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REVIEW

How to unleash mitochondrial apoptotic blockades to kill cancers?

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Abstract Apoptosis, especially the intrinsic mitochondrial cell death pathway, is regulated by the BCL-2 family of proteins. Defects in apoptotic machinery are one of the main mechanisms that cells employ to evade cell death and become cancerous. Targeting the apoptotic defects, either by direct inhibition of BCL-2 family proteins or through modulation of regulatory pathways, can restore cell sensitivity to cell death. This review will focus on the aspects of BCL-2 family proteins, their interactions with kinase pathways, and how novel targeted agents can help overcome the apoptotic blockades. Furthermore, functional assays, such as BH3 profiling, may help in predicting responses to chemotherapies and aid in the selection of combination therapies by determining the mitochondrial threshold for initiating cell death.

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Abbreviations: ASH, American Society of Hematology; ATAP, amphipathic tail-anchoring peptide; BAD, BCL-2-associated death promoter protein; BAK, BCL-2 homologous agonist; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma 2; BCL-w (BCL2L2), BCL-2-like protein 2; BCL-xL, B-cell lymphoma X long; BFL-1 (BCL2A1), BCL-2-related protein A1; BCR, B-cell receptor; BH3, BCL-2 homology 3; BID, BH3 interacting domain death agonist; BIK, BCL-2-interacting killer; BIM, BCL-2-interacting mediator of cell death; BOK, BCL-2 related ovarian killer; BTK, Bruton's tyrosine kinase; CDK, cyclin-dependent kinase; CLL, chronic lymphocytic leukemia; CHOP, cyclophosphamide, hydroxydaunorubicin, oncovin-vincristine, and prednisone; CML, chronic myelogenous leukemia; CR, complete response; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; FDA, U.S. Food and Drug Administration; GSK-3, glycogen synthase kinase-3; ITK, interleukin-2-inducible T cell kinase; MCL, myeloid cell leukemia; MOMP, mitochondrial outer membrane permeabilization; NHL, non-Hodgkin lymphoma; NIH, National Institutes of Health; NSCLC, non-small cell lung cancer; P3K, phosphatidylinositol-3-kinase; PUMA, p53 up-regulated modulator of apoptosis; SLL, small lymphocytic lymphoma; T-ALL, T-acute lymphocytic leukemia

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1. Targeted therapies fueled by the discovery of cancer genome and signaling webs

In 2003, the human genome project was pronounced completed, after thirteen years of U. S. National Institutes of Health (NIH) leading multi-national research efforts. The “finish” period has continued since the final version of sequences was published in the journal Nature in 2004, and more detailed information for the human genome has yet to be revealed. With the evolutionary development of sequencing and computing technology, which lead to a drastic decrease in costs for sequencing (from 100 million to 1000 dollars per genome), and possible interpretation of the results, the focus of current DNA sequencing is naturally oriented towards human diseases. And cancer is one of them; the discovery of important gene variants/mutations in cancer genome has changed treatment regimens and benefits many cancer patients. The most exciting examples are mutations uncovered in epidermal growth factor receptor (EGFR) and BRAF in lung cancer and melanoma, respectively.

In parallel, proteomics is expanding its web to the most of the signaling networks governing our physiological functions. New nodes and loops continue to be discovered, and novel interventions are being tested and exploited in disease treatments. In 2015, the U. S. Food and Drug Administration (FDA) approved 45 novel drugs, including four used to treat multiple myeloma. About a decade ago, most chemotherapies were limited to conventional pan-cytotoxic drugs that targeted DNA or microtubules such as the CHOP regimen (cyclophosphamide, hydroxydaunorubicin, oncovin-vincristine and prednisone), which cured millions of people of otherwise fatal diseases, but also were accompanied by severe side effects due to damage to healthy tissues. At present, targeted agents, newly approved or in clinical trials, are more precise and bring the overall response rates to much higher levels than before. This is true even for subgroups with adverse prognostic features and those who respond poorly to conventional therapies, especially in hematological cancers demonstrated at the annual meeting of American Society of Hematology—ASH 2015 (Orlando, FL, USA). In April, 2016, following promising activity in its clinical trials, the selective BCL-2 inhibitor venetoclax (AbbVie) was approved by the FDA for chronic lymphocytic leukemia (CLL) with chromosomal abnormalities, and it becomes the first-in-class direct inhibitor targeting BCL-2 family proteins that has got approved. With so many novel agents and biomarkers emerging, there is no doubt that cancer research and therapies have entered a new era, in which hopefully more cancers will become manageable in the near future.

Regardless of their categorization as conventional or targeted therapies, most of chemotherapies kill cancer cells via the apoptotic cell death pathway. In this review, the focus will be on research progress made on this intrinsic mitochondrial cell death pathway regulated by B-cell lymphoma 2 (BCL-2) family proteins; on targeted therapies that have been developed to intervene in its dysfunction in cancers; and how we can use BH3 profiling, a functional assay measuring mitochondrial priming, to predict patient responses and provide guiding information for potential combination therapies.

2. Apoptosis is regulated by BCL-2 family proteins

Apoptosis, which includes both extrinsic and intrinsic pathways, is one of the most important forms of cell death in multicellular organisms. The intrinsic cell death pathway is regulated mostly by BCL-2 family proteins residing in or recruited to the mitochondria after death insults imposed on cells. The BCL-2 family comprises both anti- and pro-apoptotic proteins. Anti-apoptotic proteins include at least BCL-2, BCL-XL, MCL-1, BCL-w and BFL-1. High expression of anti-apoptotic proteins, especially BCL-2, BCL-XL, and MCL-1, has been shown in various types of cancers, and they play important roles in tumorigenesis in different tumor models. Pro-apoptotic proteins can be further divided into two subgroups, including multi-domain proteins, like the death effectors/executors BAX and BAK; and BH3-only proteins, like activators BIM, BID and PUMA, or sensitizers including BAD, NOXA, HRK and BMF. Recently, BOK, a non-canonical BCL-2 family effector of apoptosis, has been shown to mediate cell death triggered by endoplasmic reticulum (ER)-associated degradation independent of BAX and BAK, or when BAX/BAK are absent and cells are overwhelmed by unfolded proteins.

The interactions within the BCL-2 family members are complex, and the interplay of anti- and pro-apoptotic proteins determines cell fate (see Fig. 1). The activation of BAX, BAK or BOK (in some circumstances) can lead to their oligomerization, which forms pores in the mitochondrial outer membrane and resulting the release of cytochrome 

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Thus mitochondrial outer membrane permeabilization (MOMP) is generally considered a point-of-no-return, and triggers downstream caspase activation, proteolysis and DNA fragmentation. Different apoptotic blockades, resulting from BCL-2 family protein interactions, in which pro-death signals were sequestered or counteracted by anti-apoptotic proteins, have been observed in cancer cells as a means for cells to evade apoptosis.

Apoptosis is initiated at mitochondria; however, the regulation of BCL-2 family proteins is tightly controlled by upstream signaling networks, from receptors on cell surface to transcription factors residing in the nuclei. There are usually two sets of regulations when cells face death insults. One is to upregulate pro-death signals and the other is to downregulate anti-death factors. For example, pro-apoptotic protein BIM-EL is kept low by phosphorylation (by extracellular signal-regulated kinase —ERK mediated degradation when cells are stimulated with growth signals, but its protein level can be stabilized and increased when the ERK pathway is inhibited. BIM can be induced transcriptionally by transcription factor FOXOA and when it is translocated to nuclei after PI3K/AKT inhibition. AKT can phosphorylate BAD and BAX, and regulate their pro-apoptotic functions. BID, PUMA, NOXA and BAX are targets of p53 transcription factor, and their induction by p53 in response to DNA damage or other death insults keep cells in balance between cell cycle arrest and cell death. Besides p53, PUMA can also be transcriptionally regulated by FOXOA. On the other hand, for anti-apoptotic proteins, BCL-2, BCL-XL and BFL-1 are target genes of NF-kB signaling, which is consistent with the pro-survival function of the NF-kB pathway. The anti-apoptotic function of these proteins also can be modulated by phosphorylation. Nevertheless, the best example of phosphorylation and its sub-sequential effects on apoptosis lies in MCL-1. This short half-life protein can be rapidly degraded via the proteasome pathway after phosphorylation by glycogen synthase kinase-3 (GSK-3) in the AKT pathway. The mutation of E3 ligase FBW7 and resulting stabilization of MCL-1 protein is critical in tumorigenesis of T- acute lymphocytic leukemia.
and determines the sensitivity of cancer cells to anti-microtubule drugs.31

Thus far, the genetic mutations harbored by BCL-2 family proteins are uncommon (more indeed will be discovered when more cancer genomes are sequenced), but other chromosomal abnormalities exist, which can lead to the upregulation of BCL2 family proteins. For instance, the amplification of MCL-1 gene locus is often associated with a variety of tumors, including breast cancer and non-small cell lung cancer (NSCLC)22. Chromosomal (t14;18) translocation in follicular lymphoma39,52,53 and diffuse large B cell lymphoma54 is critical for BCL-2 overexpression and oncogenesis in those types of cancers. Other means that cancer cells employ to counteract cell death include microRNAs. It has been discovered that they can negatively regulate anti-apoptotic proteins. For example, miR-15a and miR-16-1 regulate BCL-2 in CLL55, let-7 family56, miR-49157 and miR-133a58 for BCL-xL (miR-133a can also target MCL-1), and miR-19559 for BFL-1. Downregulation of these inhibitory microRNAs would elevate anti-death proteins, which then initiate tumorigenesis or drug resistance.

3. Modulating the apoptotic machinery with kinase inhibitors

As illustrated above, the regulation of BCL-2 family proteins is tightly connected with pro-survival signaling networks, including NF-κB, phosphatidylinositol-3-kinase (PI3 kinase, PI3K), and other pathways which serve as barometers for the abundance of nutrients in the microenvironment. In the last decade or so, the roles of these pro-survival pathways have been illuminated by cancer genome sequencing, proteomics and other systematic approaches. As such, kinase inhibitors have been developed to inhibit those pathways. Some of these inhibitors have brought in important clinical applications.

Indeed, each type of cancers has selected unique pathways during their tumorigenesis, and specific inhibitors will be required to target the unique pathway and trigger cell death accordingly. Erlotinib and gefitinib are inhibitors targeted the activating mutations in EGFR3 discovered in some of NSCLC patients. The pro-apoptotic proteins BIM and PUMA are induced by EGFR inhibition and trigger downstream apoptotic signaling in these cells60–62. In BRAF-mutant melanoma, BIM, PUMA, and BMF are reported contributing to the apoptosis induced by BRAF and/or MEK inhibitor treatments63. BIM and BAD trigger the apoptotic response to imatinib treatments in chronic myelogenous leukemia (CML)64. Conversely, downregulation of the anti-apoptotic MCL-1 protein by PI3K-mTORC1 inhibition can act together with BIM induction in EGFR-mutant cells to induce tumor regression65.

Most recently, inhibitors against Bruton’s tyrosine kinase (BTK), which is downstream of the B-cell receptor (BCR) pathway, have shown promising clinical activity in B-cell malignancies. Ibrutinib (developed by Pharmacycics) is the first generation BTK inhibitor, and was approved by the FDA for mantle cell lymphoma (MCL) in 2013 and for CLL in 201466. A 71% overall response rate was reported in the original clinical trial for CLL and small lymphocytic lymphoma (SLL), which was independent of clinical and genetic risk factors such as 17p deletion67. However, ibrutinib has relatively poor selectivity. Besides BTK, it also can inhibit other kinases such as interleukin-2-inducible T-cell kinase (ITK)68. Second generation BTK inhibitors were designed for better selectivity for BTK to avoid off-target effects. A recent report for a phase I–II clinical trial for one of such second generation inhibitors, acalabrutinib (ACP-196, Acerta Pharma), has demonstrated robust clinical activities68. In that trial with acalabrutinib, a 95% overall response rate was achieved in relapsed CLL patients, and it is also efficacious in patients bearing 17p deletion (100%), a subgroup that has been associated with poor prognosis.
Despite their encouraging clinical activities, it is still unclear how ibrutinib or acalabrutinib kill cancer cells. They may trigger the PI3K-mTOR pathway and upregulate pro-apoptotic BH3-only proteins like BIM. The real mechanism of action for these BTK inhibitors has yet to be fully understood since these agents don't elicit cell death quickly \textit{in vitro} (as opposed to the BCL-2 inhibitors described below). Lymphocytosis has been observed for ibrutinib and acalabrutinib, suggesting that these agents may inhibit the interaction between cancer cells and microenvironment, and displace cancer cells from protective compartments, which in turn generates pro-death signals like BIM and kills cells.

Taken together, indirect induction of BH3-only proteins or reduction of anti-apoptotic proteins by kinase inhibitors can play a central role in inducing cell death in cancers driven by constitutively activated oncogenic kinases, such as BCR-ABL, EGFR, BRAF, KRAS, BTK, some of which are described here.

4. Direct modulation of the apoptotic pathway with inhibitors for BCL-2 family proteins

The main concern for kinase inhibitors is feedback inhibition within the same or proximal pathways, which would hinder the effectiveness of these agents in killing cancer cells. Agonists or inhibitors of BCL-2 family proteins, act directly on apoptotic machinery. Such modulators can overcome the problems of inhibiting kinase pathways, and have thus been explored and developed by pharmaceutical companies.

One new approach to modulate BCL-2 family proteins is to enhance the pro-apoptotic functions. Stapled peptides based on BH3-only proteins, constructed using non-natural amino acids designed to protect peptides from proteolysis, have been tested in the pre-clinical setting. Further clinical development of these modified peptides is necessary in order to benefit patients, as they may have difficulty entering cancer cells.

For inhibitors of the anti-apoptotic proteins, early attempts included using natural products identified from chemical library screening (like gossypol and other natural polyphenols) for their inhibitory effects on BCL-2 and BCL-xL. However, none of these compounds have become drugs for clinical usage. Novel agents, based on medicinal chemistry and/or structure-activity relationship, have since been developed. These inhibitors include the antisense oligonucleotide oblimersen (Genasense, by Genta), the pan-inhibitor obatoclax (by Teva) and the ABT737/ABT263 series (by AbbVie). Oblimersen is a single-stranded 18-mer DNA molecule complementary to BCL-2 mRNA, and it had been shown to inhibit BCL-2 protein expression, presumably by inducing degradation of BCL-2 mRNA. Despite some evidence of benefits in phase I studies of CLL, myeloma and melanoma, oblimersen was not effective in a phase III study in myeloma, and only modestly beneficial when added to fludarabine in a phase III study of CLL. Thus oblimersen did not receive FDA approval and further development of the drug was not pursued. Obatoclax (GX-15-070) is considered a pan-inhibitor to the BCL-2 family since it can bind to the BH3 domain of BCL-2, BCL-xL and MCL-1. Clinical trials for obatoclax showed only modest efficacies for CLL, but no benefit for myelofibrosis or small cell lung cancer (SCLC). Furthermore, the
neurological side effects limited its further clinical development, and the drug was not approved by the FDA.

The most potent and selective BCL-2 inhibitors are those developed by AbbVie. As mentioned above, the first-in-class BCL-2 inhibitor venetoclax has just been approved by the FDA. ABT737, the first generation BCL-2 inhibitor by AbbVie, is a BH3-mimetic of the BAD BH3-only protein. ABT263 (navitoclax) is an orally bioavailable counterpart of ABT737.

Both molecules can inhibit BCL-2, BCL-xL and BCL-w with a binding affinity on the order of 10 to 10,000 times greater than other molecules, including obatoclax. These are large (nearly 1000 Da) compounds that mimic the amphipathic BH3 domain's α-helices. As such, they compete for the BH3-binding sites on anti-apoptotic proteins, freeing pro-apoptotic proteins from sequestration. These freed pro-apoptotic proteins then activate BAX/BAK, which in turn form the outer mitochondrial membrane pores, triggering downstream caspase activation and cell death.

ABT737 has poor oral bioavailability and has been limited to pre-clinical research. Navitoclax, however, has been limited in progressing through clinical trials due to thrombocytopenia, an on-target effect on BCL-xL that is required for platelet function. Even with that stated, specific BCL-xL inhibitors like WEHI-530, A-1155463 and A-1331852 are also under development. Perhaps, with better dose management, supportive care, and combination therapy, BCL-xL inhibitors can still bring benefits to patients with BCL-xL-dependent cancers, which are more common in solid tumors.

The problem of thrombocytopenia triggered by navitoclax led to the reengineered development of venetoclax, an inhibitor with better selectivity for BCL-2. It has reduced affinity for BCL-xL by three orders of magnitude. Venetoclax rapidly kills malignant cells through the intrinsic mitochondrial apoptosis pathway, and is selective for cells dependent on BCL-2, but not those dependent on BCL-xL. In preclinical models, the drug exhibited efficacy against a wide variety of tumor types, including leukemia, non-Hodgkin lymphoma (NHL), and myeloma, with no significant thrombocytopenia observed in in vivo models.

As a single agent, venetoclax has recently demonstrated robust clinical efficacy. A paper published by Roberts and colleagues reports results of a phase I clinical trial for relapsed CLL, which depends on BCL-2 for survival. Among the 116 patients who received venetoclax, 92 (79%) of them had a response. Even more encouraging is the response rate (from 71% to 79%) among patients from the subgroups with an adverse prognosis, including those with resistance to fludarabine, those with chromosome 17p deletion and those with unmutated IGHV (Immunoglobulin heavy chain variable region). In the phase-II trial reported by Stilgenbauer and colleagues, an overall response rate of nearly 80% was also achieved among patients with 17p deletion, marking venetoclax a promising new treatment option for patients with poor prognostic features.

Neither navitoclax nor venetoclax inhibits MCL-1, the anti-apoptotic protein that not only plays critical roles in multiple myeloma, mantle cell lymphoma and some solid tumors, but is also involved in drug resistance to ABT737, anti-tubulin chemotherapy and other cancer drugs. Currently, specific MCL-1 inhibitors for clinical applications are lacking. However, its short half-life feature has been exploited in the setting of kinase and cell cycle inhibition, which can lead to either translational or transcriptional repression of MCL-1 protein or transcript, as seen following treatment with PI3K-mTOR inhibitors or cyclin-dependent kinase (CDK) inhibitors like flavopiridol and roscovitine.

As described above, obatoclax is a pan-BCL-2 inhibitor that can inhibit MCL-1 as well as BCL-2, BCL-xL and BCL-w, but the clinical activity of this agent has been disappointing due to modest anti-cancer activity and neurotoxicity. Besides obatoclax, apogossypol derivative B97C1 (sabutoclax) has been reported to inhibit BH3 peptide binding to BCL-2, BCL-xL and MCL-1. Apogossypolone is a third generation gossypol derivative, designed to effectively target MCL-1. One of the structure derivatives is B97D6, which has displayed a higher selectivity for MCL-1 over BCL-2 or BCL-xL, and can overcome ABT737 resistance by acting on MCL-1. However, these inhibitors lacked the potency to induce similar extents of death in BCL-2, BCL-xL or MCL-1 dependent cells, indicating that they are weak inhibitors for BCL-2 family proteins. By analogy to the development of ABT737, several groups have generated specific small molecule inhibitors for MCL-1. Advances in pre-clinical models raise the possibility that potent and selective MCL-1 inhibitors may be available for clinical examination in the near future.

The inhibitors described in this section have been developed aiming to antagonize the up-regulation of anti-apoptotic proteins during tumorigenesis, such as BCL-2, BCL-xL or MCL-1. The effectiveness of these types of inhibitors depends on the functional intact of death executioners BAX/BAK and BOK, and depends on the overall priming of cells, which can be fluctuated by expression levels of BCL-2 family proteins. Resistant or refractory to killing can occur if anti-apoptotic factors can’t be overcome by the inhibitors. A new type of peptide, amphipathic tail-anchoring peptide (ATAP) derived from BFL-1, a bifunctional BCL-2 family member, can bear pore forming property, trigger MOMP and release cytochrome c independent of BAX/BAK and other BCL-2 family proteins, even other cellular factors. This unique feature relies on its intrinsic mitochondrial targeting sequence. Once linked to an internalized RGD peptide, selective targeting for ATAP to tumor cells can be achieved and their ability to kill cancer cells has been demonstrated in prostate cancer cell lines and their xenograft models. While these data suggest modified ATAP peptides can potentially be therapeutic agents, the mechanism of action of this type of peptides, especially their connection with BCL-2 family or other mitochondrial proteins, and the determinants of future patient response, remain to be identified.

5. Combination therapies and predicting patient response with BH3 profiling

Despite the high response rates reported in recent clinical trials in CLL for the second generation BTK inhibitor acalabrutinib (95%) and BCL-2 inhibitor venetoclax (79%), the complete response (CR) rate is low. All responses for acalabrutinib are partial, and only 20% CR is achieved by venetoclax. Furthermore, resistance to mono-therapy will inevitably occur. This raises the question of what should be combined with these novel agents in order to achieve better efficacy in treating patients. Answering this question is even more important in liquid cancers with less optimal response, or in solid tumors, which have been in general very refractory to the BCL-2 inhibitors described here (with the
exception for some SCLCs that are dependent on BCL-2 for survival\textsuperscript{87}.

For liquid cancers, another example supporting the need for combination therapy comes from the clinical trial with elderly acute myelogenous leukemia (AML) patients. Used as a single agent in elderly AML patients, venetoclax had only achieved a modest response rate (19\% objective response rate including 6\% CR\textsuperscript{102}). Indeed, AML is a much different disease than CLL, and AML in the elderly is known to have a poor response to the standard induction therapies like cytarabine and daunorubicin due to its more aggressive disease biology and toxicity from these therapies. However, combining venetoclax with hypo-methylating agents (decitabine or azacitidine) has led to overall response rates of 75\% (combining with decitabine) and 70\% (with azacitidine) in treatment-naïve elderly patients (\textgtr 65 years), who were not eligible for standard therapies\textsuperscript{105}. Thus the combination therapies have greatly improved the drug's efficacy.

So, what should be combined? Since BCL-2 family members play such a central role in regulating intrinsic mitochondrial cell death, they may have the potential to serve as biomarkers - predicting treatment responses or guiding the choice of combination therapies. A functional assay developed by the Letai group known as BH3 profiling measures mitochondrial priming resulting from the interaction of BCL-2 family proteins\textsuperscript{29,60}. Using synthetic BH3 peptides based on the BH3 domains of the BH3-only proteins, cells can be profiled to measure their distance from the apoptotic threshold\textsuperscript{106,107} (Fig. 2). Highly primed cells, containing mitochondria that can be permeabilized with relatively low doses of BH3 peptides, are those that are very close to the threshold of apoptosis. BH3 profiling, or dynamic BH3 profiling\textsuperscript{108} (DBP, a variant of BH3 profiling focusing on priming changes after drug treatments), have been tested in various preclinical models\textsuperscript{109–112}. Using pretreatment samples from patients on the venetoclax trial (M12-175), we performed BH3 profiling and found that the extent of mitochondrial depolarization induced by a BIM BH3 peptide in vitro was correlated with percentage reduction of CLL in the bone and blood marrow in vivo, while the lethal concentration (LC\textsubscript{50}) derived from standard cytotoxicity assays was not; and the mitochondrial responses to peptides were independent of 17p status\textsuperscript{113}. These data support further assessment of BH3 profiling as a predictive biomarker for this novel agent and other therapies.

In addition to baseline BH3 profiling of patient samples, DBP can be even more useful in assessing mitochondrial priming or apoptotic threshold changes after therapeutic intervention. Theoretically, two drugs from distant pathways would be less likely to develop overlapping resistance or feedback inhibition. Using DBP, we explored the possible combination of ibrutinib with venetoclax, and the mechanism underlying the efficacy of this combination. We have discovered that even though ibrutinib does not elicit frank cell death, it can increase mitochondrial BCL-2 dependence, and sensitize CLL cells to the BCL-2 inhibitor venetoclax\textsuperscript{114}. Thus, by increasing our understanding of the mechanism of action of novel agents on apoptosis, BH3 profiling can provide guiding information on what compounds to combine to achieve enhanced drug efficacy.

6. In conclusion

Cancer therapy has entered a new era, with huge data sets generated over the last decade from cancer genome sequencing and other big data molecular approaches using cancer patient samples. This provides a greater opportunity to understand the cause of tumorigenesis, and uncover novel therapeutic targets. Nonetheless, the overwhelming information could also hinder the process of turning these discoveries into clinical benefits. The ultimate goal for any therapy is to kill cancer cells, and to cure patients\textsuperscript{13}. With apoptosis converging on the BCL-2 family to execute the killing decision, understanding BCL-2 family proteins, their interactions and their context within other singling networks, will shed light on the effectiveness of chemotherapy and optimal combinations. The continued development of our BH3 profiling system and other biomarkers\textsuperscript{16} (for instance, DR_ MOMP)\textsuperscript{117,118}, perhaps can help to provide guiding information in patient and drug selection in the future.

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