cite{41,42}. Similar data exist for human chronic myelogenous leukemia (CML) cells driven by the BCR-ABL oncofusion\cite{43}. Furthermore, in these models MCL-1’s primary function is to prevent apoptosis as ectopic expression of other anti-apoptotic family members promoted leukemia survival in these models\cite{41,42}. Therefore, strategies to inhibit MCL-1’s anti-apoptotic function would be predicted to be therapeutically beneficial. However, MCL-1 is an important modulator of the survival of a number of normal cell lineages including, but not limited to hematopoietic lineages\cite{14,44,45}. Thus, it may be difficult to inhibit MCL-1 in malignant cells without inducing toxicity in normal cells. An alternative strategy may be to module MCL-1 expression levels to reduce the size of the anti-apoptotic “pool” in cancer cells to foster lethality of malignant cells without inducing toxicity to normal cell lineages.

MCL-1 is a transcriptional target of cytokine and growth factor signaling\cite{14,44,46}. Additionally, MCL-1 protein is short-lived being a proteasome substrate\cite{47,48}. MCL-1
degradation is also regulated, as post-translational modifications play a critical role in regulating its elimination\(^{49,50}\). MCL-1 can also undergo ubiquitinylation-independent protease degradation\(^{22}\). MCL-1’s labile nature raises the possibility that it may be possible to simply perturb its expression. Indeed, combining inhibitors of cellular signaling to potentiate the death induced by TKIs has already shown promise in CML models\(^{51-53}\), and in some cases these combined therapies decreased MCL-1 expression\(^{54}\). Identification of these pathways downstream of BCR-ABL signaling may identify further ways by which MCL-1 expression levels can be modulated in leukemic cells.

When used alone, navitoclax is relatively ineffective at treating BCR-ABL B-ALL cell lines \textit{in vitro}; presumably as it displaces pro-apoptotic molecules (i.e. BIM and others) from BCL-2 and BCL-X\(_L\), but the liberated pro-apoptotic molecules can still be sequestered by MCL-1\(^{32,33}\). However, when TKIs are combined with navitoclax, cell death is potentiated as there is less MCL-1 available to sequester pro-apoptotic molecules. While we cannot rule of the possibility that combining these inhibitors may have effects beyond merely modulating MCL-1 function, MCL-1 is the only anti-apoptotic molecule whose expression is significantly altered by TKI treatment. Our synergy experiments are similar to those in BCR-ABL models of CML or gastrointestinal stromal cell tumor cells in which combinatorial treatment with TKIs and BH3-mimetics like navitoclax have been shown to be synergistic\(^{26,55,56}\).

MCL-1 is a critical pro-survival molecule required to promote the survival of B cell progenitor populations during BCR-ABL transformation as well as for the continued survival of the BCR-ABL B-ALL cells. These findings highlight the potential for targeting MCL-1 protein expression and function in treating this BCR-ABL B-ALL. We
submit that while specific MCL-1 inhibitors may be effective at treating BCR-ABL leukemia, approaches that would alter the expression levels of MCL-1 should also be effective at sensitizing this leukemia to other treatment modalities including BCL-2 inhibitors such as navitoclax.
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Authorship

R.T.W. and J.T.O. conceived the study and designed the experiments. B.K. and J.M. performed the experiments, analyzed data, and prepared figures. B.K., J.M., R.M.P. and H.S. generated reagents and discussed findings. J.E.R. analyzed pathology. J.T.O. supervised the project and wrote the manuscript. The authors declare no competing financial interests.
References


Figure Legends

Figure 1. MCL-1 Expression Dictates Response Mediated by Inhibition of BCR-ABL.

(A & B) In vitro-generated BCR-ABL (p185)-expressing Arf<sup>−/−</sup> cells were treated with imatinib or dasatinib in the presence or absence or IL-7 (20 ng/mL) at (A) varying concentrations (24 hours) or (B) with the indicated imatinib (IM) or dasatinib (DAS) concentrations for different amounts of time (hours). Cell lysates were resolved and immunoblotted for indicated targets. Anti-ABL antibody was used to detect the BCR-ABL fusion protein while pBCR-ABL and pSTAT5 represent phosphorylated proteins.

(C) Immunoblot of p185-expressing cells that were derived from wild-type Mcl-1 (Mcl-1<sup>wt</sup>), haploinsufficient (Mcl-1<sup>het</sup>, one floxed Mcl-1 allele deleted using p185-IRES-Cre retrovirus), or overexpressing ectopic Mcl-1 in addition to endogenous (Mcl-1<sup>OE</sup>) on an Arf<sup>−/−</sup> genetic background. Cell lysates were resolved and immunoblotted for MCL-1 and Actin (loading control). (D & E) Cells described in (C) or p185-expressing Arf<sup>−/−</sup> pre-B cells were treated for 24 hours at indicated doses with (D) IM or (E) DAS after which cell death was determined. Cells were cultured in the presence of IL-7 (20 ng/mL) where indicated. Annexin-V and PI double-negative cells were scored as viable. Data points represent the average of three independent experiments (n=3) and the error bars denote the standard error of the mean (SEM).
**Figure 2. Requirement for MCL-1 during in vitro and in vivo BCR-ABL Leukemia Initiation**

(A&B) BM from wild-type Mcl-1 (Mcl-1*wt*), Mcl-1*fl* or Mcl-1*ff* mice on (A) Arf*wt* or (B) Arf*−/−* genetic backgrounds was transduced with p185-IRES-Cre retrovirus to transform and delete the floxed allele. Viable cell concentration was assessed every 24 hours. Each time point represents an average of three independent experiments (n=3) and the error bars denote SEM. Data represent 10 separate cultures from two independent mice.  

(C & D) Representative Mcl-1, Mcl-1-deleted, and Cre genomic genotyping preformed post outgrowth from (C) Arf*wt* and (D) Arf*−/−* genetic backgrounds.  

(E) Immunoblot analysis from cells which grew out in culture from the Arf*−/−* experiment detailed in (B) represent two independent experiments. Lysates were blotted for MCL-1, BCR-ABL (as detected by anti-ABL antibody), Cre and Ponceau stain (loading control).  

(F) Arf*−/−* BM from Mcl-1*wt* (n=10 mice), Mcl-1*fl* (n=10 mice), or Mcl-1*ff* (n=10 mice) were transduced with p185-IRES-Cre retrovirus and immediately transplanted into lethally-irradiated (1100 Rads) C57BL/6 recipients. Data represent 10 individual leukemia recipients. BM from two independent donor mice was used. Recipient mice where monitored daily and sacrificed when moribund. Asterisk (*) denotes p<0.001 by Log Rank Test when compared to Mcl-1*wt*.  

(G) Whole blood cell (WBC) counts were taken from each mouse at time of sacrifice. Each point represents one mouse and the horizontal line indicates the averages and the error bars represent SEM.  

(H) Representative Mcl-1, deleted Mcl-1, and Cre genotyping of BM cells isolated from two representative moribund mice.  

(I) Representative immunoblots of BM from three individual moribund mice were blotted for MCL-1, Cre, and Actin (loading control).
Figure 3. Requirement for Anti-Apoptotic Function in Initiation of BCR-ABL Leukemia

(A) BM from Mcl-1<sup>wt</sup>, Mcl-1<sup>ff</sup>, Bax<sup>ff</sup>Bax<sup>KO</sup>, Bax<sup>ff</sup>Bax<sup>KO</sup>Mcl-1<sup>ff</sup>, Bim<sup>ff</sup>, and Mcl-1<sup>ff</sup>Bim<sup>ff</sup> mice (on an Arf<sup>wt</sup> background) was transduced with p185-IRES-Cre retrovirus to transform and delete floxed alleles. Viable cell concentration was determined every 24 hours. Each time point represents the average of three independent experiments (n=3) each comprised of 2 separate initiations and the error bars denote the SEM. (B) Representative Mcl-1, Mcl-1-deleted, Bax, Bak, and Cre genomic genotyping post-outgrowth from experiment detailed in (A). (C) Representative Mcl-1, Mcl-1-deleted, Bim, Bim-deleted, and Cre genomic genotyping shown from post outgrowth cells detailed in (A) (D) Representative immunoblot of the cells that grew out in culture from the experiment detailed in (A). Lysates were blotted for MCL-1, BAX, BAK, Cre and BCR-ABL (as detected by anti-ABL antibody) (loading control). (E) Representative Immunoblot analysis of the cells which grew out in culture from the experiment detailed in (A). Lysates were blotted for MCL-1, BIM, Cre and BCR-ABL (loading control). (F) Mcl-1<sup>ff</sup> Arf<sup>−/−</sup> pre-B cells were co-transduced with Mcl-1, BCL-2, BCL-X<sub>L</sub>, or Mcl-1<sup>GRRL</sup> constructs and p185-IRES-Cre retrovirus. Cell viability was determined every 24 hours. Each time point represents three independent experiments (n=3) comprised of 2 separate initiations and the error bars denote the SEM. (G) Representative Mcl-1 and Mcl-1-deleted genotyping from Mcl-1<sup>ff</sup> Arf<sup>−/−</sup> pre-B cells co-transduced with indicated construct and p185-IRES-Cre retrovirus post outgrowth. (H) Lysates from Mcl-1<sup>ff</sup> Arf<sup>−/−</sup> pre-B cells co-transduced with indicated constructs and p185-IRES-Cre retrovirus post outgrowth.
were western blotted for MCL-1, BCL-XL, BCL-2 (human specific antibody) and BCR-ABL (loading control). Results indicate two independent cultures.

**Figure 4. Requirement for Anti-Apoptotic Function in Leukemia Maintenance**

(A) *In vitro* generated *Mcl-1*<sup>−/−</sup> *Arf<sup>−/−</sup>* p185<sup>+</sup> cells stably expressing either vector or ectopic *Mcl-1* were transduced with *Cre-IRES-GFP* to delete the endogenous *Mcl-1*. The total number of GFP<sup>+</sup> cells was measured every 24 hours. IL-7 was used at a final concentration of 20 ng/mL. Each point represents the average of three independent experiments (n=3) and the error bars denote the SEM. (B) Immunoblot analysis p185<sup>+</sup> *Mcl-1*<sup>−/−</sup> *Arf<sup>−/−</sup>* pre B cells before and after *Cre-IRES-GFP* transduction was performed. Lysates were western blotted for MCL-1, Cre and Actin (loading control) (C) *Mcl-1*<sup>−/−</sup> *Arf<sup>−/−</sup>* p185<sup>+</sup> pre-B cells stably expressing indicated constructs were transduced with *Cre-IRES-GFP* to delete endogenous *Mcl-1*. The total number of GFP<sup>+</sup> cells was measured every 24 hours. Each point represents the average of three independent experiments (n=3) and the error bars denote the SEM. (D) Immunoblot analysis shown for *Mcl-1*<sup>−/−</sup> *Arf<sup>−/−</sup>* p185<sup>+</sup> pre-B cells before and after *Cre-IRES-GFP* transduction for MCL-1, BCL-2 (human specific antibody), BCL-X<sub>L</sub>, Cre and Actin (loading control).

**Figure 5. Requirement for MCL-1 during in vivo BCR-ABL Leukemia Maintenance**

(A) *Mcl-1*<sup>−/−</sup> Rosa-ER<sup>T2</sup> (*Arf<sup>wt</sup>*) or *Mcl-1*<sup>wt</sup> Rosa-ER<sup>T2</sup> BM were transduced with p185-IRES-GFP and transplanted into lethally-irradiated (1100 Rads) C57BL/6 recipients that were monitored for leukemia initiation. After leukemia initiation, bone marrow from the leukemic mice (two independent leukemia donors per genotype) were
harvested and mixed 1:1 with control CD45.1 congenic bone marrow and transplanted into secondary, lethally-irradiated (1100 Rads) C57BL/6 recipients (~10 secondary recipients each for 2 separate leukemic donors). After five days, the secondary recipients were treated with five daily-doses of tamoxifen (TAM, 1 µg/day) to activate Cre (n=13 mice for Mcl-1<sup>f/f</sup> Rosa-ERCre<sup>T2</sup> and n=10 for Mcl-1<sup>wt</sup> Rosa-ERCre<sup>T2</sup> donors) or control vehicle (n=9 mice for Mcl-1<sup>f/f</sup> Rosa-ERCre<sup>T2</sup> and n=9 for Mcl-1<sup>wt</sup> Rosa-ERCre<sup>T2</sup> donors) by gavage. Mice were monitored daily and sacrificed when moribund. Asterisk (*) denotes p<0.001 by Log Rank Test. (B) White blood cell (WBC) counts were taken from moribund, secondary recipients described in (A) at time of sacrifice. Bars indicate the averages (n=6 mice for TAM-treated Mcl-1<sup>f/f</sup> Rosa-ERCre<sup>T2</sup> donors, n=9 for TAM-treated Mcl-1<sup>wt</sup> Rosa-ERCre<sup>T2</sup> donors, n=9 mice for vehicle-treated Mcl-1<sup>f/f</sup> Rosa-ERCre<sup>T2</sup> donors, and n=9 for vehicle-treated Mcl-1<sup>wt</sup> Rosa-ERCre<sup>T2</sup> donors) and error bars indicate SEM. (C) Representative immunoblot shown of GFP<sup>+</sup> bone marrow isolated from TAM-treated, moribund mice that received p185<sup>+</sup> Mcl-1<sup>f/f</sup> Rosa-ERCre<sup>T2</sup> leukemic BM (each lane represents a recipient mouse). Lysates were blotted for MCL-1, BCR-ABL (as detected by anti-ABL antibody), and ERCre<sup>T2</sup> protein as a loading control (note: ERCre<sup>T2</sup> fusion protein is present in all cells, but is only activated by TAM). (D) Representative Mcl-1, Mcl-1-deleted, and Cre genotyping of GFP<sup>+</sup> bone marrow isolated from TAM-treated, moribund mice that received p185<sup>+</sup> Mcl-1<sup>f/f</sup> Rosa-ERCre<sup>T2</sup> leukemic BM (each lane represents a recipient mouse). (E) Histopathological examination of moribund vehicle-treated or TAM-treated mice that received p185<sup>+</sup> Mcl-1<sup>f/f</sup> Rosa-ERCre<sup>T2</sup> leukemic BM. Images of indicated tissues (50X magnification) are representative of 9 vehicle-treated mice and 4 moribund, TAM-treated mice analyzed.
H&E and PAX5 immunohistochemistry are morphologically and immunophenotypically indicative of B-lineage leukemia.

Figure 6. Potentiation of Mouse BCR-ABL\(^+\) Leukemia to Combining Tyrosine Kinase Inhibitor and Small Molecule BCL-2 Inhibitors

(A) \textit{In vitro} generated \textit{Mcl-1}-deleted \textit{Arf}\(^{-}\) p185\(^+\) cells stably expressing the indicated constructs were treated with indicated doses of navitoclax (ABT-263) for 24 hours after which percent viable cells were determined (propidium iodide negative cells were scored as viable). Each point represents the average of three independent experiments and the error bars denote the SEM. p185\(^+\) \textit{Arf}\(^{-}\) cells serve as a control. (B) \textit{Arf}-null p185\(^+\) cells were treated with navitoclax for 24 hours at indicated doses. Lysates were immunoprecipitated with anti-BIM antibody and immune complexes were resolved and western blotted for MCL-1, BCL-2 (human specific antibody), BCL-X\(_L\), BIM, PARP and Actin (loading control). Endogenous BIM serves as the control for equal immunoprecipitation. (C&D) p185\(^+\) \textit{Arf}\(^{-}\) cells were treated with (C) imatinib (IM) or (D) dasatinib (DAS) for 24 hours at indicated doses. Lysates were immunoprecipitated with anti-BIM antibody and immune complexes were resolved and western blotted for MCL-1, BCL-2, BCL-X\(_L\), BIM, PARP and Actin (loading control). Endogenous BIM served as the control for equal immunoprecipitation. (E&F) p185\(^+\) \textit{Arf}\(^{-}\) cells were treated with indicated doses of navitoclax and/or (E) IM or (F) DAS for 24 hours after which percent viable cells was determined. Annexin-V and PI negative cells were scored as viable. IL-7 was added to a final concentration of 20 ng/mL. Bars represent the average of three independent experiments done in triplicate and the error bars denote the SEM. Asterisk
(*) indicates p<0.001 by 2-way ANOVA with a Bon Ferroni post-test between treatments linked with horizontal line.

**Figure 7. Potentiation of Ph+ Human Cell Lines to Combining Tyrosine Kinase Inhibitor and Small Molecule BCL-2 Inhibitors**

Human Ph+ cell lines: (A) OP-1 Ph+ B-ALL (B) TOM1 Ph+ B-ALL and (C) BV173 Ph+
CML blast crisis cell lines were treated with navitoclax, imatinib (IM) or dasatinib (DAS) at indicated doses for 24 hours after which cell lysates were analyzed for expression of MCL-1, BCL-2, BCL-w, BFL-1 and BCL-XL. For potentiation experiments, the percentage of viable cells was determined by Annexin-V and PI negativity. Bars represent the average of three independent experiments, each done in triplicate, for each cell type and the error bars denote the SEM. Asterisk (*) indicates p<0.001 by 2-way ANOVA with a Bon Ferroni post-test between treatments linked with horizontal line.
Requirement for anti-apoptotic MCL-1 in the survival of BCR-ABL B-lineage acute lymphoblastic leukemia

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