A Laboratory Guide to BH3 Profiling

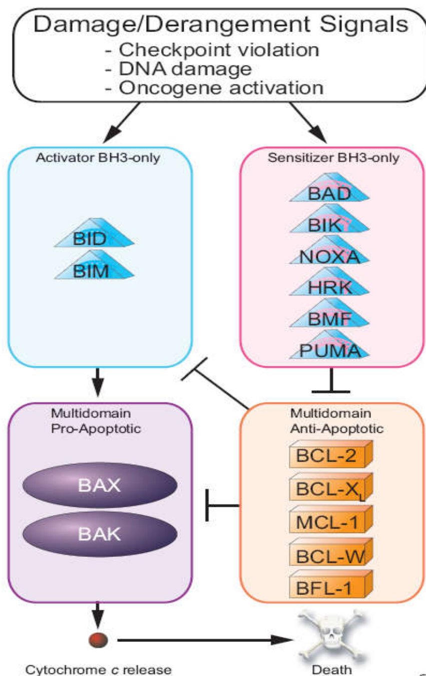

Figure 1: Classification and interactions of the BCL2 family

Why BH3 Profiling

The intrinsic pathway of apoptosis is regulated at the level of the mitochondria where more than fifteen members of the BCL-2 family of proteins interact to make this life or death decision. Many chemotherapeutic agents cause apoptosis, and the mechanism often involves changes in the levels and interactions of BCL-2 family members.

Levels of each of these proteins alone is not enough to determine how this decision will be made, and the sample size necessary to probe all possible interactions and post-translational modifications makes the task virtually impossible with cell lines and even more so with primary samples. Rather than attempt these, the Letai laboratory has developed BH3 profiling as a functional tool to determine the net response of these interactions without needing to record all of the individual interactions that determine it.

BCL-2 Family Control of Apoptosis

BH3 profiling relies on our understanding of how several broad groups of BCL2 family members interact with each other. The groups can be defined based on their structure and function as multi-domain pro-apoptotic, multi-domain anti-apoptotic, activator BH3 only, and sensitizer BH3 only. The multi-domain proteins contain BCL-2 Homology (BH) domains 1-4 in most cases while the BH3 only proteins are named because they contain only BCL2 homology domain 3, or BH3.

Functionally, the multi-domain proteins BAX and BAK are pro-apoptotic and are responsible for generating pores in the outer mitochondrial membrane when they undergo conformational changes upon activation. Mitochondrial outer membrane permeabilization (MOMP) allows for the release of cytochrome c into the cytoplasm where it can interact with pro-caspase 9 and apaf-1 to form the apoptosome, triggering downstream cleavage and activation of caspase 3 and apoptosis. Even without downstream caspase activation, MOMP will generally lead to the death of the cell even if it is no longer as efficient as with caspase activation. **All measures of response in BH3 profiling are surrogates for measuring MOMP.**

While they may not be the only proteins capable of doing so, BH3 proteins BIM and BID are capable of interacting with BAX and BAK to induce the conformational changes that leads to MOMP. This ability to activate BAX and BAK is where their designation as activator BH3s are derived. Opposing the pro-apoptotic proteins are the multi-domain anti-apoptotic proteins BCL-2, BCL-W, BCL-XL, BFL-1, and MCL-1. The proteins inhibit BIM and BID by binding them and preventing them from interacting with BAX and BAK. They can also bind the activated forms of BAX and BAK to prevent them from forming oligomers and therefore preventing MOMP. Finally, while they cannot directly activate BAX and BAK, the sensitizer class of BH3 only proteins can bind to the anti-apoptotic proteins, acting as inhibitors of inhibitors, to release activators and activated BAX and BAK. Pre-bound sensitizers leave less available

room to bind activators and activated BAX or BAK, and this makes the cell more sensitive to subsequent insults, which is where they derive their name. A summary of these interactions can be found in Figure 1. **NOTE: The BH3 domain of the BH3 only proteins is where the BH3 peptides used in profiling are derived. With few exceptions, most sequences are the native human or mouse sequence.**

The BH3 Binding Map

Not every BH3 only protein can interact with every anti-apoptotic protein, and this adds a layer of complexity that prevents a simple sum of all BH3 proteins from being used to determine the outcome of a death insult. However, the binding affinities of the BH3 domains as peptides to recombinant anti-apoptotic members is known, and this pattern also enables BH3 profiling to determine how ready a cell is to commit to apoptosis, but it also provides information regarding which anti-apoptotic proteins are being used to prevent MOMP.

	BID	BIM	BIDM	BAD	BIK	NOXA A	NOXA B	HRK	BNIP	PUMA	BMF
BCL2	**66 (6)**	**<10**	-	**11 (3)**	**151 (2)**	-	-	-	-	**18 (1)**	**24 (1)**
BCLXL	**12 (9)**	**<10**	-	**<10**	**10 (2)**	-	-	**92 (11)**	-	**<10**	**<10**
BCLW	**<10**	**38 (7)**	-	**60 (19)**	**17 (12)**	-	-	-	-	**25 (12)**	**11 (3)**
MCL1	**<10**	**<10**	-	-	**109 (33)**	**19 (2)**	**28 (3)**	-	-	**<10**	**23 (2)**
BFL1	**53 (3)**	**73 (3)**	-	-	-	-	-	-	-	**59 (11)**	-

Figure 2: EC50 of BH3-only peptides to N-terminal GST, C-terminal truncated anti-apoptotic proteins. Values listed are in nM. Grayed values are greater than 1 μM

The activator peptides BIM and BID can find to all anti-apoptotic proteins. PUMA and BMF, which can be called pan-sensitizers, share this behavior with BIM and BID. The remainder of the peptides have a more selective binding pattern. For example, HRK only binds to BCL-XL, and thus a response from the HRK peptide will indicate a dependency on BCL-XL. Similarly, the NOXA peptides only bind MCL-1. BAD can bind three proteins, and therefore it cannot discriminate between the three, but a response from a BAD peptide indicates that at least one of its binding partners is present and involved in the prevention of MOMP. When use in parallel with small molecules agents such as ABT-199 and WEHI-539, which bind to BCL2 and BCL-XL respectively, the identity of the anti-apoptotic protein in use can be better understood.

Three Classes of Priming

Priming can be divided into three major classes. Class A, competent but unprimed, is characterized by a response to activators BIM and BID but weak or no response to sensitizer peptides. This means that the mitochondria have functional BAX and/or BAK, but they are not sequestering any pro-death proteins that could be displaced to activate them. These cells are generally less sensitive to chemotherapy, but perturbations can convert them from Class A to Class C, primed, which will be described later.

Class B cells lack functional BAX or BAK and are considered apoptotically incompetent.. This may be due to mutation, insufficient expression, or homozygous deletion of both proteins. Class B cells will not depolarize or release cytochrome c even with very high doses (100 μM) of BIM and BID over long periods because they cannot form the pores necessary to release cytochrome c. Non-specific pore forming peptides, such as Alamethicin, can be used as a control to cause MOMP in these cases to ensure that a class B profile is due to the cells and not due to an omission of a profiling component such as digitonin.

Class C primed cells will show a response to both activator and sensitizer class peptides. They have functional BAX and/or BAK to cause MOMP as well as sequestered pro-death proteins including BH3 only proteins or pre-activated BAX or BAK that have exposed their N-termini. Class C cells can be subdivided based on their responses to the sensitizer peptides as dependent on one or more anti-apoptotic protein by using the binding chart as a guide. For example, A class C MCL-1 cell would respond to BIM, BID, PUMA, BMF, NOXAA but not BAD or HRK. A class C BCL-2 would respond to BIM, BID, PUMA, BMF, and BAD but not NOXAA or HRK. It is possible, and common, for cells to depend on more than one protein, and this will cause a mixed profile. In general, class C cells are the most sensitive to chemotherapy provided that other factors, such as drug efflux pumps which are upstream of mitochondria, do not skew the response.

The Cliff Model of Priming

A simple tool to understand priming is to envision cells at different distances from a cliff, a threshold, beyond which they call in to an apoptotic death. If one cell is ten units away from the cliff and another cell is fifty units away, the one ten units away is said to be more primed. If a perturbation such as a drug is applied, and this perturbation generated twenty five units of death signal, the cell only ten units away will fly off the cliff and die. The less primed cell will move, but it will end far from the threshold of apoptosis and survive. One should note, however, that this cell is now more primed than it was when it started. This is how the class A to class C conversion can be envisioned.

A class B cell can be thought of as anchored far from the cliff where nothing can move it. Instead, one would have to collapse the cliff to kill it. Such events that can kill class B include extrinsic apoptotic signaling, necrosis, or immune-mediated killing that do not necessarily rely on the intrinsic apoptotic pathway.

The Basic Components of BH3 Profiling

While BH3 profiling originally relied on isolation of mitochondria as heavy membrane preparations, modern BH3 profiling applications use permeabilized cells. This allows us to use platforms such as FACS and microscopy in addition to bulk measurements by fluorescent plate reader or cytochrome c western blots. Every BH3 profiling experiment contains a few key elements:

1. **Profiling Buffer:** This is a solution that contains non-ionic solutes to maintain osmolarity because high salt concentrations tend towards spontaneous release of cytochrome c. They also contain a carbon source, such and succinate, and often low concentrations of EGTA and/or EDTA to sequester calcium in particular.
2. **Digitonin:** This is a mild detergent made of a steroid head and a polysaccharide tail. Use at 10-50 μg/mL final, it makes pore large enough to bleed the cytoplasm out of the cell within minutes

but leaves the organelles intact. This is a necessary to ensure all cells and their mitochondria get even exposure to the peptides so that they can be compared to each other.

3. **Peptides and/or small molecules:** These are the heart of the assay. Because of their unique interaction patterns, the BH3 peptides and some small molecules make it possible to determine priming class and subclass. The relative response of mitochondria to a fixed dose or a series of doses allows priming to be ranked across cell types or primary samples.

All profiling systems will rely upon the first three components. However, plate-based JC-1 fluorescence requires the following additional agents.

1. **JC-1:** Used between 100 nM and 1 μM final concentration, JC-1 makes the plate-reader based assay possible. As a monomer it fluoresces green, but it can form aggregates that fluoresce red when concentrated. Because it is a lipophilic cation, it accumulated in the matrix of the mitochondria when they are polarized and diffuses away when the mitochondria depolarize. By monitoring the red fluorescence, we can measure mitochondrial potential as a surrogate for MOMP.
2. **Oligomycin**: This blocks the ATP synthase from running in reverse and repolarizing the mitochondrial membrane. This generally works as a signal clarifying agent, and its importance can be sample dependent. It is possible to profile without it, but the curves may not be as sharp.
3. **2-mercaptoethanol**: This is a signal enhancing agent that appears to work by breaking up non-specific aggregates of JC-1 and this ensures that the fluorescence measured is from the mitochondria and not from the solution as a whole. Omission can cause a dominant exponential decay curve that can obscure other curves.

All BH3 profiling platforms consist of three stages.

1. Create the profiling template with peptides
2. Add cells and expose to peptides
3. Measure MOMP

Steps 1 and 2 are similar for all methods while measuring MOMP will vary based on the method. For each method, the most current protocols as of the revision date have been provided. A list of reagents used in these methods and how to prepare them, including the buffers and peptides, are included in the appendix at the end of this document.