Diagnosing and exploiting cancer’s addiction to blocks in apoptosis

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Abstract | Cancer cells survive despite violating rules of normal cellular behaviour that ordinarily provoke apoptosis. The blocks in apoptosis that keep cancer cells alive are therefore attractive candidates for targeted therapies. Recent studies have significantly increased our understanding of how interactions among proteins in the BCL2 family determine cell survival or death. It is now possible to systematically determine how individual cancers escape apoptosis. Such a determination can help predict not only whether cells are likely to be killed by antagonism of BCL2, but also whether they are likely to be sensitive to chemotherapy that kills by the intrinsic apoptotic pathway.

Defining apoptosis

Interest in apoptosis was greatly enhanced when it was recognized that a genetically controlled programme governed commitment to and execution of apoptosis in a wide range of multicellular organisms spanning worms, flies and humans. Over the past two decades an impressive amount of research has resulted in a better understanding of the genes and biochemical events responsible for the process of apoptosis, including the morphological changes that first alerted researchers to this form of cell death (FIG. 1). Cells destined for apoptosis are at best useless, and at worst harmful to the organism. For the benefit of the organism as a whole, it is important that once a cell is no longer useful the commitment and execution of its death be rapid and irreversible. Otherwise, the organism might have to endure ailing, useless and deleterious cells using resources that could be more efficiently expended. The combined processes of nucleosomal fragmentation by endonucleases, widespread proteolysis by caspases and cell surface tagging to induce phagocytosis are the key elements of apoptosis. Thus a cell that is committed to undergo programmed cell death (PCD) by the intrinsic apoptotic pathway is rapidly converted to a metabolically inactive remnant that cannot replicate its DNA. It is engulfed by phagocytes to prevent potentially harmful exposure of intracellular antigens to the immune system.

The crucial event that commits a cell to death by the intrinsic apoptotic pathway is permeabilization of the outer mitochondrial membrane with the subsequent release of multiple pro-apoptotic factors that direct the physiological changes described above. Although
At a glance

- Cancer cells exhibit many phenotypes, such as genomic instability or oncogene activation, that ought to induce apoptosis, but they nonetheless survive. A block in apoptosis is a likely requirement for cancer maintenance.
- In cancer cells that overexpress BCL2, the protein itself is often largely bound to pro-apoptotic BH3-only proteins like BIM. In such circumstances we describe BCL2 and the cell as being ‘primed’.
- BH3 profiling is a novel tool that exploits selective interaction between BH3 domains and anti-apoptotic BCL2 proteins to reveal the different ways cancer cells escape apoptosis. Certain cancer cells escape apoptosis by expression of BCL2; BH3 profiling can specifically identify these primed, BCL2-dependent cells.
- BCL2 expression does not necessarily confer a chemoresistant phenotype to cancer cells when selected for in previously untreated cells. If the expressed BCL2 is primed, sequestering large amounts of pro-apoptotic proteins such as BIM, it may actually relate to increased chemosensitivity.
- Primed cells are selectively sensitive to antagonists of anti-apoptotic BCL2 proteins like ABT-737. Primed cells might also be selectively more sensitive to conventional chemotherapy agents compared with cancer cells that use a different apoptotic block.
- It has long been suspected that cancer cells are more susceptible to cell death than normal cells. That some cancer cells appear to be primed for death in comparison with normal cells offers a possible biochemical explanation for this clinical observation.
- Small-molecule drugs that target BCL2 and related anti-apoptotic proteins are currently in early-phase clinical trials.

Necrosis
A type of cell death distinct from apoptosis, characterized by breakdown in the plasma membrane and release of intracellular contents. Though previously thought to be a passive process, it might also be regulated by a genetically encoded programme.

Mitotic catastrophe
A death resulting from failure of a cell to arrest before mitosis following DNA damage, resulting in severe aberrancies in chromosomal structure and segregation. It might share downstream events with apoptosis.

Autophagy
Literally, a cellular response in which the cell metabolizes its own contents and organelles to maintain energy production. Although such a process can eventually result in cell death, it can also be used to maintain cell survival under conditions of limiting nutrients.

In the decade following the cloning of BCL2, an entire family of proteins that are related by sequence homology and participation in the control of apoptosis was identified. Some of these proteins share with BCL2 the ability to oppose PCD: BCL-X (also known as BCL2-like 1 (BCL2L1))\(^{12}\), BCL-w (also known as BCL2L2)\(^ {13}\), myeloid cell leukemia sequence 1 (MCL1)\(^ {14}\) and BFL1 (also known as BCL2A1)\(^ {15}\). These proteins have sequence homology in four α-helical BCL2 homology (BH) regions, BH1–4. BCL2-associated X protein (BAX)\(^ {16}\), BCL2-antagonist/killer (BAK)\(^ {17}\) and BCL2-related ovarian killer (BOK)\(^ {18}\), sharing only the BH1–3 domains, promote cell death. A third class of proteins was later established\(^ {19}\). These proteins, including BCL3-interacting domain death agonist (BID), BCL2 antagonist of cell death (BAD), BCL2-interacting killer (BIK), PUMA (also known as BCL2-binding component 3 (BBC3))\(^ {20}\), NOXA (also known as phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1)), Bcl2-modifying factor (BMF) and HRK, demonstrate homology only in the BH3 region, and are often referred to as BH3-only proteins\(^ {21}\). Like BAX and BAK, the pro-apoptotic BH3-only proteins require an intact BH3 domain to promote apoptosis\(^ {22,23}\) (FIG. 2).

Death at the mitochondria. The BCL2 family of proteins governs the commitment to PCD at the mitochondrion — the intrinsic apoptotic pathway\(^ {22,23}\). Myriad death signals, including DNA damage, growth-factor deprivation, oncogene activation, cell signalling pathway perturbation and microtuble disruption, are communicated through the intrinsic apoptotic pathway. In response to the initial insult, incompletely understood signalling events are eventually converted into active BH3-only family members, which may be considered the cellular sentinels of PCD\(^ {24}\). These BH3-only family members become active through many mechanisms, including increased transcription, protein stabilization and post-translational modification\(^ {25–28}\). Once active, certain BH3-only proteins, including BIM (also known as BCL2L11) and BID, induce the activation of BAX and BAK\(^ {29–34}\). BAX and BAK are absolutely essential for death induced by BH3-proteins. BOK expression is restricted to reproductive tissues, so deletion of BAX and BAK provides a profound inhibition of apoptosis in most tissues\(^ {35,36}\).

We term BH3-only proteins that have the ability to activate BAX and BAK activators\(^ {31}\) (FIG. 3). Notably, there is no reason to think that BID and BIM are the only activators of BAX and BAK. Experiments addressing the properties of the isolated BH3 domain of PUMA have indicated that this domain cannot function as an activator\(^ {32,33}\). However, studies using the whole protein suggest that PUMA may indeed act in this way\(^ {39,37}\), and in one study PUMA has been shown to directly bind BAX in a manner dependent on its BH3 domain\(^ {38}\). Therefore, PUMA might be an example of a molecule in which the BH3 domain gains additional properties in the context of the entire protein.

As only three amino acids out of roughly twenty in the BH3 domain are highly conserved across the family, it is not possible, even with a complete knowledge of the
how apoptosis kills and clears cells. Apoptosis can be described morphologically as causing cell shrinkage, convolution and blebbing of the cell surface, and marked condensation of the nucleus. Inversion of the plasma membrane occurs so that the phosphatidyl serine that is usually restricted to the cytoplasmic face of the inner leaflet is exposed to the extracellular environment. In vivo, the externalized phosphatidyl serine and other induced signals allow recognition by phagocytic cells, which phagocytose apoptotic cells. Phagocytosis can rapidly remove apoptotic cells from their environment, which can contribute to their underestimation when investigated in vivo. The entire programme, once initiated, can be completed on a timescale of minutes to a few hours.

Figure 1

Macro...
Figure 2 | Summary figure of the structures of BCL2 family proteins by group.
There are three main classes of proteins in the BCL2 family. The anti-apoptotic proteins contain four α-helical BCL2 homology (BH) domains. The BH1, BH2, and BH3 domains form a hydrophobic cleft that binds the hydrophobic face of pro-apoptotic BH3 domains. Multidomain pro-apoptotic proteins have theBH1, BH2 and BH3 domains. The pro-apoptotic BH3-only proteins share only a BH3 domain in common with the BCL2 family. Flanking regions are unrelated, and probably relate to non-apoptotic cellular functions. Whether PUMA is a member of the sensitizer or activator class of BH3 proteins still remains to be clarified, as shown by the question mark. BAX-related proteins include BID, BCL2 antagonist of cell death; BAK, BCL2-antagonist/killer; BAX, BCL2-associated X protein; BID, BH3-interacting domain death agonist; BIK, BCL2-interacting killer; BMF, Bcl2-modifying factor; BOK, BCL2-related ovarian killer; MCL1, myeloid cell leukaemia sequence 1; TM, transmembrane domain.

might be already bound or binding free anti-apoptotic proteins so that their binding sites are no longer available to sequester activator proteins. A key property of the sensitizers is that they do not interact equivalently with all of the anti-apoptotic proteins. Instead, each anti-apoptotic protein interacts with a subset of the sensitizer BH3-only proteins.30,32,65 Below, we show how this selectivity pattern can be exploited to develop an important tool, a test that can provide a summary interpretation of the participation of all of the BCL2 family members to reveal how ‘primed’ a cell is for death, and what anti-apoptotic protein(s) are required for the ongoing survival of the cell in question. We call this strategy BH3 profiling (BOX 2).

An alternative model of activation of BAX and BAK. An alternative, ‘indirect’ model holds that direct activation of BAX and BAK does not take place.61-68. Rather, BAX and BAK are continually kept at bay by anti-apoptotic proteins such as BCL2 and MCL1. Inhibition of all of the anti-apoptotic proteins is necessary and sufficient to cause commitment to PCD. Yet even proponents of this model acknowledge that in order to execute MOMP, some change in BAX and BAK must be brought about.69 The changed BAX or BAK is referred to as ‘primed’ rather than activated, but the concept is the same. Left unanswered in this model is the question of what events are responsible for priming or activating BAX and BAK.

Proponents of the indirect model cite as support the interesting observation that activation of BAX and death consistent with apoptosis can occur even in the absence of BID and BIM.64 However, as explained above, there is no reason to believe these are the only activators. The same proponents also point out that if BIM and BID truly interact with BAX and BAK, it should be easier to observe complexes of these proteins by immunoblot. Such complexes are indeed difficult to observe, but they have been reported20,29,38,50,69. The difficulty in observing them might be due to the transient quality of their interaction: it is hypothesized that the interaction of activators with BAX or BAK is a ‘hit-and-run’ catalytic interaction in which the transient complex comes apart following the conformational change induced in BAX or BAK. It should also be remembered that BID, the first protein to be identified as an activator, was originally cloned through its interaction with BAX as well as BCL2 (REF: 20).

Proponents, like me, of the activator–sensitizer model point to several key findings. In a simplified liposomal system, it has been clearly shown that recombinant BAX is unable to induce liposomal permeabilization without the presence of an activator like BID or BIM.12,23. Definite biochemical measurements can be made of a stable interaction between BAX and a stabilized BID or BIM BH3 peptide.25. In certain systems, at least, inhibition of death by BCL-XL does not depend on binding BAX or BAK, but it does depend on binding BH3-only proteins.30,62.

A position that can accommodate much of this data is that BID and BIM can also activate BAX and BAK under many circumstances. In other circumstances, other activators, proteins or otherwise may fulfill this role. BAX and BAK might also be able to undergo relatively slow spontaneous activation independent of interaction with any activator molecule. In this instance, activators may be seen as acting as catalysts to greatly accelerate a slow spontaneous conversion of BAX and BAK to activated forms. This model conforms to the apparently general agreement that an activated form of BAX and BAK must be attained somehow to accomplish apoptosis.30,32,51,58,68. Anti-apoptotic proteins prevent death by sequestering activator proteins, but also in some circumstances monomeric activated BAX or BAK.

BCL2 dependence and ‘primed for death’
Cancer cells exhibit many abnormalities, such as genomic instability or oncogene activation, which should normally induce death signalling through the intrinsic apoptotic pathway. For example, cell death can be induced by the deregulated expression of an oncogene, such as MYC, which triggers the tumour suppressor ARF (encoded by cyclin-dependent kinase inhibitor 2A (CDKN2A)) that activates p53. In turn, p53 can increase the transcription of pro-death BH3-only proteins PUMA and NOXA, resulting in apoptosis. It seems likely that this pathway is used as an important form of cell-autonomous tumour surveillance at the single-cell stage. Of course, cancer cells find a way to survive such signalling, suggesting that a block in apoptosis might be a requirement of oncogenesis. Given our outline of the intrinsic, or mitochondrial, apoptotic pathway, one can envision three distinct ways this block could be effected. One way they might escape apoptosis is by reducing or eliminating the activation of pro-death BH3-only proteins that otherwise would be activated by the aberrant phenotype (FIG. 4). This might theoretically
be accomplished by deletion of key BH3-only genes or by genetic modulation of any of the poorly understood interactions functioning upstream of BH3-only upregulation. An example would be inactivation of p53, which controls expression of BH3-only proteins PUMA and NOXA. We refer to this as a class A block.

Alternatively, the cancer cell might eliminate the effector arm of this pathway by reducing or eliminating BAX and BAK. It has been shown that loss of BAX and BAK confers a profound apoptotic block, and that BH3-only proteins depend absolutely on BAX or BAK for their pro-death function. We have found tumour cells that use this route of escape, and refer to this as a class B block.

Finally, the apoptotic fate may be foiled by the increased expression of an inhibitor such as BCL2, MCL1 or a related anti-apoptotic protein. We refer to this as a class C block. In cells employing this block, pro-apoptotic activator BH3-only proteins that would otherwise activate BAX or BAK and cause MOMP are bound and sequestered by the anti-apoptotic proteins. Thus the anti-apoptotic proteins are largely bound to, or primed with death signals in the form of, pro-apoptotic activator proteins. We refer to cells in this state as primed, or primed for death. Primed cells depend on the continuous function of anti-apoptotic proteins to maintain survival. If anti-apoptotic proteins are lost, or their functions antagonized, the pro-death BH3-only proteins are released to activate BAX and BAK and commit the cell to PCD. The discussion that follows describes priming in terms of activator BH3-only proteins, as that is what we have consistently observed. However, adherents to the indirect activation model may mentally substitute priming with activated BAX or BAK monomers and maintain much of the substance of the discussion.

**BH3 profiling — detecting escape from apoptosis**

As discussed above, in theory, cancer cells can be classified into three distinct groups on the basis of how apoptosis has been suppressed. To move beyond a merely theoretical treatment of this construct, it was necessary to develop a technique for determining what type of block a given cell employs. To this end, we have developed a strategy we call BH3 profiling (BOX 2). BH3 profiling is based on the selective interactions that take place between the anti-apoptotic proteins and the BH3 domains of sensitizer BH3-only proteins. In **Table 1** we summarize the interaction pattern of anti-apoptotic proteins with oligopeptides that are derived from the BH3 domains of BH3-only proteins. It can be seen that, although all of the anti-apoptotic proteins interact with the activators BID and BIM, each of the anti-apoptotic proteins may be distinguished from the others based on its pattern of interaction with the BH3 domains of sensitizer BH3 proteins.

We used these observations to generate BH3 profiling, a method to classify types of apoptotic block. We have now validated this strategy in model systems, cell lines and primary cancer cells and have found it to be a useful assessment of the apoptotic blocks employed by cancer cells. This functional assay, which can be performed in an afternoon, can be thought of as integrating the function of the myriad BCL2 family proteins that are simultaneously expressed in most cells to derive a comprehensive picture of the arrangement of the cell death pathway.

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**Figure 3** | **A model of BCL2 family control over mitochondrial apoptosis.** In response to cellular damage or deregulation, activator BH3-only proteins such as BH3-interacting domain death agonist (BID) or BIM are activated by transcription or post-translational modification. Activator BH3-only proteins activate effectors such as BCL2-associated X protein (BAX) and BCL2-antagonist/killer (BAK), inducing homo-oligomerization, resulting in mitochondrial permeabilization and commitment to death. Anti-apoptotic proteins sequester activators to prevent them contacting effectors, and also might sequester activated monomeric BAX or BAK. Sensitzers act as selective antagonists of anti-apoptotic proteins. Whether PUMA is a member of the sensitizer or activator class of BH3 proteins remains to be shown clearly, so it is placed in between the two classes in this figure. BAD, BCL2 antagonist of cell death; BID, BH3-interacting domain death agonist; BIK, BCL2-interacting killer; BMF, Bcl2-modifying factor; BOK, BCL2-related ovarian killer. Modified, with permission, from REF. 72 © Elsevier Science (2007).
Box 1 | Caspase activation and phagocytosis

Activation of the cysteine proteases from the family known as caspases is an important hallmark of apoptotic death. Mitochondrial outer membrane permeabilization allows the release of cytochrome c from the mitochondrial intermembrane space. Once in the cytosol, cytochrome c interacts with apoptotic peptidase activating factor 1 (APAF1) and the initiator procaspase 9 to form, with dATP, a holoenzyme known as the apoptosome. Activated caspase 9 in the apoptosome then cleaves procaspase 3 into the effector caspase caspase 3 (REFS 5,55). The activated caspases cause cell-wide specific proteolysis and dysfunction. Resulting effects include a decrease in ATP production and a labelling of the cell with ‘eat me’ signals, allowing the apoptotic cell to be recognized and consumed by phagocytic cells56,57. This results in a minimization of resources wasted by damaged cells, and a reduction in counter-productive immunological stimulation by dead cells.

Box 2 | BH3 profiling

The basic strategy is to incubate mitochondria isolated from the cell of interest with the panel of BH3 peptides from TABLE 1, and observe which peptides cause permeabilization of the mitochondrial outer membrane. We have used release of cytochrome c as a measurement of mitochondrial outer membrane permeabilization. Based on the pattern of response to the panel of peptides, we can then assign the cell to one of the classes defined in FIG. 4. For instance, if a cell has eliminated the upregulation of activator BH3-only proteins, but maintains adequate BCL2-associated X protein (BAX) and BCL2-antagonist/killer (BAK), its mitochondria will respond to BH3-interacting domain death agonist (BID) and BIM, but not to any of the sensitizer BH3 peptides. Note that this is also the pattern that might be expected of normal cells that are not undergoing active death signalling. This gives a pattern (FIG. 4) that defines a class A block. If, however, the effector arm is eliminated by loss of BAX and BAK, none of the peptides will cause mitochondrial outer membrane permeabilization, resulting in a flat pattern characteristic of a class B block. Finally, if the cell is primed, and dependent upon anti-apoptotic BCL2 proteins to sequester activator BH3-only proteins, we would see response to both activator and sensitizer peptides. Moreover, by reference to the interaction patterns in TABLE 1, the specific pattern of activity of the sensitizer peptides would reveal the identity of the anti-apoptotic protein(s) that are most responsible for keeping the cell alive. Note that, regardless of whether the priming is mainly by activators, as the direct activation model would suggest, or BAX and BAK, as the indirect model would suggest, BH3 profiling will accurately identify primed cells.

Drugs targeting anti-apoptotic proteins

The understanding and identification of cancer cells that depend on BCL2 or related anti-apoptotic proteins is of great practical significance owing to the recent emergence of drugs targeting BCL2 from preclinical studies into clinical trials (TABLE 2). The first attempt to introduce an agent that specifically targeted BCL2 was made by Gentex with their antisense DNA agent, oblimersen (Genasense). On the basis of preclinical studies that found that antisense inhibition of BCL2 levels could induce death in cancer cell lines, they designed a phosphorothioate DNA molecule complementary to BCL2 (REF. 74). Preclinical studies with this agent supported its ability to reduce BCL2 in certain cells. However, numerous clinical studies failed to reach their designated clinical endpoints. More recently, in a study of chronic lymphocytic leukaemia (CLL), evidence emerged indicating that an improvement in response rate could be observed in relapsed CLL patients when oblimersen was added to fludarabine and cyclophosphamide25. However, it is not clear that this will be enough to prompt approval by the United States Food and Drug Administration.

If dependence on BCL2 is found selectively in cancer cells, as I have argued above, why was this drug not more successful? It may have to do with its mechanism of action. It did not inhibit BCL2 function — rather it attempted to reduce BCL2 levels. There is evidence that this down-regulation can induce other anti-apoptotic proteins in response, which would mitigate the drug’s effectiveness26. Furthermore, it is not clear what level of reduction of BCL2 could be obtained in vivo as little data to address this question is publicly available. It is possible that the cytotoxic effects that are seen are due to off-target activity. For instance, CpG dinucleotides, which are found in the oblimersen molecule, have been shown to stimulate an immune response independent of interaction with BCL2 (REF. 77). Off-target mechanisms independent of immune system stimulation have also been proposed28. Finally, with the exception of CLL29, it is not clear which cancers tested in clinical trials required BCL2 for survival. In short, though certainly somewhat disappointing, oblimersen does not represent a sufficient test of BCL2 antagonism to induce general pessimism about the approach.

Subsequent approaches have focused on inhibiting BCL2 function with small molecules rather than on decreasing BCL2 protein levels. Prominent among these is the Abbott drug ABT-263, which is now in clinical trials for small cell lung cancer, lymphoma and CLL. ABT-263 is an orally available modification of the ABT-737 compound that has been studied extensively in preclinical studies30,80–84. A strategy employed by Abbott Laboratories that was nicknamed ‘SAR by NMR’ (structure–activity relationships by NMR) ultimately yielded ABT-737, a molecule that binds the BCL2, BCL-XL and BCL-w binding pockets with sub-nanomolar affinity. By contrast, it shows low affinity for MCL1 or BFL1, two other anti-apoptotic proteins. It can be seen that ABT-737 in fact mimics the binding pattern of the BH3 domain of BAD, suggesting that both the compound and the peptide are recognizing features common to the hydrophobic cleft in all three molecules.

ABT-737 is toxic to a subset of cancer cell lines. There are numerous studies that support the contention that the mechanism of action is indeed inhibition of BCL2 (REFS 30,80–84). It is important to understand what makes certain cells sensitive to ABT-737 and others resistant. One hypothesis is that response to ABT-737 depends primarily on the amount of MCL1 present30,31. ABT-737 does not efficiently target MCL1, it seems reasonable to suggest that presence of excess MCL1 would maintain survival even if BCL2 were inhibited. Indeed, we also have found that systems defined as MCL1-dependent by BH3 profiling or by genetic design are resistant to ABT-737 (REFS 50,79). Others have shown that, in
Clinical use

A question that often arises is whether antagonists of BCL2 and related anti-apoptotic proteins are likely to find a clinical niche as single-agent therapy or as components of a multiagent chemotherapy regimen. I would suggest that majority opinion would predict that BCL2 antagonists will have little effect as single agents. This opinion comes from the idea that, in cancer, overexpressed BCL2 serves to provide resistance to therapeutic drugs rather than endogenous death signalling. The idea is that BCL2 antagonists will serve mainly to counteract BCL2-mediated resistance, allowing killing by conventional agents. In this view, BCL2 antagonism by itself is unlikely to cause much cancer cell death. Indeed, many, if not most, successful anti-cancer agents eventually find their primary use in combination with other effective agents, so it is quite likely that anti-BCL2 agents will be used similarly.

Yet the ability of BCL2 antagonists like ABT-737 to kill cells as monotherapy in preclinical models has been impressive. Primary C.I.L. cells are killed at concentrations <20 nM within 4 h, and primary acute lymphoblastic leukaemia cells are killed in the 10–400 nM range. In addition, many sensitive cancer cell lines are killed in the nanomolar range in vitro. Of course it remains to be seen whether such impressive anti-cancer activity will be replicated in vivo. However, these in vitro observations demonstrate that single-agent therapy is at least theoretically feasible, an observation that might have surprised some. Of course, before successful results from clinical trials of these agents are obtained, discussing final clinical use is somewhat premature. Moreover, the use will depend on complicated balancing of toxicity to the tumour versus toxicity to normal tissues, a balance that cannot be estimated outside of clinical trials. Suffice it to say that current knowledge is insufficient to rule out the optimal use of BCL2 inhibitors as monotherapy or combination therapy.

Another question that often arises is whether the ideal drug would be a selective inhibitor of anti-apoptotic proteins (that is, inhibiting only a subset), or one that inhibited all of the anti-apoptotic proteins (a ‘pan-inhibitor’). The feasibility of a synthesizing a pan-inhibitor drug is not far-fetched. Indeed, the PUMA BH3 peptide serves as proof that a single molecule can antagonize at least five cellular anti-apoptotic proteins, though in a peptide form that is pharmacologically useless. Certain small molecules in pre-clinical and clinical development show the ability to bind all anti-apoptotic family members, but the affinity appears to be relatively low in these cases. If priming is indeed entirely specific to cancer cells, a pan-inhibitor may be preferable. It would also prevent what seems to be an obvious route of escape from a selective inhibitor, overexpression of an anti-apoptotic protein not targeted by the selective inhibitor. However, it would be no benefit if it was also toxic to non-malignant cells. Already some toxicity is seen in BCL2-specific inhibitors such as ABT-737 in the form of thrombocytopenia and lymphopenia. Only a clinical trial can evaluate the complicated balance between tumour and normal cell toxicity adequately enough to answer this question.
BH3 profiling might have a useful role in the clinic as well. By identifying cells that are dependent on anti-apoptotic proteins, it has the potential to select patients that are most likely to respond to targeted inhibitors of anti-apoptotic proteins. Treating tumour cells isolated from tumour biopsies with a number of drugs to ascertain drug efficacy might well be unsatisfactory, owing to the long observed problem of \textit{ex vivo} culture altering tumour cell biology and, hence, sensitivity to anti-tumour agents. One could consider simply treating mitochondria isolated from the patient’s tumour cells with a drug directly and observing effect. However, we have found this to be less reliable than assessing anti-apoptotic protein dependence using peptides. This might be because proteins and membranes in the concentrated mitochondrial preparations interact non-specifically with the drugs. In addition, direct mitochondrial drug treatment works only for the subset of drugs with a mitochondrial target.

As with any summary assay based on an aggregate of many cells, it is quite possible that heterogeneity in tumours could influence results. For instance, if there were a mixture of primed and unprimed cells in a tumour population, BH3 profiling might yield results showing intermediate priming that really did not apply to either subset alone. We are pursuing two ways to address this potential issue. One is to prospectively identify such populations and to separate them by cell sorting before mitochondrial purification. The other is to modify BH3 profiling to a single cell-based assay so that individual cells may be evaluated by fluorescence-activated cell sorting. In this way, individual subsets could be prospectively identified in the case of heterogeneity.

**BCL2 as a marker of chemosensitivity?**

Above we described how cancer cells can be segregated into three classes based on the type of apoptotic block they harbour. We also discussed how membership of a subgroup of one of these classes, class C, confers sensitivity to antagonists of BCL2. However, many other cancer therapeutics make use of the intrinsic apoptotic pathway to kill cancer cells. It is worth considering whether sensitivity to these conventional agents might be predicted on the basis of membership of one of these classes.

\textit{A priori}, one might expect that cells in class C might be most sensitive to agents killing through the intrinsic pathway. These cells possess all the required elements for apoptosis to occur — activator BH3-only proteins and effectors BAX and BAK. They are kept alive in a tenuous dynamic equilibrium only by a balancing amount of anti-apoptotic proteins. After treatment with drug, the BH3-only proteins that are generated, whether sensitizer or activator, can either activate BAX and BAK or displace pre-bound activators to do the same. By contrast, class A or B cells lack key components of the pro-death signaling pathway: activators or BAX and BAK, respectively. Of course, these blocks might be reversible, and the treatment in question might theoretically contribute to reversing them. However, it would seem that this process would probably nonetheless result in a less robust response than that expected by class C cells (\textbf{Fig. 5}).

We tested this hypothesis in a limited set of lymphoma cell lines\textsuperscript{72}. We found that the primed cells of class C were indeed more sensitive than unprimed class A or B cells to treatment with several drugs that operate in the intrinsic apoptotic pathway, including vincristine, doxorubicin and etoposide. As a control, we also tested sensitivity to N-methyl-N′-nitro-N-nitrosoguanidine, an alkylating agent that has been shown to kill efficiently by a non-apoptotic pathway in cells that have profound blocks in apoptosis. As expected, there was no distinction found between the primed and unprimed cells in this case. Thus, membership in an unprimed class (A or B) predicted an inferior \textit{in vitro} response to one group of drugs, but not to another. Of course, these results need to be confirmed in a larger cohort of cells and drugs. The clinical implications are worth considering, however. One could potentially evaluate the suitability of drugs for individual cancers on the basis of knowing how apoptosis is blocked. As BH3 profiling can be performed on fresh tissue, the problem of \textit{ex vivo} cell culture, which has plagued former attempts to predict sensitivity, would be avoided.

It should be noted that our observation has been made on a fairly limited number of cell lines. The consistency of the finding surprised us, and it is possible that when we compare BH3 profiling and drug response on a larger number of cell lines we will discover cell lines that...
have resistance mechanisms outside the mitochondrion, so that the correlation with class C and chemosensitivity may be less tight for certain cells. However, in our extension of BH3 profiling to several dozen cell lines, we find multiple examples of all three classes of apoptotic block, at least raising our confidence that all three are indeed commonly exploited in cancer (Ryan, J. and A.G.L., unpublished data).

So it appears that membership of the primed class C might augur greater sensitivity to numerous chemotherapy agents. However, when one considers what it means to be primed, an apparent paradox is confronted. High expression of BCL2 or a related anti-apoptotic protein is necessary for a cell to be primed. Can we really provide support for the heretical notion that overexpression of BCL2 can imply chemosensitivity? One of the reasons why this idea may seem surprising is that most of our knowledge of BCL2 function in chemoresistance comes from overexpression models in tissue culture cells. In these models, additional BCL2 expression is added to a cell that has already come to terms with its environment, so that the additional BCL2 provides extra anti-apoptotic reserve. Such a cell is consistently found to be less sensitive than the parental cell to agents that induce the intrinsic apoptotic pathway. The appropriate interpretation is that BCL2 confers chemoresistance in these models. However, expression of BCL2 in cancer cells is driven by selection rather than exogenously derived overexpression. High BCL2 expression is selected for during oncogenesis to buffer the death signals generated by the aberrant phenotypes required for illegitimate growth. Here this extra BCL2 does not necessarily provide an additional anti-apoptotic reserve to ensure chemoresistance. Rather, the BCL2 selected for is already occupied by pro-death proteins. In other words, in overexpression tissue culture models, the BCL2 is largely ‘empty’; the BCL2 selected for in cancer is already ‘full’ or ‘primed’.

In fact, there are some fairly obvious clinical models that support this interpretation. CLL and follicular lymphoma are both well-established as consistently over-expressing BCL2. Both cancers consist of relatively slowly dividing cells. By conventional estimation, therefore, one would expect these cancers to be quite resistant to chemotherapy. The consistent clinical observation, however, is that both cancers are extremely sensitive to chemotherapy. When treated with conventional multi-agent regimens, the majority of patients with either disease will enter a complete remission. In support of our biochemical interpretation of this behaviour, it has been shown that cells from both cancers are sensitive in vitro to BCL2 antagonism by ABT-737, and hence likely to be primed. Indeed, we have shown explicitly that the BCL2 in CLL is consistently primed with pro-apoptotic BIM79. Moreover, several studies have shown that expression of BCL2 confers a superior prognosis to patients with breast cancer [86–91].

It is true that, despite a robust initial response, neither CLL or follicular lymphoma are generally cured by chemotherapy, or any other modality, but this may be because of either the existence of a minority stem-like cell with different properties to the bulk population or the evolution of cells within the original tumour. It is worth noting that on treatment with chemotherapy, cancer cells are exposed to selection pressures that can select for expression of anti-apoptotic proteins at levels greater than before. These levels may then provide excess anti-apoptotic reserve to cancer cells between treatments, and treatment resistance when the treatment cycle resumes.

If the primed cells that are best targeted by antagonists of anti-apoptotic proteins like ABT-263 are also killed more readily by conventional chemotherapy, is it possible that such strategies will only treat cancers that are already adequately treated? I think that there exists ample opportunity for the anti-apoptotic antagonists to provide significant clinical benefit. All too many cancers that respond to conventional agents have an unsatisfactory clinical response. In this case, addition of a tolerable agent that efficiently kills the cancer cell might be expected to improve clinical outcome. In addition, the testing I describe attempts to predict sensitivity to conventional or targeted drugs only as single agents. It seems likely that use of conventional agents in combination with the

### Table 2 | Clinical development of drugs targeting anti-apoptotic proteins

<table>
<thead>
<tr>
<th>Drug</th>
<th>Company</th>
<th>Clinical phase</th>
<th>Function</th>
<th>Additional comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABT-263</td>
<td>Abbott Laboratories</td>
<td>Phase I and I/II clinical trials in NHL, CLL and SCLC</td>
<td>BH3 mimetic, targets BCL2, BCL-Xl, BCL-w</td>
<td>ABT-263 is an orally available compound closely related to ABT-737</td>
</tr>
<tr>
<td>Obatoclax (GX15-070)</td>
<td>Gemin X</td>
<td>Multiple phase I and phase II clinical trials in haematological malignancies and non-small cell lung cancer</td>
<td>BH3 mimetic</td>
<td>Might be a pan-inhibitor of anti-apoptotic proteins</td>
</tr>
<tr>
<td>Oblimersen (G3139)</td>
<td>Genta</td>
<td>Many clinical trials including phase III in melanoma and CLL</td>
<td>Antisense DNA targeting BCL2</td>
<td>None</td>
</tr>
<tr>
<td>AT-101 Ascenta Therapeutics</td>
<td>Phase II clinical trials in a variety of cancers</td>
<td>BH3 mimetic</td>
<td>AT-101 is the (-) enantiomer of gossypol</td>
<td></td>
</tr>
</tbody>
</table>

BH, BCL2 homology; CLL, chronic lymphocytic leukaemia; NHL, non-Hodgkin lymphoma; SCLC, small-cell lung cancer.
that can then activate BAX and BAK at the mitochondrion, causing MOMP. 

are primed, and therefore are more prone to displacing excess pre-existing activators 
molecules with mitochondrial outer membrane permeabilization (MOMP). Class C cells 
BCL2-antagonist/killer (BAK), and so are less prone to respond to excess activator 
molecules or BH3 mimetics. Class B 
proteins like BCL2, and thus are relatively resistant to new burdens of BH3-only 
molecules. Class A cells lack pre-existing activator bound to anti-apoptotic 
proteins like BCL2 and thus are relatively resistant to new burdens of BH3-only 
molecules or BH3 mimetics. Class B cells lack BCL2-associated X protein (BAX) and 
BCL2-antagonist/killer (BAK), and so are less prone to respond to excess activator 
molecules with mitochondrial outer membrane permeabilization (MOMP). Class C cells 
are primed, and therefore are more prone to displacing excess pre-existing activators 
that can then activate BAX and BAK at the mitochondrion, causing MOMP.

anti-apoptotic agents might expand the spectrum of sensitive 
cells into the class A or even class B group, as the conven-
tional agents may provide the extra priming necessary 
for anti-apoptotic antagonists to work in cancers in these 
classes. Furthermore, ABT-263 has a fairly restricted 
pattern of toxicity, without widespread genotoxicity 
or neurotoxicity, so a greater therapeutic window might 
be observed than for most conventional agents. Finally, I 
do not mean to suggest that the mitochondrion is the sole 
determinant of response, just one that has been somewhat 
overlooked. There are doubtlessly many mechanisms of 
resistance to conventional agents that lie upstream of mito-
chondria. However, cells resistant to chemotherapy on 
the basis of these upstream blocks might still be sensitive 
to drugs like ABT-263.

An explanation for chemotherapy efficacy?
In the decades of chemotherapy that modern oncol-
ogy has witnessed, it has been observed that cancer 
cells that are resistant to one agent are often resistant 
to many agents, even those targeting sites distal to that 
targeted by the initial agent. On the other hand, many 
cancers that are sensitive to single agents are sensitive 
to many agents working by different mechanisms. This 
can be clearly seen in the fact that most, if not all, cura-
tive chemotherapy regimens use a combination of drugs 
working on different targets. These observations have led 
to investigation of whether there is not some common 
mechanism defining resistance in pan-resistant cells. 
At the very start of the pathway of drug-induced death is 
the necessity that the drug gain cell entry. One obvious 
mechanism that has been explored is that of entry of 
drug into the cancer cell. Indeed, p-glycoprotein-type 
multi-drug resistance pumps might account for a subset 
of pan-resistance observed. However, the proportion is 
probably less than initially thought and, furthermore, 
intervention by drug pump inhibitors has been disappointing.

I would like to suggest that the specific class of block 
in apoptosis found in a cancer cell might be a heretofore 
unappreciated and crucial factor in determining the 
sensitivity of cancer cells to multiple diverse chemother-
apy agents simultaneously. The death signalling 
pathways of a wide variety of different cytotoxic agents 
converge at the intrinsic cell death pathway. Therefore, 
a particular class of block, such as a class C block, might 
broadly confer sensitivity to a wide variety of agents. 
Another block, such as a class B block, might on the 
other hand confer resistance to a wide variety of agents.

Differential blocks in apoptosis might be an explana-
tion for chemotherapy efficacy? Why certain cancers, such as leukaemias, are 
intrinsically more sensitive to almost all conventional 
agents than other cancers, such as pancreatic cancer or 
melanoma. Moreover, the fact that being primed for 
death (class C block) is apparently much more common 
in tumours than in non-malignant tissues might explain 
why chemotherapy is often more toxic to cancers than 
normal tissues. The idea that cancer cells might be more 
prone than normal cells to die is not a new one, but here 
we present a biochemical explanation for that clinical 
suspicion. With newly discovered tools we can system-
atically explore how often the mechanism of apoptotic 
escape determines chemosensitivity. Moreover, with 
novel therapeutic agents, we already have promising 
evidence that we will be able to selectively target some 
of the barriers constructed by cancer cells to evade their 
rightful death sentence.
13. In this anti-apoptotic proteins transcribed by the bcl-x locus were first described.
19. The concept of priming of BCL2 proteins and mitochondria with pro-death proteins is demonstrated. Bax expression and validated in several test systems.
22. This report uses a completely defined system of liposomes, recombinant proteins, and synthetic liposomes to demonstrate that BAX requires BHS domains to exert its permeabilizing function.
25. This paper first presents the activator versus sensitizer dichotomy in the function of BHS domains. Certain BHS domains are shown to act as activators of apoptosis.
This report is a presentation of the mature indirect model of apoptosis induction.


83. This paper introduces ABT-737, a high affinity small-molecule antagonist of BCL2, BCL-XL, and BCL-w.


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I apologize to those many authors whose important work could not be included in this review owing to space constraints.

Competing interests statement
The author declares competing financial interests: see web version for details.

DATABASES
APAF1 | BAD | BAX | BAK | BIRC3 | BCL2 | BCL2A1 | BCL2L1 | BCL2L3 | BCL11B | BID | BIK | BIM | BOK | BCL2L7 | BID | BIK | BIM | BOK | BCL2L7 | BID | BIK | BIM | BOK

breast cancer | leukemia | melanoma | pancreatic cancer

cyclophosphamide | doxorubicin | etoposide | fludarabine | oblimersen | vinorelbin

FURTHER INFORMATION
Anthony G. Letai’s homepage: http://research.dtf.hartford.edu/letai/
ALL LINKS ARE ACTIVE IN THE ONLINE PDF