MCL-1–dependent leukemia cells are more sensitive to chemotherapy than BCL-2–dependent counterparts

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Myeloid cell leukemia sequence 1 (MCL-1) and B cell leukemia/lymphoma 2 (BCL-2) are anti-apoptotic proteins in the BCL-2 protein family often expressed in cancer. To compare the function of MCL-1 and BCL-2 in maintaining cancer survival, we constructed complementary mouse leukemia models based on Eμ-Myc expression in which either BCL-2 or MCL-1 are required for leukemia maintenance. We show that the principal anti-apoptotic mechanism of both BCL-2 and MCL-1 in these leukemias is to sequester pro-death BH3-only proteins rather than BAX and BAK. We find that the MCL-1–dependent leukemias are more sensitive to a wide range of chemotherapeutic agents acting by disparate mechanisms. In common across these varied treatments is that MCL-1 protein levels rapidly decrease in a proteosome-dependent fashion, whereas those of BCL-2 are stable. We demonstrate for the first time that two anti-apoptotic proteins can enable tumorigenesis equally well, but nonetheless differ in their influence on chemosensitivity.

Introduction

Cancer cells exhibit many properties that have been shown to cause apoptosis in normal cells. Among these is the activation of proliferative oncoproteins like MYC. Activation of MYC induces a transcriptional program that can result in increased proliferation, but often at the expense of cell death via the apoptotic pathway. Whether the net effect is increased proliferation or apoptosis appears to vary depending on cell context. Conditions which often occur in cancer, such as growth factor and/or oxygen deprivation, can sensitize MYC-expressing cells to apoptosis (Evan et al., 1992; Graeber et al., 1996; Brunelle et al., 2004). To survive in these conditions, the cells must find a way to prevent cell death. Cancer cells can select for ways to block the apoptotic signaling from MYC in order to tip the balance in favor of proliferation. A seminal observation of this principle was made when it was found that overexpression of the anti-apoptotic protein BCL-2 (B cell leukemia/lymphoma 2) could rescue cells from MYC-induced apoptosis (Bissonnette et al., 1992; Fanidi et al., 1992). However, the molecular mechanisms responsible for the apoptotic induction by MYC and its rescue by BCL-2 remain incompletely understood.

The BCL-2 family proteins control apoptosis at the mitochondrion. They are related by sequence homology and by participation in control of apoptosis (Danial and Korsmeyer, 2004). They contain one or more BCL-2 homology (BH) domains that are involved in protein–protein interactions. BCL-2 family proteins containing only a BH3 domain can be activated in response to critical aberrations in cellular physiology, including DNA damage, growth factor withdrawal, and oncogene activation (Puthalakath and Strasser, 2002). These BH3-only proteins are activated by mechanisms involving transcriptional up-regulation, subcellular localization, and/or posttranslational modification. In one model of apoptotic control, the so-called “direct” model, certain BH3-only proteins (including BID, BIM, and perhaps PUMA) are known as “activators.” These proteins activate the pro-apoptotic BCL-2 family proteins BAX and BAK (Wei et al., 2000; Certo et al., 2006; Letai, 2008). Activated BAX and BAK then homo-oligomerize and cooperate in the permeabilization of the outer mitochondrial membrane. Pro-apoptotic contents of the mitochondrial intermembrane space, including cytochrome c,
are released to the cytosol. These contents drive the activation of caspases, which are proteases that cleave and disable critical proteins throughout the cell. Anti-apoptotic proteins (BCL-2, MCL-1, BCL-XL, BFL-1, BCL-w) inhibit cell death by intercepting activator BH3-only proteins and preventing their interaction with BAX and BAK (Cheng et al., 1996; Certo et al., 2006). Anti-apoptotic proteins may also bind activated, monomeric BAX and BAK before they can oligomerize (Willis et al., 2005).

Other BH3-only proteins called sensitizers (including BAD, NOXA, and BIK) cannot activate BAX and BAK directly (Letai et al., 2002; Certo et al., 2006). Sensitizers promote death by displacing activators from the hydrophobic binding pocket of anti-apoptotic proteins. PUMA is a pro-apoptotic BH3-only protein first identified as a transcriptional target of p53 (Nakanou and Vousden, 2001). Whether PUMA acts primarily as a sensitizer or an activator remains the subject of debate. An alternative model of apoptosis control, the so-called “indirect” model, holds that BAX and BAK are not activated by activator BH3-only proteins. A key principle of this model is that the anti-apoptotic function of proteins like BCL-2 resides solely in their binding to BAX and BAK (Willis et al., 2007).

Certain cancers depend upon BCL-2 for survival. Understanding the mechanism of this dependence not only provides information to guide therapeutic targeting of such dependence, but also provides important insights into the function of BCL-2 (Deng et al., 2007; Letai, 2008). We have previously studied BCL-2–dependent cancers including human chronic lymphocytic leukemia (CLL), lymphoma cell lines, small cell lung cancer cell lines, and a murine lymphocytic leukemia (Certo et al., 2006; Del Gaizo Moore et al., 2007; Deng et al., 2007). We have found that BCL-2 dependence can be identified in cancer cells using a strategy we call BH3 profiling (Certo et al., 2006; Letai, 2008). The main principle of BH3 profiling is to quantify mitochondrial dysfunction after treatment with a panel of BH3 peptides. This affords the opportunity to isolate the mitochondrial precondition as a contributor to subsequent death decisions. By treating with measured death signals, in the form of BH3 peptides, we can identify mitochondria that more readily undergo apoptosis than others. Because anti-apoptotic proteins each have a distinct pattern of interaction with the BH3 peptides, the pattern of response to individual BH3 domains can identify the anti-apoptotic protein required to maintain survival in a cell.

A common property of the BCL-2 protein in BCL-2–dependent cells is that it is largely bound to pro-apoptotic proteins, specifically activator BH3-only proteins like BIM (Del Gaizo Moore et al., 2007; Deng et al., 2007). We refer to cells in this state as “primed for death,” or more simply “primed.” These proteins can be displaced by competition with peptides derived from the BH3 domains of BH3-only proteins. Mitochondrial dysfunction ensues and its measurement is the readout for BH3 profiling. We have previously found that primed cells are more sensitive to chemotherapy than unprimed cells (Deng et al., 2007).

The anti-apoptotic MCL-1 (myeloid cell leukemia sequence 1) protein is gaining attention as a potential therapeutic target in cancer (Reynolds et al., 1994). Although MCL-1 has been implicated in both tumorigenesis and chemoresistance, there is little known about MCL-1 dependence in cancer. There is evidence that MCL-1 is important for survival of myeloma cells, but the mechanism behind this requirement is obscure (Derenne et al., 2002). An MCL-1 transgenic murine model exists that demonstrates an increased risk of lymphoma, but the long latency (>18 mo on average) renders such a model inconvenient for mechanistic studies (Zhou et al., 2001). In murine bone marrow viral infection models, MCL-1 was found to accelerate both myeloid and lymphoid malignancies (Wendel et al., 2007; Beverly and Varmus, 2009).

To better understand MCL-1 dependence in cancer, we compared MCL-1– and BCL-2–dependent leukemia models. We find that BH3 profiling confirms the dependence in these tumors, and that the molecular basis to the dependence involves binding of BH3-only proteins rather than BAX or BAK. Furthermore, we find that MCL-1–dependent leukemias are more sensitive to a wide variety of treatments than are their BCL-2–dependent counterparts.

Results
MCL-1 facilitates myc-mediated leukemogenesis
It has been established previously that targeting Bcl-2 expression in mice to lymphoid cells that also overexpress the c-myc oncogene results in rapid onset of a lymphoid leukemia (Strasser et al., 1990). This leukemia is less mature and more rapidly fatal than the lymphoma generated by the c-myc oncogene alone (Adams et al., 1985). By using a Bcl-2 promoter that could be turned off when doxycycline was added to the drinking water, we showed that the resulting leukemia was explicitly dependent on BCL-2 for survival (Letai et al., 2004). An important question remains whether other BCL-2 family anti-apoptotic transgenes could function similarly to facilitate lymphoid leukemogenesis driven by c-Myc in this transgenic model. To test whether MCL-1 overexpression results in a leukemia, the H2K promoter was used to target MCL-1 expression to many tissues, including blood, spleen, bone marrow, thymus, and lymph nodes. As expected, MCL-1 protein levels were significantly increased in spleen and bone marrow samples of H2K-Mcl-1 mice when compared with those of wild-type mice (Fig. 1 A). H2K-Mcl-1 mice were crossed with Eμ-Myc mice and a cohort was analyzed, henceforth called Mcl-1/Eμ-Myc mice. Mcl-1/Eμ-Myc mice survived only an average of 72 ± 21 d. Mice bearing the Eμ-Myc transgene survived an average of 134 ± 35 d, whereas both mice bearing only the H2K-Mcl-1 transgene and wild-type mice survived well beyond 200 d (Fig. 1 B).

Given the results from prior models noted above, we suspected that the early mortality in the Mcl-1/Eμ-Myc mice was due to the development of a lymphoid leukemia. White blood cell counts for 4–6-wk-old Mcl-1/Eμ-Myc mice averaged 367,000 ± 156,000 per microliter of blood. Counts from Eμ-Myc only, H2K-Mcl-1 only, and wild-type mice of the same age were significantly lower and nearly normal: 12,000 ± 2,000, 12,000 ± 3,000, and 7,900 ± 1,600 per microliter of blood, respectively (Fig. 1 C). White blood cells isolated from Mcl-1/Eμ-Myc mice
were positive for B220 and CD19, negative for CD4, IgG, IgM, and IgD, and variable for AA4.1 and CD43 (unpublished data). This pattern confirms their B lymphocyte origin. Their cell surface marking corresponds to a pre- or pro-B cell, class C or D in the Hardy scheme (Hardy and Hayakawa, 2001), consistent with the majority of leukemias derived from previously reported combination of BCL-2 and c-Myc (Letai et al., 2004). The earliest white blood cell counts were taken after weaning and genotyping. Therefore, Mcl-1/E\(\mu\)-Myc mice show evidence of leukemia by 4 wk of age and may well have leukemia even earlier.

Spleen and bone marrow samples from 1–3-mo-old Mcl-1/E\(\mu\)-Myc mice contained a monotonous population of lymphoblasts crowding out the normal trilineage hematopoiesis, similar to the BCL-2/E\(\mu\)-Myc marrow samples (Fig. 2). Note that the bone marrow and spleen populations of comparably aged H2K-Mcl-1, E\(\mu\)-Myc, or wild-type mice are essentially normal. These results show that MCL-1 facilitates c-MYC-driven leukemogenesis in a manner clinically and morphologically indistinguishable from BCL-2. The bone marrow from leukemic mice is, however, quite distinct from that of E\(\mu\)-Myc mice.

**BH3 profiling distinguishes MCL-1- and BCL-2-dependent leukemias**

We have previously used BH3 profiling to detect dependence on individual anti-apoptotic family proteins in several systems (Certo et al., 2006; Del Gaizo Moore et al., 2007; Deng et al., 2007). An important test of this technique is whether it can distinguish between dependence on two different anti-apoptotic proteins in primary cancer cells. Our murine leukemia models provided an ideally controlled test case for performance of BH3 profiling. Briefly, mitochondria were isolated from the white blood cells of Mcl-1/E\(\mu\)-Myc mice. The mitochondria were treated with peptides corresponding to the BH3 domain of several BCL-2 family proteins: BIM, BID, BAD, BIK, NOXA A, NOXA B, HRK, BNIPI, PUMA, and BMF. After peptide treatment, release of cytochrome c was measured. The resulting profile showed high levels of cytochrome c release when treated with BIM, BID, NOXA, PUMA, and BMF peptides (Fig. 3 A). This matches fluorescence polarization data indicating that MCL-1 has high affinity for BH3 domains derived from BIM, BID, NOXA, PUMA, and BMF (Certo et al., 2006). This pattern thus corresponds to one of MCL-1 dependence. We also performed BH3 profiling on leukemia cells derived from the leukemia in BCL-2/E\(\mu\)-Myc mice. We had previously explicitly shown that these leukemias were BCL-2 dependent, as they vanished when BCL-2 was removed using the tet-off switch (Letai et al., 2004). The BH3 profile of BCL-2/E\(\mu\)-Myc white blood cells shows high levels of cytochrome c release when treated with BIM, BID, BAD, PUMA, BMF (Fig. 3 D). This pattern is consistent with mitochondrial BCL-2 dependence and consistent with the cellular BCL-2 dependence already established. Thus, using BH3 profiling we have verified that we have engineered two distinct models of murine leukemia, differing in that one model is dependent on BCL-2 and the other model is dependent on MCL-1. We detected no other difference between the two leukemia models. These results increase our confidence in the ability of BH3 profiling to make the subtle distinction between dependence on different anti-apoptotic proteins in primary cancer samples.

Although MCL-1- and BCL-2-dependent primary leukemia models were identified, it is convenient to perform replicate experiments with the same cell over time and primary leukemia cells are not useful reagents for such work. Thus, we established cell lines, three from each genotype that spontaneously immortalized in culture from these primary leukemias. The BH3 profiles of these cell lines correspond to the BH3 profile from the primary leukemias (examples in Fig. 3, B and E). Therefore, the cell lines were validated as cancer cell line models of MCL-1 and BCL-2 dependence.

The BH3 profiling in Fig. 3, A, B, D and E relies on the performance of a heavy membrane preparation that is enriched for mitochondria at the expense of lighter membranes like...
endoplasmic reticulum. However, these lighter membranes can also harbor MCL-1 and BCL-2, albeit usually at lower quantities. To better test the contribution of MCL-1 and BCL-2 at all membrane locations, we turned to a modification of BH3 profiling using whole cells. In this modification, cells were permeabilized with low concentrations of digitonin to permit peptide access to the cell interior, and mitochondrial permeabilization was measured with the JC1 dye. We have found across dozens of cell lines that there is a tight correlation between MOMP measured by cytochrome c release and by JC1 by this method (unpublished data). Dependence on MCL-1 and BCL-2 are again demonstrated. As expected, the Bad peptide caused a rapid decrease in mitochondrial membrane potential in the putatively Bcl-2–dependent cells (Fig. 3 F), whereas the Noxa-specific peptides (Noxa A and Bim 2A) caused a decrease in mitochondrial membrane potential in the putatively Mcl-1–dependent cells (Fig. 3 C). The results using whole cells confirm the initial BH3 profiling results obtained

Figure 2. Pathology reveals that the normal architecture of spleen and bone marrow is replaced by a monotonous population of lymphoblasts in Mcl-1/Em-Myc mice, similar to BCL-2/Em-Myc mice. (A) Wildtype. (B) H2K-Mcl-1 only. (C) Em-Myc only. (D) Mcl-1/Em-Myc. (E) BCL-2/Em-Myc. Bar for the spleen samples is 100 µm, and bar for the bone marrow samples is 50 µm.
likely because the MCL-1 and BCL-2 are sequestering pro-death molecules that would induce death if MCL-1 or BCL-2 function were lost or abrogated. In this situation, we describe the pro-death proteins as "priming" MCL-1 or BCL-2, and cells or mitochondria or anti-apoptotic proteins in that situation to be "primed" (Certo et al., 2006; Deng et al., 2007). To determine what pro-death molecules are priming MCL-1 in Mcl-1/Eμ-Myc leukemia, coimmunoprecipitation was performed from CHAPS lysates of white blood cells from two primary samples and one cell line. MCL-1 did indeed coimmunoprecipitate with BIM and PUMA, but not with BID, BAX, or BAK (Fig. 4 A). Similar to the Mcl-1/Eμ-Myc leukemia and to prior results (Letai et al., 2004), BCL-2/Eμ-Myc white blood cell lysates show that BCL-2 coimmunoprecipitated with BIM and PUMA, but not with BID, BAX, or BAK in two primary samples and one cell line (Fig. 4 B). In summary, it appears that anti-apoptotic proteins BCL-2 and MCL-1 are primed with BH3-only proteins BIM and PUMA. In contrast, priming by BAX and BAK is not detectable. Others have found that BAK and MCL-1 interact, and we and others have found that BAX and BCL-2 interact in from isolated mitochondria. Results from all six cell lines can be seen in Fig. S1.

To further ensure that key apoptotic phenotypes were preserved, we treated the cell lines with doxycycline, which turns off BCL-2 expression and induces killing of leukemia cells in vivo. We found that doxycycline efficiently induced killing of BCL-2/Eμ-Myc leukemia cell lines, but not Mcl-1/Eμ-Myc leukemia cell lines (Fig. 3 G). These cell lines provided us with a pair of models which differed only in whether they were dependent on BCL-2 or MCL-1. We are unaware of any other comparable pairing of primary or cancer cell line models contrasting defined BCL-2 and MCL-1 dependence. BH3 profiling demonstrated a remarkable ability to distinguish these two models.

MCL-1 and BCL-2 are primed with pro-death BIM and PUMA

Anti-apoptotic proteins inhibit apoptosis by binding and sequestering pro-apoptotic BH3-only proteins and possibly monomeric BAX or BAK, especially when BAX or BAK have been activated. If cells are dependent on MCL-1 or BCL-2, it is likely because the MCL-1 and BCL-2 are sequestering pro-death molecules that would induce death if MCL-1 or BCL-2 function were lost or abrogated. In this situation, we describe the pro-death proteins as "priming" MCL-1 or BCL-2, and cells or mitochondria or anti-apoptotic proteins in that situation to be "primed" (Certo et al., 2006; Deng et al., 2007). To determine what pro-death molecules are priming MCL-1 in Mcl-1/Eμ-Myc leukemia, coimmunoprecipitation was performed from CHAPS lysates of white blood cells from two primary samples and one cell line. MCL-1 did indeed coimmunoprecipitate with BIM and PUMA, but not with BID, BAX, or BAK (Fig. 4 A). Similar to the Mcl-1/Eμ-Myc leukemia and to prior results (Letai et al., 2004), BCL-2/Eμ-Myc white blood cell lysates show that BCL-2 coimmunoprecipitated with BIM and PUMA, but not with BID, BAX, or BAK in two primary samples and one cell line (Fig. 4 B). In summary, it appears that anti-apoptotic proteins BCL-2 and MCL-1 are primed with BH3-only proteins BIM and PUMA. In contrast, priming by BAX and BAK is not detectable. Others have found that BAK and MCL-1 interact, and we and others have found that BAX and BCL-2 interact in.
other cell lines (Willis et al., 2005; Deng et al., 2007). Our results here do not rule out such interactions, but instead point out that many of the interactions between pro- and anti-apoptotic family proteins will be very dependent on the context, and specifically dependent on the abundance of other BCL-2 family proteins that might be competing as heterodimerization partners.

**Heterogeneous dependence on BCL-2 and MCL-1 in lymphomas from Eµ-Myc mice**

Forced transgenic expression of BCL-2 and MCL-1 apparently buffers death signaling in a way that fosters the development of a MYC-driven leukemia that is more aggressive than the lymphoma that appears in Eµ-Myc mice later in life. Yet even the less aggressive lymphomas in the Eµ-Myc mice likely have death signals to buffer. It is unknown how Eµ-Myc lymphomas block apoptosis. We asked whether the lymphomas in Eµ-Myc mice also showed dependence on anti-apoptotic proteins, or whether they selected an alternative type of block in apoptosis (Deng et al., 2007). To answer this question, we turned again to BH3 profiling. This technique shows that dependence on anti-apoptotic proteins in these lymphomas varies from case to case. The lymphoma from mouse #859 demonstrates MCL-1 dependence (Fig. 5 A). Combinations of BCL-2, BCL-XL, and/or MCL-1 dependence were discovered in the lymphomas of three other mice tested (Fig. 5, B–D). To test whether patterns of protein expression bore a discernable relationship to the dependence observed in BH3 profiling, we examined immunoblots of whole-cell lysates of the lymphomas (Fig. 5 E). Consistent with the MCL-1 dependence seen by BH3 profiling, MCL-1 protein levels are high and BCL-2 protein levels are relatively lower in tumor #859 (Fig. 5 A). In the remaining lymphoma cells with a more mixed picture of dependence by BH3 profiling, expression of BCL-2, MCL-1, and BCL-XL appeared more mixed. In the absence of a transgene driving overexpression of a particular anti-apoptotic protein, c-MYC–driven lymphomas demonstrate a heterogeneous pattern of dependence on anti-apoptotic proteins. It is a formal possibility that some of this heterogeneity might derive from oligo- or poly-clonality in the tumors. However, although we did not directly demonstrate clonality for the tumors studied here, others have shown that tumors from this model are almost always monoclonal (Adams et al., 1985).

Western blotting was also used to compare BCL-2 family protein levels among BCL-2/Eµ-Myc−, Mcl-1/Eµ-Myc−, and Eµ-Myc−only tumors (Fig. 6 A). Notably, BIM and PUMA levels were much higher in the leukemias in which BCL-2 or MCL-1 was overexpressed. This suggests the possibility that the anti-apoptotic buffering capacity permitted a more aggressive phenotype by fostering tolerance of greater level of pro-death signaling.

**Leukemias that rely on MCL-1 for survival are more chemosensitive than those that rely on BCL-2**

Although biochemical details are important in understanding leukemogenesis, it is of paramount practical clinical interest to understand how these changes affect response to chemotherapy. Although both MCL-1 and BCL-2 can cooperate with c-MYC to form leukemias, it has not been tested whether cellular exploitation of one over the other might confer greater chemosensitivity. To put this question another way, with all other things being equal, do leukemia cells that depend on MCL-1 for survival have a different sensitivity to conventional chemotherapy agents than those that depend on BCL-2 for survival? To answer this question, we compared the sensitivity of our Mcl-1/Eµ-Myc leukemia cell lines with those of our BCL-2/Eµ-Myc cell lines to a range of chemotherapeutic agents that work via diverse mechanisms (Fig. 7). The agents used included etoposide (topoisomerase II inhibitor), vincristine (microtubule disruptor), staurosporine (broad spectrum

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**Figure 4.** MCL-1 and BCL-2 are primed with BIM and PUMA in leukemia cells. (A) Coimmunoprecipitation of MCL-1 from CHAPS lysate of white blood cell primary sample or cell line from Mcl-1/Eµ-Myc mouse. Blot was probed for BIM, PUMA, BID, BAX, BAK, and MCL-1. (B) Coimmunoprecipitation of BCL-2 from CHAPS lysate of white blood cell primary sample or cell line from BCL-2/Eµ-Myc mouse. Blot was probed for BIM, PUMA, BID, BAX, BAK, and BCL-2.

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**Table 1.** BH3 profiling of lymphomas from Eµ-Myc mice. | BH3 Sensitizer | Mcl-1/Eµ-Myc | Bcl-2/Eµ-Myc |
|----------------|---------------|---------------|
| BIM            | #977          | #766          |
| PUMA           | #1780         |               |
| BAX            |               | #977          |
| BAK            |               | #766          |
| MCL-1          |               |               |

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**Figure 5.** MCL-1 and BCL-2 are primed with BIM and PUMA in leukemia cells. (A) Coimmunoprecipitation of MCL-1 from CHAPS lysate of white blood cell primary sample or cell line from Mcl-1/Eµ-Myc mouse. Blot was probed for BIM, PUMA, BID, BAX, BAK, and MCL-1. (B) Coimmunoprecipitation of BCL-2 from CHAPS lysate of white blood cell primary sample or cell line from BCL-2/Eµ-Myc mouse. Blot was probed for BIM, PUMA, BID, BAX, BAK, and BCL-2.

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**Figure 6.** Western blotting was also used to compare BCL-2 family protein levels among BCL-2/Eµ-Myc−, Mcl-1/Eµ-Myc−, and Eµ-Myc−only tumors (Fig. 6 A). Notably, BIM and PUMA levels were much higher in the leukemias in which BCL-2 or MCL-1 was overexpressed. This suggests the possibility that the anti-apoptotic buffering capacity permitted a more aggressive phenotype by fostering tolerance of greater level of pro-death signaling.

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**Figure 7.** MCL-1 and BCL-2 are primed with BIM and PUMA in leukemia cells. (A) Coimmunoprecipitation of MCL-1 from CHAPS lysate of white blood cell primary sample or cell line from Mcl-1/Eµ-Myc mouse. Blot was probed for BIM, PUMA, BID, BAX, BAK, and MCL-1. (B) Coimmunoprecipitation of BCL-2 from CHAPS lysate of white blood cell primary sample or cell line from BCL-2/Eµ-Myc mouse. Blot was probed for BIM, PUMA, BID, BAX, BAK, and BCL-2.
We examined MCL-1 levels after treatment with vincristine, etoposide, and flavopiridol and found that in each case MCL-1 levels were dramatically reduced over the first 24 h (Fig. 8, A–C), the time over which almost all cells were killed. In addition, it can be seen that the quicker MCL-1 levels were reduced, the more rapidly cells died. For instance, in cells treated with flavopiridol, MCL-1 levels were nearly undetectable at 8 h (Fig. 8 A) and nearly all cells were killed by 8 h (Fig. 9 B). Etoposide was next-most efficient at reducing MCL-1 levels, followed by vincristine, again corresponding to how rapidly the cells were killed (Fig. 8, B and C; Fig. 9 B). Note that although the MCL-1 decrease for etoposide-treated 2640 cells appears modest, it was consistent, as demonstrated by densitometric analysis of four independent experiments. MCL-1 levels (average [standard deviation]) at 8, 16, and 24 h were 95% [18%], 76% [25%], and 72% [33%] of starting levels. In contrast, treatment with these same three agents had little effect on BCL-2 levels in the BCL-2–dependent cell lines, with concomitantly slower killing kinetics and reduced toxicity to the drugs (Fig. 8, D–G; Fig. 9 C). It is important to note that although Mcl-1 levels are driven by the H2K promoter in the Mcl-1/Eμ-Myc mouse

Figure 5. BH3 profiling of Eμ-Myc tumors reveals variable dependence on anti-apoptotic proteins. Data presented are from one single experiment. (A) Eμ-Myc tumor #859 shows a MCL-1 profile. (B) Eμ-Myc tumor #1107 shows a profile where MCL-1 > BCL-2/BCL-XL. (C) Eμ-Myc tumor #1433 shows a profile where MCL-1 > BCL-2/BCL-XL. (D) Eμ-Myc tumor #1656 shows a profile where BCL-2/ BCL-XL > MCL-1. (E) Western blot containing CHAPS lysates of the Eμ-Myc tumors in order to compare protein levels of anti-apoptotic proteins BCL-2, BCL-XL, and MCL-1. Note low BCL-2 protein level in Eμ-Myc tumor #859.
important to distinguish whether MCL-1 loss occurs upstream or downstream of cellular death. To that end, it is important to note that the experiments in Fig. 8 were performed in the presence of the broad spectrum caspase inhibitor Q-VD-OPH, which was used at concentrations sufficient to inhibit manifestations of death including membrane permeabilization as measured by propidium iodide staining and phosphatidylserine exposure as measured by Annexin V staining (Fig. 9 A). Thus, MCL-1 loss is seen in the absence of overt manifestations of cell death, suggesting that MCL-1 loss is a cause, rather than a consequence, of cell death in these experiments.

MCL-1 protein half-life remains constant after treatment

Like all proteins, MCL-1 levels depend on an equilibrium between production and degradation. A decrease in MCL-1 can thus be due either to an increase in degradation or a decrease in production, or both. It has been shown that MCL-1 is subject to proteosomal degradation, both tonically and in response to numerous cellular perturbations (Nijhawan et al., 2003; Maurer et al., 2006). This can be seen here in the increase in MCL-1 levels caused by treatment with the proteosome inhibitor bortezomib (Fig. 8 A). Loss of MCL-1 after chemotherapy treatment could therefore be due to an induced increase in proteosomal degradation. To test whether degradation was increasing, we compared MCL-1 half-life in cells treated with cycloheximide in the presence and absence of flavopiridol, the drug that most rapidly caused MCL-1 loss. Fig. 10, A and B shows that the half-life is unaffected by flavopiridol, indicating that an increase in degradation is unlikely to play an important role in MCL-1 loss. This suggests that the loss of MCL-1 after chemotherapy treatment in these leukemias is not due to an increase in its rate of degradation, but rather due to a decrease in its production caused by the toxic agents.

It has been found that NOXA can bind to MCL-1 and increase the rate of proteosomal degradation of MCL-1 (Czabotar et al., 2007). Although our results here suggest that the rate of MCL-1 degradation is not increasing, we nonetheless tested whether changes in NOXA levels correlate to changes in MCL-1 levels. We were unable to identify any antibody to recognize murine NOXA, so we used RT-PCR to quantitate NOXA message levels. We found that although NOXA transcript levels increased in response to bortezomib, etoposide, and vincristine, they decreased in response to flavopiridol (Fig. S2), where the most rapid MCL-1 decrease was seen. Thus, although we cannot rule out a role for NOXA, an increase in NOXA transcript levels are not a requirement for MCL-1 loss.

MCL-1 and BCL-2 have similar a priori anti-apoptotic potency in the leukemias

The Mcl-1/ Eμ-Myc and BCL-2/ Eμ-Myc leukemias were constructed by the same basic method, and leukemias of identical morphology, cell surface phenotype, and clinical behavior were obtained. However, as the proteins were untagged, we could not directly compare absolute levels of BCL-2 and MCL-1. We could, however, test their relative “potency.” That is, we could test whether Mcl-1/Eμ-Myc cells contained mitochondria that

Figure 6.  Higher BIM and PUMA expression observed in MCL-1– and BCL-2–overexpressing leukemias. Western blot comparing protein levels among three Eμ-Myc tumors, two BCL-2/Eμ-Myc leukemias, and three Mcl-1/ Eμ-Myc leukemias. (A) The proteins blotted were MCL-1, BCL-2, BIM, PUMA, BID, BAD, BAX, and BAK. Please note that the BCL-2 transgene in the BCL-2/Eμ-Myc lymphomas is human. (B) The proteins blotted were BAD, Phospho-Bad (Ser112), and Actin.

and MCL-1–dependent cell lines, endogenous levels of MCL-1 are found in the BCL-2/Eμ-Myc mouse and BCL-2–dependent cell lines. MCL-1 levels do decrease in the Bcl-2–dependent cells when treated with etoposide, vincristine, or flavopiridol (Fig. 8, E–G), suggesting that the changes in MCL-1 levels are not dependent on the promoter from which protein expression derives.

After death, one might expect the levels of many short half-life proteins to decrease, potentially including MCL-1. Thus, it is important to distinguish whether MCL-1 loss occurs upstream or downstream of cellular death. To that end, it is important to note that the experiments in Fig. 8 were performed in the presence of the broad spectrum caspase inhibitor Q-VD-OPH, which was used at concentrations sufficient to inhibit manifestations of death including membrane permeabilization as measured by propidium iodide staining and phosphatidylserine exposure as measured by Annexin V staining (Fig. 9 A). Thus, MCL-1 loss is seen in the absence of overt manifestations of cell death, suggesting that MCL-1 loss is a cause, rather than a consequence, of cell death in these experiments.

MCL-1 protein half-life remains constant after treatment

Like all proteins, MCL-1 levels depend on an equilibrium between production and degradation. A decrease in MCL-1 can thus be due either to an increase in degradation or a decrease in production, or both. It has been shown that MCL-1 is subject to proteosomal degradation, both tonically and in response to numerous cellular perturbations (Nijhawan et al., 2003; Maurer et al., 2006). This can be seen here in the increase in MCL-1 levels caused by treatment with the proteosome inhibitor bortezomib (Fig. 8 A). Loss of MCL-1 after chemotherapy treatment could therefore be due to an induced increase in proteosomal degradation. To test whether degradation was increasing, we compared MCL-1 half-life in cells treated with cycloheximide in the presence and absence of flavopiridol, the drug that most rapidly caused MCL-1 loss. Fig. 10, A and B shows that the half-life is unaffected by flavopiridol, indicating that an increase in degradation is unlikely to play an important role in MCL-1 loss. This suggests that the loss of MCL-1 after chemotherapy treatment in these leukemias is not due to an increase in its rate of degradation, but rather due to a decrease in its production caused by the toxic agents.

It has been found that NOXA can bind to MCL-1 and increase the rate of proteosomal degradation of MCL-1 (Czabotar et al., 2007). Although our results here suggest that the rate of MCL-1 degradation is not increasing, we nonetheless tested whether changes in NOXA levels correlate to changes in MCL-1 levels. We were unable to identify any antibody to recognize murine NOXA, so we used RT-PCR to quantitate NOXA message levels. We found that although NOXA transcript levels increased in response to bortezomib, etoposide, and vincristine, they decreased in response to flavopiridol (Fig. S2), where the most rapid MCL-1 decrease was seen. Thus, although we cannot rule out a role for NOXA, an increase in NOXA transcript levels are not a requirement for MCL-1 loss.

MCL-1 and BCL-2 have similar a priori anti-apoptotic potency in the leukemias

The Mcl-1/ Eμ-Myc and BCL-2/ Eμ-Myc leukemias were constructed by the same basic method, and leukemias of identical morphology, cell surface phenotype, and clinical behavior were obtained. However, as the proteins were untagged, we could not directly compare absolute levels of BCL-2 and MCL-1. We could, however, test their relative “potency.” That is, we could test whether Mcl-1/Eμ-Myc cells contained mitochondria that
were intrinsically more sensitive to apoptotic stimuli, even before perturbation with toxic agents. To test whether this was the case, we isolated mitochondria from Mcl-1/Eμ-Myc and BCL-2/Eμ-Myc leukemia cell lines, and compared their response to the PUMA BH3 peptide. The PUMA BH3 peptide behaves as a sensitizer and interacts with all of the anti-apoptotic proteins (Certo et al., 2006). Thus, it can be used alone as an index of how primed for death a mitochondrion is. This test provides a functional comparison that interrogates simultaneously the possible differential contribution of many BCL-2 family proteins in the two leukemias. In Fig. 10 C, we show that there is no significant difference in the priming of Mcl-1/Eμ-Myc and BCL-2/Eμ-Myc at the heavy membranes. In Fig. 10 D, we show a similar result using the whole-cell BH3 profiling technique. This implies that before treatment, the intrinsic sensitivity of the Mcl-1/Eμ-Myc and BCL-2/Eμ-Myc mitochondria to apoptotic...
In summary, we observed that MCL-1–dependent cancer cells were more sensitive to treatment with a range of chemotherapeutic agents than were matched BCL-2–dependent counterparts. The mechanism behind the difference appears to be that MCL-1 is a short half-life protein subjected to ongoing proteosome-dependent degradation. Although the rate of this degradation does not change after chemotherapy treatment, chemotherapy apparently disrupts efficient MCL-1 protein production, resulting in a rapid net loss of MCL-1 protein. BCL-2 has a longer half-life and seems to be spared chronic proteosomal degradation, so its levels are relatively stable after treatments, resulting in less chemosensitivity.

**Discussion**

Here we report a comparison of murine models of MCL-1– and BCL-2–dependent lymphoid leukemias. MCL-1 dependence has not been previously demonstrated in a murine cancer model. We now have a system of cell lines and murine leukemia models that are apparently identical except for their different dependence signaling is the same. This is consistent with the hypothesis that net loss of MCL-1 after drug treatment is responsible for the selective sensitivity of the MCL-1–dependent leukemias.

Our data suggest that Mcl-1–dependent cells are killed easier than those that are Bcl-2 dependent, all other things being equal. However, the ARF/Mdm2/p53 pathway can also play an important role in apoptosis, and it has previously been found that selection of alterations of this pathway can be altered by anti-apoptotic protein expression in a myc-driven lymphoma model (Schmitt et al., 2002). We measured expression of ARF and p53 to test whether MCL-1 expression systematically resulted in a different proportion of p53- or ARF-overexpressing tumors compared with BCL-2 expression. In Fig. S3, we show that MCL-1 and BCL-2 expression result in similar proportions of p53 and ARF up-regulation (6/12 vs. 7/12, 5/12 vs. 5/12, respectively). Note that p53 up-regulation, indicating a defect in the ARF/Mdm2/p53 tumor suppressor pathway, was found in 10 of 11 Eμ-Myc samples tested, a higher proportion than was reported previously (Eischen et al., 1999). These results are consistent with prior results which found that forced expression of an anti-apoptotic protein decreased the rate of selection for abnormalities in the ARF/Mdm2/p53 pathway in a myc-driven lymphoma model (Schmitt et al., 2002).

In summary, we observed that MCL-1–dependent cancer cells were more sensitive to treatment with a range of chemotherapeutic agents than were matched BCL-2–dependent counterparts. The mechanism behind the difference appears to be that MCL-1 is a short half-life protein subjected to ongoing proteosome-dependent degradation. Although the rate of this degradation does not change after chemotherapy treatment, chemotherapy apparently disrupts efficient MCL-1 protein production, resulting in a rapid net loss of MCL-1 protein. BCL-2 has a longer half-life and seems to be spared chronic proteosomal degradation, so its levels are relatively stable after treatments, resulting in less chemosensitivity.

**Figure 8. Varied chemotherapeutics induce MCL-1 before cell death.** All cell lines pretreated with 20 μM of caspase inhibitor Q-VD-OPH for 1 h. CHAPS lysates were prepared and MCL-1, BCL-2, BIM, and Actin protein levels were determined using Western blotting. (A) Mcl-1/Eμ-Myc cell lines treated with 1 μM Bortezomib or 1 μM Flavopiridol for 0, 8, and 16 h. (B) Mcl-1/Eμ-Myc cell lines treated with 1 μM Etoposide for 0, 8, 16, and 24 h. (C) Mcl-1/Eμ-Myc cell lines treated with 1 μM Vincristine for 0, 8, 16, and 24 h. (D and E) BCL-2/Eμ-Myc cell lines treated with 1 μM Etoposide or 1 μM Vincristine for 0, 8, 16, and 24 h. (F) BCL-2/Eμ-Myc cell lines treated with 1 μM Bortezomib for 0, 8, and 16 h. (G) BCL-2/Eμ-Myc cell lines treated with 1 μM Flavopiridol for 0, 8, and 16 h.
on either BCL-2 or MCL-1. Such a system has potential utility as in vivo and in vitro test systems for putative selective antagonists of either BCL-2 or MCL-1 that are currently in preclinical and clinical development for cancer therapy. In addition, as we have exploited it in this paper, such a system is useful in understanding the mechanisms by which cancer cells escape apoptosis, and in comparing the similarities and differences in biological function between BCL-2 and MCL-1.

We have previously reported testing BH3 profiling in model systems in which BH3 profiling accurately identified dependence on different individual anti-apoptotic proteins, but have not previously identified a purely MCL-1–dependent cancer. Here we find that BH3 profiling could usefully validate the

dependence on different individual anti-apoptotic proteins, but have not previously identified a purely MCL-1–dependent cancer. Here we find that BH3 profiling could usefully validate the
distinction between MCL-1 and BCL-2 dependence in leukemia models based on MCL-1 or BCL-2 overexpression in combination with the Eμ-Myc transgene. However, the Eμ-Myc transgene by itself drives formation of lymphomas, leading us to ask how these tumors escaped apoptosis. Although the Eμ-Myc lymphoma model has been studied for decades, the tools were not previously available to investigate its dependence on anti-apoptotic proteins (Adams et al., 1985). Using BH3 profiling, we find that in the absence of forced overexpression of any single anti-apoptotic protein, Eμ-Myc lymphomas are nonetheless dependent on anti-apoptotic proteins for survival. The specific anti-apoptotic protein dependence varies from lymphoma to lymphoma, and can be mixed in a single lymphoma. The source for the differences may lie in a simple stochastic selection for greater or lesser expression of one or the other of the anti-apoptotic proteins, as seems to be the case for lymphoma 859 (Fig. 5). Alternatively, the differences in expression may reside in inherent differences in differing initiating cells selected for tumorigenesis in each animal.

We also asked if the anti-apoptotic proteins in our leukemias are primed, what pro-death molecules are they primed with? We were unable to observe sequestration of BAX or BAK in our leukemias. Instead, we observed sequestration of the pro-death BH3-only proteins BIM and PUMA. This observation is more consistent with a model in which the most important anti-apoptotic function of anti-apoptotic proteins like BCL-2 or MCL-1 is to sequester BH3-only proteins (Cheng et al., 1996; Cheng et al., 2001; Letal et al., 2002; Kuwana et al., 2005; Certo et al., 2006). The pure indirect model would suggest that we should observe sequestration of BAX and BAK. Although we observed sequestration of both BIM and PUMA, it is yet possible that in these leukemias BCL-2 and MCL-1 sequester other BH3-only proteins, perhaps some not yet even identified as BH3-only proteins. If indeed the sequestration of BIM or PUMA is critical to the function of MCL-1 or BCL-2, one would expect that loss of PUMA or BIM might also be able to cooperate with Eμ-Myc to drive development of the less mature leukemias. In fact, loss of PUMA or BIM combined with the Eμ-Myc transgene does cause development of the immature leukemia, supporting their sequestration as a key role performed by MCL-1 and BCL-2 (Egle et al., 2004; Michalak et al., 2009).

Despite the similarity in phenotype and in biochemical properties, the BCL-2/Eμ-Myc and Mcl-1/Eμ-Myc leukemias differed consistently in their sensitivity to cytotoxic agents. Our results suggest that this is because proteosomal degradation of MCL-1 persists while new synthesis of MCL-1 is reduced by treatment with a wide variety of agents. This changes the steady state so that there is a net loss of MCL-1, resulting in accelerated death of MCL-1-dependent cells. A similar observation has been made in the response of HeLa cells to ultraviolet radiation (Nijhawan et al., 2003). Here, we show that MCL-1 loss is a more general phenomenon following a wide variety of insults. Such a net loss does not occur for the longer half-life BCL-2 protein, which is more stable. Killing the BCL-2-dependent cells thus requires more time and more drug (Figs. 7 and 9). Note that our studies cannot address issues of which tumors are cured because this depends on issues like fractional kill and stem cell biology, still poorly understood for individual tumors. Yet it is important to understand chemosensitivity because this is a necessary, though not sufficient, condition for cure by chemotherapy.

Chemosensitivity of the Eμ-myc and Eμ-myec/Eμ-BCL-2 leukemia/lymphoma models was compared in a recent manuscript (Mason et al., 2008). In contrast with our BCL-2/Eμ-Myc model, the expression of BIM in the Eμ-myec/Eμ-BCL-2 cancers was similar to that in the Eμ-myc cancers. Consistent with our expectation that tumors from the Eμ-myc model would be more frequently MCL-1 dependent (see Fig. 5), MCL-1 levels were higher in these malignancies than in the Eμ-myec/Eμ-BCL-2 malignancies. Both models were subjected to treatment with cyclophosphamide, an alkylating agent. Consistent with our findings that Eμ-myc tumors are frequently MCL-1 dependent, and the leukemias based on myc and BCL-2 transgenes BCL-2 dependent, and that MCL-1-dependent tumors tend to respond better to chemotherapy, the Eμ-myc mice demonstrated vastly superior survival after therapy, with a 50% long-term survival compared with 0% in the Eμ-myec/Eμ-BCL-2 mice. These results support an in vivo drug sensitivity consequence to MCL-1 dependence versus BCL-2 dependence in cancers.

It may well be that using BH3 profiling or other strategies, other tumors, perhaps even primary tumors, will be revealed as MCL-1 dependent. Indeed, there is already reason to believe that many myeloma cells may be MCL-1 dependent (Derene et al., 2002). Superficially, our results may suggest that such cells will be readily killed by a wide variety of agents. More exactly stated, however, our prior results and those here suggest that primed cells that are dependent on any anti-apoptotic protein will be killed easier that unprimed, and that primed cells that are MCL-1 dependent will be killed easier that those that are BCL-2 dependent, all other factors being equal. Achieving comparisons where all other factors are equal, outside of purposefully constructed models, will indeed be difficult, but perhaps with sufficient numbers, informative testing of the hypothesis may be possible. It will be interesting to test this hypothesis in the coming years as relevant cancers are identified. However, we already know that our use of cytotoxic drugs is limited by toxicity, so that more directly targeted therapy may be superior. Thus, selective antagonists of MCL-1 may eventually play an important role in treating MCL-1-dependent cancers.

A key generalizable finding of this study is that the type of block in apoptosis selected by a cancer during tumorigenesis can significantly influence chemosensitivity. Though two different anti-apoptotic proteins might both enable leukemogenesis, the leukemias derived differ critically in their chemosensitivity. As shown here and previously, BH3 profiling can identify types of blocks in apoptosis, and thus may be useful in prediction of chemosensitivity.

Materials and methods

Transgenic mice
The MMTV-HA/tetBCL-2/Eμ-Myc mouse has been described previously (Letal et al., 2004).

Mcl-1 transgenic mice
A minigene containing the H2K promoter/enhancer and the Moloney MuLV enhancer/poly(A) site (a gift from Dr. Jos Domen, Medical College
of Wisconsin, Milwaukee, WI) driving the expression of mouse Mcl-1 cDNA was injected into zygotes obtained from crosses between F1 (C57BL/6 3 C3H) mice. Resulting progeny were tested by Southern blotting, and positive mice were backcrossed to C57BL/6 mice. Four independent founder lines were generated with different expression levels as tested by Western blot. The transgenic mice were generated, housed, and bred in the Dana-Farber Cancer Institute (DFCI) animal facility. All animal experiments were approved by the Dana-Farber Cancer Institute IACUC (protocol #05-001).

Mice containing the H2K-Mcl-1 transgene (usually female) were bred to Eμ-Myc mice (usually male).

Monitoring leukemia
Blood from a razor nick was diluted in buffer (saponin 0.3%, Hoechst 33258 1 μg/ml, EDTA 25 mM in PBS) and manual counts of white blood cells were performed.

Pathology
Spleen and bone marrow samples were stored in formalin and sent to the Dana Farber Harvard Cancer Center Pathology Core for processing with hematoxylin and eosin stain.

Microscopy and image analysis
The microscope used was a Nikon Eclipse E600. To view the bone marrow samples the objective lens was a Nikon Plan Fluor 20x/0.75 NA. To view the spleen samples the objective lens was a Nikon Plan Fluor 10x/0.30 NA. The camera was a SPOT RT SE from Diagnostic Instruments, Inc. (model 9.0 monochrome-6) with SPOT software for image acquisition.

Mouse cell lines
White blood cells were isolated from blood using Ficoll gradient. Spleens were removed from the mouse and smashed between two glass slides. Smashed spleens and white blood cells were placed in 50 mL of RPMI, 10% FBS, 1% PenStrep, and 25 mM HEPES (pH 7.4). Single suspension was washed once in T-EB and resuspended at 2–3 x 10^6 cells/ml. One volume of the above cell suspension was added to one volume of a 4x dye solution containing 2 μM A23187 and 0.1 mM EGTA to bring the final density to 4x their final density. One volume of the above cell suspension was added to one volume of a 4x dye solution containing 2 μM A23187 and 0.1 mM EGTA to bring the final density to 4x their final density. Images were captured using a Nikon Microphot-FXA with a Nikon Plan Fluor 20x/0.75 NA objective lens and a SPOT RT SE 14 megapixel camera.
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