Apoptosis and Cancer

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Abstract

Cancer is a disease involving the abnormal accumulation of cells resulting from an imbalance of proliferation and programmed cell death. This review focuses on the mitochondrial apoptotic pathway, a mechanism of programmed cell death with particular relevance to cancer. Starting over 30 years ago, basic findings in model organisms have been combined with findings in clinical cytogenetics to uncover a family of proteins, the BCL-2 family, that regulates the commitment to apoptosis by controlling permeabilization of the mitochondrial outer membrane. Cancer cells are generally more poised to engage the apoptotic machinery than normal cells are, a fact that likely underlies much of the therapeutic index exploited by many types of cancer chemotherapy. More recently, small molecules directly targeting the antiapoptotic proteins of the BCL-2 family have entered the clinic for testing in cancer. One therapeutic, venetoclax (ABT-199), has recently gained FDA approval in a landmark achievement for the apoptosis community. Important future efforts will be directed at building combinations of agents that selectively induce apoptosis in cancer cells.

INTRODUCTION

Genetically encoded programs of cell death have been identified across metazoans (Bender et al. 2012). This suggests that there is a broad evolutionary advantage to be gained by multicellular animals in forcing individual cells to eliminate themselves when they are harmful to the organism as a whole. Whereas many different genetically programmed pathways of cell death have been identified, including apoptosis, necroptosis (Zhou & Yuan 2014), and ferroptosis (Dixon et al. 2012, Green & Llambi 2015), there are two distinct pathways of apoptosis: the intrinsic (or mitochondrial) and extrinsic (or death receptor) pathways. Although they share the end result of activating cysteine proteases known as caspases, the mechanisms by which this occurs are quite distinct. This review focuses on the mitochondrial pathway of apoptosis. It discusses the early history of apoptosis, the fundamental molecular biology of the regulation of apoptosis, and the relevance to cancer.

IMPORTANT LANDMARKS IN THE HISTORY OF APOPTOSIS

Although apoptosis and the BCL-2 family now appear to be inextricably linked to human cancer, this link was not clear in early studies of apoptosis. Kerr et al. (1972) proposed associating the neologism apoptosis (from the Greek "falling off," as in leaves off a tree) with a morphologically distinct pattern of cell death that they observed in a variety of mammalian tissue sections. The apoptosis they observed was characterized by nuclear condensation and cellular fragmentation, followed by phagocytosis of the fragments by nearby cells. Although there was as yet no appreciation that apoptosis was produced by genetically encoded proteins, it was noted that an invariant and coordinated program appeared to be initiated in these dying cells.

Sulston (1976) noted a similar form of programmed cell death occurring during development of the roundworm *Caenorhabditis elegans*. While meticulously mapping the developmental lineage of each of the 1,028 cells in the mature *C. elegans*, he noted that cell death occurred invariantly in specific cells at specific times during development. In subsequent studies, Horvitz et al. (1983) identified mutations in two genes, *ced-3* and *ced-4*, which impaired this programmed cell death. This represented the first evidence that programmed cell death pathways were genetically encoded. This work was recognized by the 2002 Nobel Prize in Physiology or Medicine awarded to John Sulston, Sydney Brenner, and H. Robert Horvitz.

In parallel work that initially had no immediate evident connection to the above, in the 1970s, investigators began to identify recurrent chromosomal translocations associated with specific cancer histologies. In one example, a translocation between chromosomes 14 and 18 was associated with small, noncleaved cell lymphoma, now more commonly called follicular lymphoma (Rowley 1973, 1988). In the mid-1980s, the region on chromosome 14 involved in the translocation was shown to be the enhancer of the immunoglobulin heavy chain gene, likely driving B cell–specific expression of a gene on chromosome 18, putatively a novel oncogene of unknown function (Bakhshi et al. 1985; Cleary & Sklar 1985; Tsujimoto et al. 1984, 1985). The first evidence of the prosurvival function of BCL-2 came when Vaux et al. (1988) observed that BCL-2 expression promoted proliferation of c-myc-overexpressing cells, as well as survival of leukocytes upon growth factor withdrawal. An in vivo prosurvival function was demonstrated in the expansion of the B-lymphoid compartment in mice expressing a bcl-2-Ig minigene (McDonnell et al. 1989). That *BCL-2* might function independently as an oncogene was confirmed with the observation that these mice frequently progressed to a malignant lymphoma (McDonnell & Korsmeyer 1991).

Hockenbery et al. (1990) forged a direct link between BCL-2 and apoptosis with the observation that BCL-2 did not simply have just a prosurvival function, but rather specifically prevented apoptotic cell death in cells deprived of growth factor. These authors were the first to connect

apoptosis to mitochondria, with the observation that the BCL-2 protein was localized to mitochondria (although they incorrectly assigned it to the inner, rather than the outer, mitochondrial membrane). The link of BCL-2 to apoptosis and oncogenesis was strengthened when Strasser et al. (1990) identified that BCL-2 blocked an apoptotic death induced by myc, explaining the prior observation of the facilitation of myc-induced transformation by BCL-2. The important observation that there were shared mechanisms of cell death across much of the animal kingdom was made by Vaux et al. (1992), who showed that enforced expression of human BCL-2 prevented developmental cell death in *C. elegans*. Subsequently, Hengartner & Horvitz (1994a,b) demonstrated that the antiapoptotic gene of *C. elegans*, ced-9, showed structure and sequence homology to bcl-2. Thus, by 1994, it was known that apoptosis was a genetically programmed function conserved across a broad swath of the animal kingdom, that this regulation likely took place at the mitochondrion (by as yet mysterious means involving BCL-2 in mammals), and that dysregulation of this pathway could facilitate oncogenesis.

MOLECULAR BIOLOGY OF THE BCL-2 FAMILY

Important questions regarding the molecular mechanisms by which BCL-2 regulated apoptosis remained unanswered in the early 1990s. What was BCL-2 interacting with at the mitochondrion? What did the mitochondrion, theretofore recognized primarily as a source of energy and a crucible for innumerable metabolic reactions, have to do with cell death?

An important first step in answering these questions was the identification of a prodeath protein that bound to BCL-2. This protein, named BAX, shared structural and sequence homology with BCL-2 (Oltvai et al. 1993). This finding opened up the discovery over the next decade of an entire family of structurally related proteins, both pro- and antiapoptotic, that regulated apoptosis, the so-called BCL-2 family (Boise et al. 1993, Chittenden et al. 1995, Choi et al. 1995, Gibson et al. 1996, Han et al. 1996, Kozopas et al. 1993, Lin et al. 1996, Nakano & Vousden 2001, O'Connor et al. 1998, Oda et al. 2000, Sedlak et al. 1995, Yang et al. 1995, Zhou et al. 1997). An important finding was that the release of cytochrome *c* from mitochondria was a central event in the activation of caspases (Kluck et al. 1997, Liu et al. 1996). This was rapidly followed by the observation that BCL-2 prevented the release of cytochrome *c* from mitochondria (Yang et al. 1997). Over the next few years, investigation from several laboratories demonstrated that antiapoptotic BCL-2 family proteins (including BCL-2, BCL-XL, BCL-w, MCL-1, and BFL-1/A1) act to prevent mitochondrial permeabilization, whereas proapoptotic proteins (including BAX, BAK, BIM, BID, BAD, BMF, and PUMA) act to promote mitochondrial permeabilization. Although many competing models were initially developed to explain how these many BCL-2 family members coordinated commitment to apoptosis, including those excluding activation of BAX or BAK by activator BH3-only proteins (Willis et al. 2007), in recent years consensus has coalesced around a model bearing the features demonstrated in **Figure 1** and described below (Kim et al. 2006, Letai et al. 2002, Llambi et al. 2011).

Mitochondrial outer membrane permeabilization (MOMP), with its attendant release of proapoptotic factors, including cytochrome *c*, can in most cases be considered the point of irreversible commitment to programmed cell death. However, exceptions to this generalization exist, and the survival of cells following the permeabilization of a minority of a cell's mitochondria is an area of active investigation (Ichim et al. 2015). In addition, there is complex regulation of caspase activation following MOMP, involving the formation of an apoptosome consisting of cytochrome *c*, APAF-1, caspase-9, caspase-3, and ATP acting to activate caspase-3, which is beyond the scope of this review (Zou et al. 1999). Moreover, the production of "eat me" signals that facilitate the recognition and phagocytosis of apoptotic cells is not discussed here, though ably presented elsewhere (Suzuki et al. 2013, Toda et al. 2012). The inhibition of caspase activity

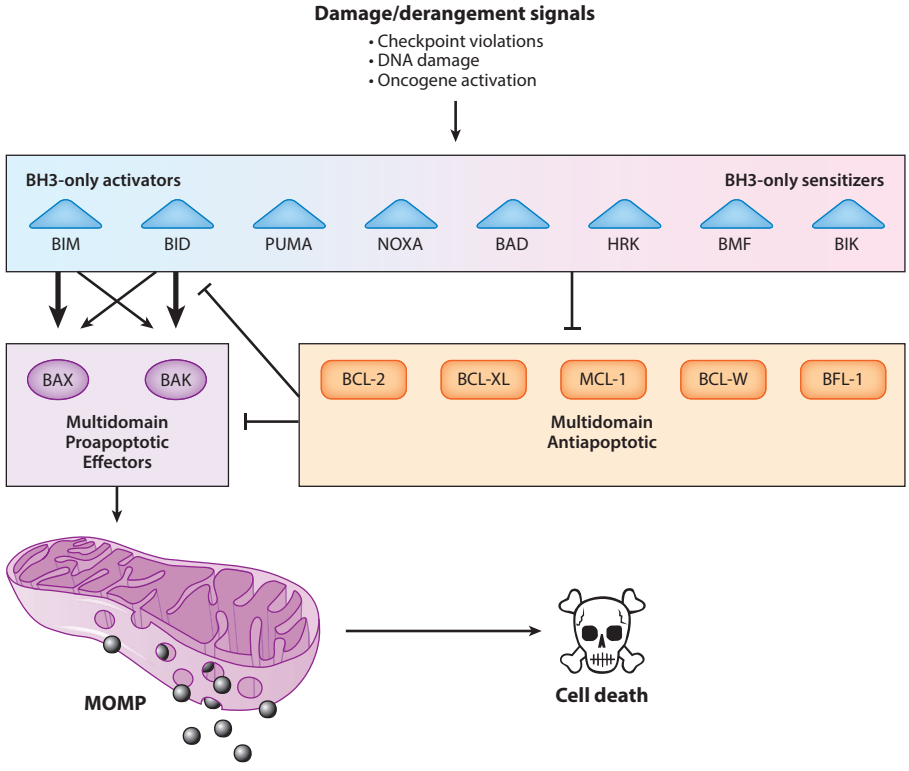

Figure 1

The basic circuitry of how the BCL-2 family regulates apoptosis. In response to many types of perturbations, BH3-only activators such as BIM and BID stimulate the oligomerization of effectors BAX and BAK to induce mitochondrial outer membrane permeabilization (MOMP), release of cytochrome *c*, and cell death. Antiapoptotic multidomain members may inhibit activation by sequestration of activators or activated, monomeric BAX and BAK. BH3-only sensitizers can bind to the BH3 binding sites of and inhibit antiapoptotic multidomain members, but cannot activate BAX or BAK on their own. If the antiapoptotic proteins are already binding to activators or effectors, these can be displaced by sensitizers so that the activators and effectors can proceed with mitochondrial outer membrane permeabilization and commitment to cell death. Note that among BH3-only proteins, activator function appears to be present along a gradient, with BIM and BID being the most potent activators, and PUMA and NOXA also exhibiting activator function, though apparently less potent than BIM or BID. Also, BIM preferentially activates BAX over BAK, and BID preferentially activates BAK over BAX. Figure redrawn from Certo et al. (2006).

eliminates many of the morphologic characteristics of apoptosis, including efficient phosphatidyl serine exposure, DNA fragmentation, and PARP cleavage. However, caspase inhibition does not necessarily inhibit clonogenic death, which may often result from mitochondrial dysfunction alone (Cheng et al. 2001, Ekert et al. 2004, Janssen et al. 2007, Marsden et al. 2006).

MOMP is caused by the formation of pores that result from the homo-oligomerization of BAX or BAK, so-called proapoptotic effector proteins (Antonsson et al. 2000, Korsmeyer et al. 2000, Lindsten et al. 2000, Saito et al. 2000, Wei et al. 2001). This pore is likely distinct from the mitochondrial permeability transition pore triggered by calcium and inhibited by cyclosporine A (Eskes et al. 1998). Although there may be other unknown factors that also participate in the pore, either BAX or BAK is necessary and sufficient for pore formation (Kuwana et al. 2002, Saito et al.

2000). Cells deficient in BAX and BAK therefore exhibit profound resistance to cell death via the mitochondrial pathways of apoptosis (Lindsten et al. 2000, Wei et al. 2001).

Homo-oligomerization of BAX and BAK is induced by interaction with so-called activator BH3-only proteins (Cheng et al. 2001, Desagher et al. 1999, Eskes et al. 2000, Letai et al. 2002). The term BH3-only proteins arises from their sharing only a single BCL-2 homology (BH) domain, the BH3 domain, with the rest of the BCL-2 family. Proteins with the most potent activator function include BIM and BID, but PUMA and NOXA also have been found to activate BAX and BAK (Cartron et al. 2004; Dai et al. 2014; Du et al. 2011; Kim et al. 2006, 2009; Letai et al. 2002; Shangary & Johnson 2002). It is likely that BIM and BID are the most potent, however (Du et al. 2011, Letai et al. 2002). The BH3 domains of activator BH3-only proteins interact with BAX and BAK noncovalently to induce an allosteric change that facilitates their homodimerization (Brouwer et al. 2014, Lovell et al. 2008, Sarosiek et al. 2013, Suzuki et al. 2000, Zhang et al. 2016). Homodimerization is followed by the formation of higher-ordered oligomers that eventually reach a size sufficient to cause pore formation (Antonsson et al. 2001, Czabotar et al. 2013, Mikhailov et al. 2003). Recent findings suggest that the related multidomain pro-apoptotic protein BOK may not require activation by activators to oligomerize and cause MOMP. Instead, its proapoptotic function may be regulated at the level of protein stability, although this remains under investigation (Carpio et al. 2015, Einsele-Scholz et al. 2016, Fernandez-Marrero et al. 2016, Ke et al. 2013, Llambi et al. 2016, Schulman et al. 2016).

Antiapoptotic proteins, including BCL-2, BCL-XL, MCL-1, BCL-W, and BFL-1/A1, interrupt this proapoptotic cascade in two ways. First, antiapoptotic proteins can bind activator BH3-only proteins, preventing their interaction with and activation of BAX or BAK (Certo et al. 2006, Cheng et al. 2001, Letai et al. 2002). In addition, antiapoptotic proteins can bind the monomeric, activated forms of BAX and BAK, which have their BH3 domains exposed (Cuconati et al. 2003; Ding et al. 2014; Willis et al. 2005, 2007). This prevents homo-oligomerization of BAX and BAK, and thus prevents MOMP and apoptosis.

BH3-only proteins with a sensitizer function, including PUMA, NOXA, BAD, BMF, and BIK, can be thought of as inhibitors of the antiapoptotic proteins (Certo et al. 2006, Deng et al. 2007, Letai et al. 2002). BH3 domains of sensitizers compete with the BH3 domains of activators and effectors to prevent their binding to antiapoptotic proteins. Alternatively, if activator or effector proteins are already bound to an antiapoptotic protein, they can be displaced by a competing sensitizer protein, facilitating MOMP (Certo et al. 2006). The net effect of a sensitizer therefore greatly depends on the pre-existing conditions of the BH3 family at the mitochondrion. Venetoclax and other small molecule inhibitors of BCL-2 family antiapoptotic proteins function as mimetics of sensitizer BH3 peptides (Ni Chonghaile & Letai 2008). We refer to mitochondria in which there is an abundance of prodeath proteins sequestered by antiapoptotic proteins as "primed for death." Such mitochondria permeabilize readily in response to BH3 domains, including sensitizer BH3 domains. Cells bearing highly primed mitochondria are sensitive to chemotherapy, as discussed in more detail below (Ni Chonghaile et al. 2011, Vo et al. 2012).

The BH3 domain is a roughly 20 amino acid amphipathic α-helical segment possessed by all BCL-2 family members. It is required for the proapoptotic function of all proapoptotic proteins (Huang & Strasser 2000). When forming a heterodimer with antiapoptotic proteins, the BH3 domain is bound by a hydrophobic cleft formed by the BH1, BH2, and BH3 domains of the antiapoptotic protein. BH3 domains demonstrate limited conservation of primary amino acid sequence among BCL-2 proteins, but there is conservation of some features of hydrophobicity, size, and charge nonetheless. Likewise, BH3 binding clefts are distinct among antiapoptotic proteins (**Figure 2*a***). The result is that there is a binding code that governs interactions among pro- and antiapoptotic proteins (**Figure 2*b***) (Certo et al. 2006, Chen et al. 2005, Kuwana et al. 2005).

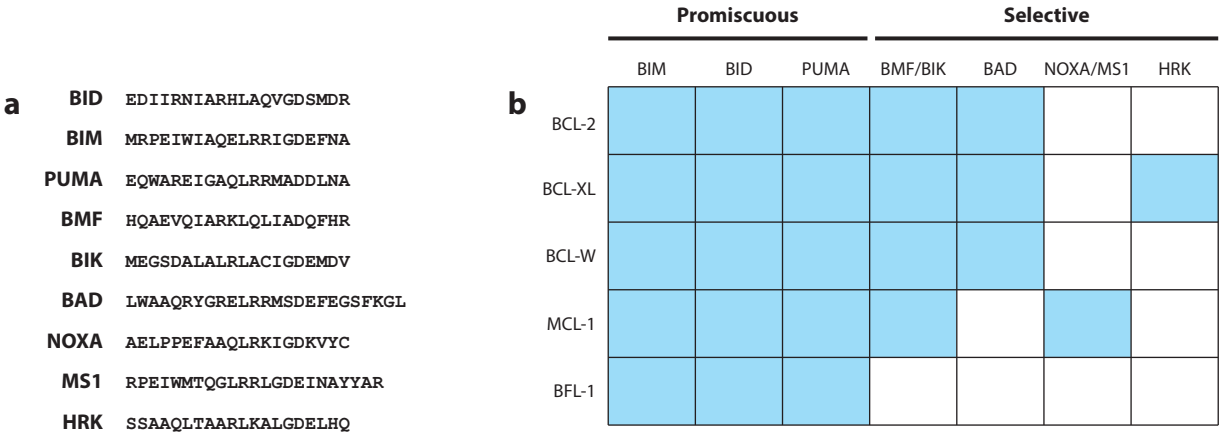

Figure 2

(*a*) Amino acid sequences for BH3 domains in the BCL-2 family. (*b*) Interaction map for BH3 peptides with the antiapoptotic Bcl-2 members. Blue spaces indicate a K_d of less than 100 nM as determined by fluorescence polarization. The unique binding signatures of antiapoptotic Bcl-2 proteins can be used to identify when an individual cell is dependent on an individual antiapoptotic protein. For instance, HRK is a selective probe for BCL-XL dependence, whereas NOXA or MS1 is a selective probe for MCL-1 dependence. Response to peptides for which binding is more promiscuous (e.g., BIM, BID, PUMA) is useful for measuring how close a cell is to the threshold of apoptosis, or how primed a cell is. Panel *a* adapted from Certo et al. (2006) and Letai et al. (2002), and panel *b* adapted from Certo et al. (2006).

There is also specificity of interaction of activators with effectors: BID preferentially activates BAK, whereas BIM preferentially activates BAX (Sarosiek et al. 2013).

EVADING APOPTOSIS AS A HALLMARK OF CANCER

Evading apoptosis or resisting cell death has been proposed as a hallmark of cancer (Hanahan & Weinberg 2000, 2011). This concept requires some clarification. It is likely that many of the events of becoming a cancer, including oncogene activation, growth independent of growth factors, and invasion and metastasis beyond the site of origin, provoke apoptotic signaling. This signaling must be somehow endured for a cell to survive to eventually become a cancer. In several model systems, blocks in apoptotic signaling facilitate oncogenesis. It is therefore quite safe to state that the process of oncogenesis subjects cells to proapoptotic stresses that must be buffered or blocked for oncogenesis to produce a cancer cell. Indeed, Hanahan & Weinberg's description of evading apoptosis as a hallmark of cancer was made mainly in the context of tumorigenesis.

However, some have extended this concept to assert that cancer cells are generally resistant to subsequent proapoptotic signaling that befalls them even after oncogenesis has produced a cancer cell. This assertion provokes the question, resistant compared to what? There is little evidence that cancer cells are more resistant to apoptosis than normal cells are. Indeed, in many cancers, there is ample evidence of continual spontaneous apoptosis. Furthermore, the presence of a therapeutic index for conventional chemotherapy in most cancers relies on the increased sensitivity to apoptosis in cancer cells than in normal cells (Ni Chonghaile et al. 2011, Vo et al. 2012). Although cancer cells may select for evasion of apoptosis under the stress of oncogenesis, they cannot presciently select for resistance to apoptotic signals they have yet to encounter, such as those provoked by chemotherapy. Although it is true that cancer cells are more resistant to apoptosis than many of us wish they were, it is not generally true that they are more resistant to apoptosis than normal cells are.

Inhibition of apoptosis can facilitate carcinogenesis. Perhaps the first evidence of this effect is simply that the t(14;18) translocation from which BCL-2 was originally cloned is found in nearly all cases of follicular lymphoma and many additional cases of diffuse large B cell lymphoma, suggesting a selective advantage for apoptosis inhibition during carcinogenesis (Fukuhara et al. 1979, Rowley 1988). BCL-2 blocks myc-induced apoptosis, and overexpressed BCL-2 greatly accelerates the induction of a lymphoid leukemia/lymphoma induced by overexpressed c-myc in a murine model (Bissonnette et al. 1992, Fanidi et al. 1992, Strasser et al. 1990). However, although inhibition of apoptosis can facilitate the carcinogenic action of other oncogenes, it is by itself only weakly oncogenic. This can be seen by the slow kinetics and low penetrance of lymphoma in a murine model of BCL-2 overexpression (McDonnell & Korsmeyer 1991). Moreover, the BAX/BAK double knockout mouse, deeply resistant to the mitochondrial pathway of apoptosis, only rarely develops tumors (Lindsten et al. 2000, Wei et al. 2001).

When cancer cells are subjected to chemotherapy, there is selection for reduced sensitivity to apoptosis, likely an important contributor to the pan-resistant phenotype of many relapsed tumors (Davids et al. 2012, Ni Chonghaile et al. 2011, Vo et al. 2012). In this respect, it is curious that the combined deletion or loss of BAX and BAK function has been only rarely reported in cancer cells, as such a block would be an excellent strategy for surviving many kinds of anticancer treatments. One can speculate that it is difficult to select for the loss of the four alleles that is required for the full, apoptosis-resistant phenotype, or perhaps that loss of BAX and BAK is intolerable due to functions of BAX and BAK that are as yet unknown. Further clarification of this issue awaits a more focused and comprehensive analysis of cancer genomes.

APOPTOSIS IN CANCER TREATMENT

Most approved cancer therapies can kill cells via the mitochondrial apoptotic pathway. This can be seen directly by the induction of apoptotic markers, such as phosphatidyl serine exposure, caspase activation, and PARP cleavage by diverse anticancer agents in in vitro cell line experiments. Moreover, apoptotic blocks caused by overexpression of antiapoptotic proteins such as BCL-2 or BCL-XL, or loss of BAX and BAK, lend resistance to anticancer agents (Wei et al. 2001).

Whether anticancer agents kill cancer cells via apoptosis in vivo is more difficult to state categorically, and the answer probably depends greatly on the agents and cancers being considered. Blocks in apoptosis can prevent sensitivity to a wide variety of agents in vivo in murine experiments (Wei et al. 2001). Moreover, measures of apoptotic signaling predict in vivo killing via a wide variety of agents, including conventional chemotherapy, kinase inhibitors, HDM2 inhibitors, and nuclear export inhibitors (Davids et al. 2012, Etchin et al. 2016, Montero et al. 2015, Ni Chonghaile et al. 2011, Townsend et al. 2016, Vo et al. 2012). However, tumor cells can also die a necrotic cell death in vivo. In addition, some tumor cell death is likely immunologic, with the cell death induced by treatment acting as an adjuvant to stimulate an innate or adaptive immune response (Aymeric et al. 2010, Ma et al. 2010, Michaud et al. 2011). Apoptotic cell death is generally considered to be less immunogenic than other types of cell death such as necrosis, as the rapid phagocytosis of apoptotic cells results in less exposure of intracellular contents to the immune system. However, apoptotic cell death can nonetheless be immunogenic, perhaps by facilitating antigen presentation. In general, however, the more rapid and complete a response in vivo, the more likely it is that the main type of cell death is apoptosis, best documented in hematologic malignancies. One challenge in measuring apoptosis in vivo is that the rapid phagocytosis of apoptotic cells can result in a dramatic underestimation of the magnitude of apoptotic cell death.

Ionizing radiation is another important modality of cancer treatment. There is clear evidence that radiation can directly induce apoptosis in some cancer cells, and blocks in apoptosis can inhibit cell death following irradiation (Debbas & White 1993, Jeffers et al. 2003, Lowe et al. 1993). However, in other instances, alternate cell death pathways have been implicated, including what is termed mitotic catastrophe. In mitotic catastrophe, badly damaged chromosomes are present during mitosis, leading to impaired chromosomal segregation. The cell is trapped in M phase, and eventually dies. It is possible, however, that even this type of cell death occurs ultimately via the mitochondrial apoptotic pathway (Castedo et al. 2004, Eom et al. 2005, Imreh et al. 2016, Skwarska et al. 2007).

p53 is the most commonly deleted or mutated tumor suppressor in cancer. Among its myriad functions is its ability to cause apoptosis in response to genotoxic insults (Lowe et al. 1993). As such, its loss often is accompanied by clinically important resistance to standard genotoxic chemotherapy regimens. The proapoptotic effect of p53 is generally attributed to its transactivating function, in which p53 activates the transcription of proapoptotic proteins, most prominently PUMA, NOXA, and BAX (Nakano & Vousden 2001, Oda et al. 2000, Villunger et al. 2003). These proteins then interact with other BCL-2 family members to tilt the mitochondrion toward the cell fate of apoptotic cell death. Alternatively, anti-apoptotic proteins can block p53-mediated cell death by sequestering the newly translated proapoptotic proteins (Chiou et al. 1994). There is also evidence that cytoplasmic p53 can interact directly with the BCL-2 family, inducing apoptosis even in the absence of new transcription (Chipuk et al. 2004, 2005; Green & Kroemer 2009; Mihara et al. 2003). Although the tumor suppressive function of p53 is often attributed to its ability to induce apoptosis, mouse models have shown that, at least in some contexts, it is instead other functions of p53 that are most necessary for tumor suppression (Valente et al. 2013).

DIRECTLY TARGETING THE MITOCHONDRIAL APOPTOTIC PATHWAY IN CANCER

Because antiapoptotic proteins function to keep cells alive, including cancer cells, efforts have been made to target antiapoptotic BCL-2 family proteins. The first of these efforts to make it to the clinic was Genasense (oblimersen sodium), an antisense DNA intended to reduce BCL-2 expression. It had limited clinical activity in several settings in which it was tried, including chronic lymphocytic leukemia (CLL) (O'Brien et al. 2005, 2007). A significant challenge was that decreased BCL-2 protein levels in tumor cells, an important pharmacodynamic marker for this strategy, were not systematically measured, so it was not clear that the drug was even having its intended primary effect.

Following this, others focused on developing so-called BH3 mimetic small molecules that would compete for binding in the hydrophobic BH3-binding pocket of antiapoptotic proteins. Various efforts have been reviewed elsewhere (Letai 2008, Vogler et al. 2009). The general pattern is that a small molecule lead was identified and its binding optimized against a truncated and soluble form of the protein. The observed binding affinity was often in the roughly 100-nM range. The compound that emerged killed some cells but not others, and clinical activity was inconsistent when tested (O'Brien et al. 2009, Parikh et al. 2010, Varadarajan et al. 2013). It became clear that better methods were needed to credential BH3 mimetics as well as to identify truly antiapoptotic-dependent cancers if the BH3 mimetic approach was to succeed.

BH3 PROFILING

BH3 profiling is an approach pioneered by the Letai laboratory to identify tumors selectively dependent on individual antiapoptotic proteins (Certo et al. 2006; Deng et al. 2007; Ryan &

Letai 2013; Ryan et al. 2010, 2016). BH3 profiling takes advantage of the fact that synthetic BH3 domain oligopeptides of roughly 20 amino acids can mimic the proapoptotic functions of BH3-only proteins. For instance, the BH3 domains of BIM and BID act as potent activators, whereas the BH3 domains of sensitizer BH3-only proteins can function as sensitizers (Letai et al. 2002). The essence of BH3 profiling is to systematically expose mitochondria to known concentrations of BH3 peptides and measure the resulting MOMP. If selective peptides are used, BH3 profiling can be very useful as a probe of the dependence on individual antiapoptotic proteins (**Figure 2*b***). For instance, mitochondria that are sensitive to the NOXA BH3 peptide are MCL-1 dependent. Mitochondria that are sensitive to the HRK BH3 peptide are BCL-XL dependent. A comparison of results of the HRK BH3 peptide with those of the BAD BH3 peptide can be used to impute BCL-2 dependence. These BH3 peptides act as model selective BH3 mimetic drugs, albeit drugs with very poor pharmacologic properties, such as very poor intracellular penetration and poor in vivo stability. Nonetheless, if they are applied directly to mitochondria, they are very effective probes of antiapoptotic dependence. The dependence on BCL-2 or MCL-1 is not categorical, but rather graded. For instance, two cells can be dependent on BCL-2, but one more so, and hence more sensitive to BCL-2-specific BH3 mimetics. Additionally, cells can be dependent on more than one antiapoptotic, so they might be sensitive to BH3 mimetics that inhibit either (Touzeau et al. 2016).

In practice, we no longer purify mitochondria for BH3 profiling. Instead, we gently permeabilize the plasma membranes of the cell of interest with concentrations of digitonin that are too low to permeabilize the mitochondrial membranes (Ryan & Letai 2013; Ryan et al. 2010, 2016). BH3 peptides gain access to mitochondria by simple passive diffusion so that cell-type-dependent factors of drug accumulation are eliminated, allowing better comparison of results among different cell types. After an incubation of 30–90 min, MOMP is measured one of several ways. We can use potential sensitive dyes, such as JC-1, to read out loss of the potential gradient across the inner mitochondrial membrane, which correlates with MOMP. This signal can be read at the bulk population level on a simple fluorometer. Alternatively, single-cell information can be obtained using a FACS (fluorescence-activated cell sorting) machine. Another way to measure MOMP is to fix the cells following incubation and perform immunofluorescent staining for proteins lost during MOMP, such as cytochrome *c*. Because the cells are permeabilized with digitonin, cytochrome *c* diffuses away from the cell completely once it is released from the mitochondrial intermembrane space. Cytochrome *c* loss can then be measured by FACS or microscopy. Either of these two methods lends itself to the study of heterogeneous clinical samples, as subsets of interest can be distinguished by additional immunofluorescent staining for discriminating antigens.

IDENTIFYING ANTIAPOPTOTIC DEPENDENCE: PRECLINICAL DATA AND CLINICAL EXPLOITATION

BH3 profiling has been used on sets of samples from a variety of human cancers to identify selective antiapoptotic dependence. CLL was shown to be quite homogeneously BCL-2 dependent (Del Gaizo Moore et al. 2007). Acute myelogenous leukemia (AML) was found to be heterogeneously BCL-2 dependent, with significant subsets of MCL-1 and BCL-XL dependence (Pan et al. 2014, Vo et al. 2012). Multiple myeloma was found to be heterogeneously dependent on different antiapoptotic proteins, often with mixed dependence, with the plurality showing dependence on MCL-1, followed by BCL-2 and BCL-XL (Touzeau et al. 2016). B cell acute lymphoblastic leukemia has been found by several laboratories to be quite BCL-2 dependent (Benito et al. 2015, Del Gaizo Moore et al. 2008, Suryani et al. 2014). T cell acute lymphoblastic leukemia is largely BCL-XL dependent, whereas a particularly poor-risk subset that exhibits myeloid and

immature markers, early T cell progenitor acute lymphoblastic leukemia, appears consistently BCL-2 dependent (Anderson et al. 2014, Ni Chonghaile et al. 2014).

Among candidate inhibitors of antiapoptotic BCL-2 family inhibitors, the series of BH3 mimetics developed by AbbVie Pharmaceuticals has progressed furthest in human clinical trials. These compounds, developed using fragment-based screening and optimized exploiting high-throughout nuclear magnetic resonance evaluation, bind to their antiapoptotic targets with subnanomolar affinity (Oltersdorf et al. 2005, Souers et al. 2013, Tse et al. 2008). ABT-737 and ABT-263 can be described as BAD BH3 mimetics, as they share with BAD BH3 the selective binding of BCL-2, BCL-XL, and BCL-w. ABT-737 has never been used to treat humans, but instead has functioned as a proof-of-concept preclinical reagent. ABT-263 (navitoclax) is orally available, and has been tested in several clinical trials, with mixed efficacy in CLL, small-cell lung cancer, and other solid tumors (Gandhi et al. 2011, Roberts et al. 2012, Wilson et al. 2010) An on-target toxicity of thrombocytopenia based on the BCL-XL dependence of mature circulating platelets limited dose escalation (Gandhi et al. 2011, Mason et al. 2007, Zhang et al. 2007). Nonetheless, cases of impressive activity were observed, spurring the development of the orally available ABT-199 (venetoclax), which selectively binds only BCL-2 with high, subnanomolar, affinity.

Based on preclinical data suggesting the BCL-2 dependence of CLL (Del Gaizo Moore et al. 2007) as well as the observation of clinical activity in CLL in the navitoclax trials, CLL has received the greatest focus thus far in clinical trials of venetoclax. Initial single-agent testing in CLL was delayed when investigators observed tumor lysis syndrome, which resulted in the death of two patients. Clinical testing was resumed with increased surveillance and a more conservative dose-escalation strategy. Although serious tumor lysis syndrome is an important adverse event, it was driven by activity in CLL that exceeded expectations and resulted in very high (80%) response rates even in a population of heavily pretreated patients. Based on single-agent clinical trial results, in April 2016 venetoclax received FDA approval for use in CLL with the 17p chromosomal deletion (Anderson et al. 2016, Roberts et al. 2016, Stilgenbauer et al. 2016). This chromosomal deletion results in loss of p53, and has previously portended very poor prognosis and response to chemotherapy. The poor prognosis of 17p patients likely results from the p53-dependent mechanisms of apoptotic signaling on which prior CLL treatments have depended. Venetoclax apparently escapes this defect by applying an apoptotic signal directly to mitochondria, obviating the need for p53 signaling to induce apoptosis. As such, venetoclax offers an intriguing opportunity in other cancers for which loss of p53 function is a barrier to effective therapy. Current trials in CLL focus on combining venetoclax with other agents with demonstrated activity in CLL.

The mechanism underlying the special dependence of CLL on BCL-2 relates to the high expression of BCL-2. This abundant BCL-2 does not afford much protection from additional proapoptotic signaling, however, because it is already occupied by large amounts of the proapoptotic protein BIM (Del Gaizo Moore et al. 2007). When a small molecule BH3 mimetic such as venetoclax binds to BCL-2, it displaces BIM, allowing it to activate BAX or BAK, inducing oligomerization, MOMP, and cell death. This is one of the very rare instances in which the mechanism of killing of a cancer cell by a drug is understood all the way from drug binding target to the commitment and execution of programmed cell death (**Figure 3**). The high levels of BCL-2 expression are shared by other lymphoid cells, so lineage is probably an important reason for the BCL-2 expression in CLL. Additionally, microRNAs mir15 and mir16, which negatively regulate BCL-2 transcripts, are often lost in CLL, and this loss might drive BCL-2 expression in some cases of CLL (Cimmino et al. 2005). However, there is no evidence that CLL cases in which mir15 or mir16 is retained express any less BCL-2 than those in which it is lost, so this is unlikely to be the sole explanation for BCL-2 expression in CLL. Notably, the BCL-2 gene has not been found to be altered in CLL. Similarly, the Bruton's tyrosine kinase, CD20, and PI3 kinase

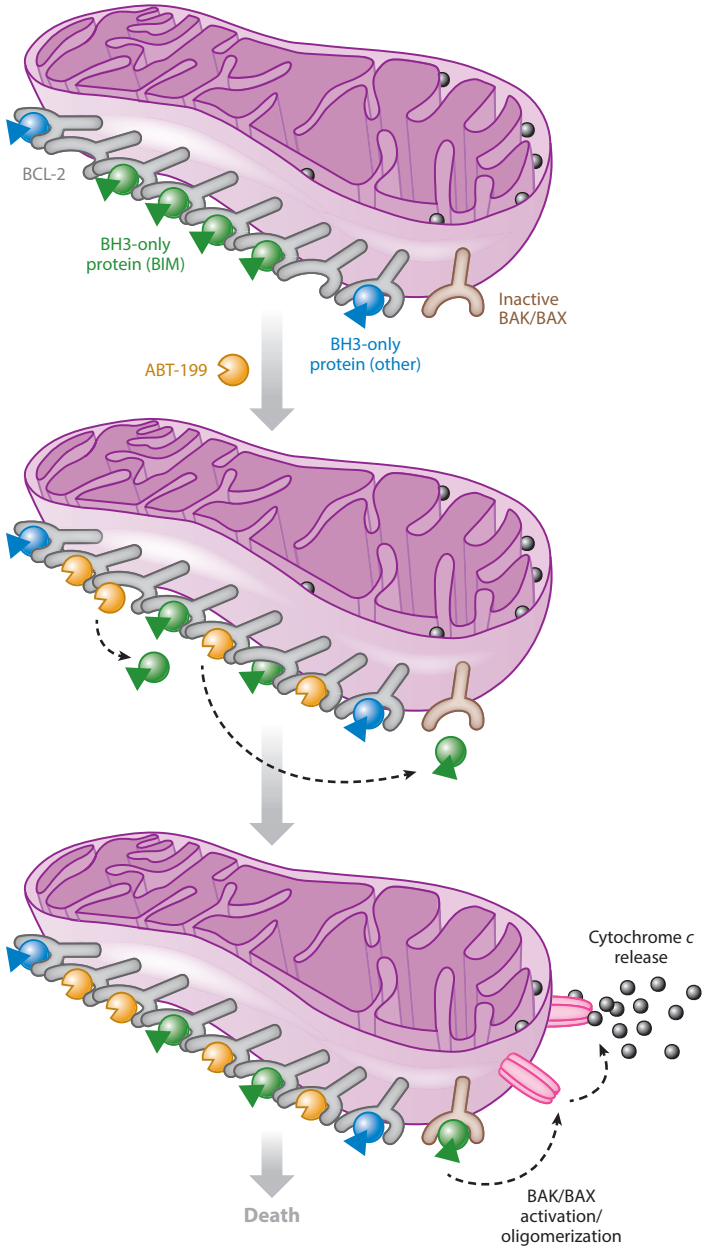

Figure 3

How BCL-2 inhibitors kill chronic lymphocytic leukemia (CLL) cells. In CLL, the abundant BCL-2 (*gray*) is largely occupied by the activator BIM (*green*). Small molecule BH3 mimetics such as ABT-737, ABT-263, and ABT-199 (*yellow*) compete for binding in the hydrophobic cleft in BCL-2, displacing BIM, which can activate BAX (*brown*), which in turn undergoes an allosteric change, initiates oligomerization, and forms pores (*pink*) to permeabilize the mitochondrial outer membrane. This allows the release of proapoptotic factors such as cytochrome *c*, resulting in caspase activation and cell death. Figure redrawn from Del Gaizo Moore et al. (2007).

isoform δ genes, all targets for which exciting agents have been recently approved for therapy in CLL, lack genetic alterations in CLL. CLL is thus an important cautionary tale highlighting the challenges in depending solely on genetic information to direct therapy in cancer.

A clinical trial of single-agent venetoclax in non-Hodgkin's lymphoma has been performed. The greatest clinical activity was observed in mantle cell lymphoma. Notably, the sensitivity of follicular lymphoma was less impressive, despite the presence of the t(14;18) translocation (Roberts et al. 2016).

Most of the clinical effort in targeting BCL-2 has been in the area of lymphoid malignancies. After all, BCL-2 was discovered in a lymphoid cancer, and the t(14;18) translocation is found only in lymphoid cancers. However, based on the preclinical demonstration of functional BCL-2 dependence in AML, clinical trials began in this disease, first as single agents and now as combination therapies (Konopleva et al. 2006, Pan et al. 2014, Vo et al. 2012). The initial single-agent clinical trial demonstrated significant biological activity, and even complete remissions, in a population of pretreated patients who were relapsed or refractory to highly aggressive conventional chemotherapy induction regiments (Konopleva et al. 2016). This trial prompted ongoing studies in combination with agents commonly used in treating AML patients, hypomethylating agents (azacitidine or decitabine) or low-dose cytarabine. Preliminary results for these trials in treatment-naïve elderly patients are available, and reveal a response rate (>70%) that exceeds response rates provided historically by even very toxic induction chemotherapy regimens in this population (DiNardo et al. 2015).

BH3 mimetics are being developed now by several pharmaceutical companies. Servier and Amgen have a BCL-2 and MCL-1 inhibitor, respectively, in clinical trials of hematologic malignancies. Given their perturbation of a key cell death node present in all cancer cells, there will likely be opportunities for clinical testing in a wide variety of liquid and solid tumors, both as single agents and in combination with other active agents.

APOPTOTIC PRIMING: A KEY DETERMINANT OF SENSITIVITY TO CONVENTIONAL CHEMOTHERAPY

Despite appropriate contemporary excitement about novel targeted pathway inhibitors, it is important to recognize that, by far, the most effective medicines we have for cancer patients continue to be conventional chemotherapy agents. These agents are responsible for curing tens of millions of cancer patients over the past five decades. As they target ubiquitous elements, mainly DNA and microtubules, why they kill cancer cells better than normal cells is not immediately clear. Traditional explanations rely on the conjecture that the rapidity of cancer cell division leaves them susceptible to such agents. However, clinical data supporting this conjecture are lacking. Cancer cells often do not divide very rapidly in vivo compared to normal tissues, and some cancers, such as indolent lymphomas, are chemosensitive while dividing very slowly (Komlodi-Pasztor et al. 2011, 2012).

In considering mechanisms for the therapeutic index for conventional chemotherapy in cancer, it is important to acknowledge that sensitivity to one agent often means sensitivity to several. For instance, the curability of childhood ALL relies not just on the sensitivity of this cancer to steroids, but also on its sensitivity to anthracyclines, alkylating agents, vinca alkaloids, l-asparginase, and 6-mercaptopurine. In contrast, the lack of chemotherapy-induced remissions in renal cell carcinoma results not just from resistance to platins, but also from resistance to taxanes, alkylating agents, anthracyclines, vinca alkaloids, and topoisomerase inhibitors. These observations suggest that there exists a node that broadly regulates chemosensitivity.

We have proposed that this node is the mitochondrial apoptotic pathway. One hypothesis is that all cells endure proapoptotic signaling in response to DNA or microtubule poisons.

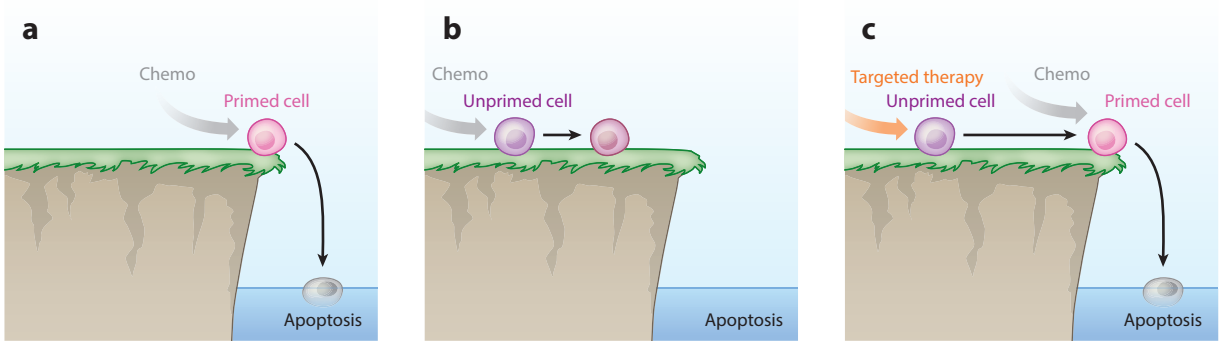

Figure 4

Apoptotic priming and cellular response to chemotherapy. Apoptosis can be considered to occur once a threshold has been exceeded, such as going over the edge of a cliff. One can imagine that some cells are closer than others to the edge of the cliff. BH3 profiling with promiscuous BH3 peptides (**Figure 2*b***) can provide a measure of the proximity to the cliff's edge. (*a*) Cells whose mitochondria are relatively sensitive to BH3 peptides are primed for apoptosis, and close to the edge. When they receive proapoptotic signaling as a result of chemotherapy, they are forced to commit to cell death. (*b*) Cells whose mitochondria are relatively insensitive to BH3 peptides are unprimed for apoptosis and farther from the cliff's edge. When they receive a proapoptotic signal from chemotherapy, they move closer to the edge, but are not compelled to commit to apoptosis. (*c*) With a series of proapoptotic pulses from different agents, unprimed cells can first be primed, and then compelled to commit to cell death. The key will be to identify targeted agents that selectively induce cancer cell proapoptotic signaling with minimal toxicity to normal cells.

However, some cells perch close to the threshold of commitment to apoptosis, whereas others perch further away (**Figure 4**). The former cells commit to apoptosis, whereas the latter survive. To test this hypothesis, one would need a tool that can measure the proximity of a cell to the apoptotic threshold. We have used BH3 profiling with so-called promiscuous BH3 peptides (see **Figure 2*b***), peptides that bind all antiapoptotic proteins with high affinity. Mitochondrial sensitivity to such peptides gives a measure of overall antiapoptotic reserve, and hence proximity to the threshold. We refer to cells or mitochondria that are more sensitive to these peptides as being more primed. We have now conducted clinical experiments in AML, ALL, CLL, multiple myeloma, and ovarian cancer. In each case, the pretreatment apoptotic priming of patient cancer samples predicted sensitivity to conventional chemotherapy regimens, suggesting that differential priming was an explanation underlying differential chemosensitivity among tumors (Davids et al. 2012, Ni Chonghaile et al. 2011, Vo et al. 2012). Moreover, we also found that most normal cells were very poorly primed for apoptosis, affording an explanation for the therapeutic index of chemotherapy that has been exploited for so many decades. One exception to this observation was provided by white blood cells, which are relatively highly primed. In this respect, it is notable that loss of white blood cells is a very common dose-limiting toxicity of most chemotherapeutic regimens. Although there are doubtless additional relevant drug-specific and cell-specific factors, it seems likely that differential mitochondrial priming is an important determinant of chemosensitivity and an explanation for therapeutic index in cancer chemotherapy.

FUTURE DIRECTIONS

While it may not always have been appreciated, oncologists have been exploiting differences in the apoptotic pathway between cancer cells and normal cells for decades. More recently, direct targeting of the mitochondrial apoptotic pathways with BH3 mimetics such as venetoclax has proven its clinical utility. One approach to achieving deeper and more durable responses in cancer

may be in the combination of agents that selectively provoke apoptotic signaling in cancer cells (**Figure 4*c***). Even if none of these can kill many cells as a single agent, combinations may prove efficacious. Conventional chemotherapy has proven its ability to cure certain cancers for decades. Time will tell whether ideal combinations can truly dispense with less selective conventional chemotherapy, or whether the broadly proapoptotic effects of conventional chemotherapy are instead an essential part of combinations to obtain durable responses in certain contexts.

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