BH3 profiling as a research and prognostic tool in the detection and manipulation of de-regulated apoptosis.

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ABSTRACT

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BH3 profiling is a technique developed to assess how close a cell is committing to the apoptosis pathway and, ultimately, death. This technique has mostly been utilized to study cancer, specifically in the context of various subtypes of lymphoma. In short, this technique utilizes controlled inputs in the form of peptides designed after the specific BH3 domains from various members of the Bcl-2 family of proteins which is largely responsible for mediating the cellular apoptotic response. These inputs allow for mitochondrial membrane permeabilization and the release of cytochrome C, the event considered to be the point at which the cell is committed to apoptosis. By controlling which peptides are administered to the cell, one can assess how close the cell is to making this commitment. Furthermore, it is also possible for scientists to utilize this method to see how specific types of cancer cells evade apoptosis despite the chaotic cellular environment which would normally lead to cell death. This information may also lead to predictions about which agents may work to effectively kill cancer cells, making this technique potentially useful as tool for assigning personalized therapies appropriately. This technique is simple and powerful and could be potentially modified for many different applications within the basic, translational, and clinical research contexts.

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To Mom and Dad.

Thank you for investing in me.

INTRODUCTION

Apoptosis is essential for the proper development of tissues and organs, and for the maintenance of those tissues in the body. Regulation for this counter of cell growth is therefore essential to avoid pathogenesis and to maintain a proliferative homeostasis. When left unchecked, unnecessary apoptosis can result in neurodegenerative diseases such as Alzheimer's and Huntington's disease. (Mattson, 2000) Conversely, if cells can escape apoptosis, uncontrolled cell proliferation may be the result, as in the case of many cancers. Mutations which either allow the tumor cells to protect themselves from apoptosis or disrupt the apoptotic pathway altogether, result in increased cell proliferation and ultimately in conditions which pose a threat to the organism as whole. (Reed, 1999)

Apoptosis is activated within a cell by two distinct, but related pathways. These pathways are termed the intrinsic and the extrinsic apoptotic pathways. Apoptosis via the extrinsic pathway involves activation via the Fas activated Death receptor. In this mechanism, the Fas ligand binds to the death receptor, resulting in the activation of caspase 8 and the subsequent cleavage of Bid into the active T-Bid. (Li et. al., 1998) T-Bid can then go on to the mitochondria to activate Bax and Bak and cause the formation of pores in the mitochondrial membrane, allowing for the release of cytochrome C. From here, a cascade of caspases is activated, culminating in widespread proteolytic cleavage by caspase 3 and cell death. (Walczak and Krammer, 2000)

While the intrinsic apoptotic pathway results in a similar endpoint, it is distinct in that it is not activated by the Fas ligand. Contrary to the extrinsic apoptotic pathway, the intrinsic pathway is activated by cellular stress such as DNA damage or the accumulation of mis-folded peptides. This pathway is also distinct in that it is mediated mostly by the

mitochondria. (Letai, 2008) Inside the mitochondria, the Bcl-2 family of proteins includes over 12 members that function as both pro- and anti-apoptotic agents. Interactions within the family push the cell closer or farther away from the apoptotic threshold which, when crossed, typically signifies irreversible commitment to programmed cell death. (Letai, 2008)

The Bcl-2 family of proteins is named as such because of the mutually conserved BH3 domain (Bcl-2 Homology Domain 3). This domain is the only one to be conserved in all family members, but it is important to note that some members have multiple domains in common with the founder of the family, Bcl-2. (Adams and Cory, 1998) *In vivo,* the BH3 domain tends to form an amphipathic alpha helix in pro-apoptotic members whose role is to either further sensitize the cell towards apoptosis or to activate the components which form pores in the mitochondria. Proteins which share multiple domains in common with the Bcl-2 protein (multidomain proteins) are able to form hydrophobic binding pockets which facilitate interaction with the amphipathic helices of molecules that only contain the BH3 domain. (Petros et. al., 2003)

BH3 profiling is a technique which monitors the status of the Bcl-2 family of proteins within the cell and determines how close it is to apoptosis. In the context of cells which are resistant to death, BH3 profiling can inform users as to what factors may be preventing the cell from committing suicide. These two insights into cell status are obtained by using defined inputs in the form of peptides derived from the BH3 domain of pro-apoptotic sensitizing and activating proteins. These peptides interact with multidomain pro- and anti-apoptotic proteins in a manner identical to the interactions facilitated by their parent proteins, as evidenced by the peptides' ability to induce

permeabilization of the outer mitochondrial membrane and dissipation of the gradient across that membrane. (Del Gaizo Moore and Letai, 2012) Although it is relatively simple in practice, the technique is immensely powerful in translational application. It has already been utilized in *in vivo* disease research and has also been implemented into clinical trials. (Deng et. al., 2007; Pierceall et. al., 2007) While these are the main applications, the technique may be modified to adapt to many different studies. Its versatility along with its ability to provide a simple, physiologic analysis of a rather complicated signaling mechanism, make it a truly useful tool for the study and manipulation of mechanisms of disease pathogenesis.

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Bcl-2 FAMILY OF PROTEINS

Bcl2, the founding member of the family, was originally discovered as the proto-oncogene in human follicular lymphoma which underwent a t(14;18) translocation, placing the gene under control of the immunoglobulin heavy chain promoter region. This translocation resulted in the increased production of the Bcl2 protein. The oncogene was unusual in that, rather than providing a growth advantage to the cells which expressed increased levels of the protein, it provided some sort of protection under conditions which would normally cause cell death. (Kelekar and Thompson, 1998) Proteins that shared varying amounts of homology with Bcl-2 have been discovered and functionally associated with the protein into its own superfamily.

All Bcl2 proteins have the BH3 domain, which is the only domain necessary for pro-death function, in common. In addition to this structural feature, it is also important to note that many of the anti-apoptotic proteins in this family share a carboxy terminal hydrophpobic domain which is thought to be the means by which some members of the family are localized to specific membranes. (Borner et. al., 1994) Bcl-2, for example, has been shown to be localized to the cytoplasmic side of the mitochondrial membrane and this specific localization may play an important role in its ability to protect a cell against apoptosis. (Krajewski et. al., 1993) Pro-apoptotic proteins such as Bax and Bid however, are found mainly in the cytosol before activation, whereupon they are recruited to the mitochondria. (Wolter et. al., 1997; Li et. al., 1998) The different patterns of localization between the pro and anti-apoptotic proteins may serve as delicate nuances which have consequences on the ability of the cell to undergo apoptosis via the intrinsic apoptotic pathway.

Classifications of the Bcl-2 Family

The Bcl2 family of proteins is organized based upon the amount of homology that is shared with Bcl2. The family is comprised of 2 main classes. (Figure 1) The first class is made up of members that share the BH1, BH2, BH3, and transmembrane domains with Bcl2. Within this class, both multidomain anti-apoptotic proteins and multidomain pro-apoptotic proteins exist. The anti-apoptotic group includes the proteins Bcl-2, Mcl-1, Bcl-XL and Bcl-W, whose presence generally resists apoptosis induction, but it should be noted that these members may be converted to pro-death molecules by effector caspases. (Li et. al., 1998; Michels et. al., 2004) The pro-apoptotic group includes Bax, Bak, and also Bok which is expressed specifically in reproductive tissues. (Hsu et. al. 1997) Studies have shown that at least one of these proteins is necessary to carry out apoptosis.(Letai et. al., 2008) These proteins are able to form oligomers with themselves or with each other which is thought to allow these proteins to permeabilize the mitochondrial membrane, resulting in the release of apoptotic factors, cytochrome C, SMAC, and DIABLO. Interestingly, it has been shown that these members of the Bcl-2 family share sequence and structural similarity with a pore forming bacterial toxin, providing further evidence for their accepted mechanism of action. (Muchmore et. al., 1996) In addition to allowing for the release of pro-apoptotic factors via permeabilization of the mitochondrial membrane, it is thought that these proteins may have some function on the membrane of endoplasmic reticulum as well. (Szegezdi et. al., 2009)

The following class of proteins lack BH1 and BH2 domains and act as pro death agents by inactivating multi-domain anti-apoptotic molecules (i.e. Bcl-2, Bcl-XL, Mcl-1,

etc...). Proteins belonging to this class are named" BH3 only proteins" because they only share the BH3 domain with Bcl2.

The BH3 domain forms an amphipathic alpha helix which binds with high affinity to a pocket that is formed by the BH1, BH2, and BH3 domains of multi-domain proteins. This binding is facilitated by both hydrophobic and electrostatic interactions. (Sattler et. al., 1997) Essential to the BH3 domain is an eight residue core, which is conserved at two positions in all members of the BH3 superfamily. A leucine residue at position 1 and an aspartic acid at position 6 of the core are crucial to heterodimer formation as well as death induction. This homologous sequence can be expressed as LXXXXD. (Ryan and Letai, 2013) Some members of this group also contain a hydrophobic transmembrane domain which may serve as a localization tool. (Kelekar and Thompson, 1998)

The BH3 only class includes the proteins Bid, Bim, Bad, Bik, Puma, Noxa, Bmf, and Hrk (Harakiri). (Wang et. al., 1996) These proteins can be further subdivided into 2 groups, activators and sensitizers. Bim and Bid are activators because of their ability to directly induce conformational change in Bax and Bak resulting in mitochondrial membrane permeabilization. The role of BH3 only activators is to directly induce death, but their actions can be attenuated by multi-domain anti-apoptotic proteins through sequestration. Anti-apoptotic proteins physically restrain the activator proteins through amphipathic helix/hydrophobic pocket interactions which are not exclusive to the association with the activator subclass. Sensitizer proteins with an amphipathic helix, Bad, Bik, Puma, Noxa, Bmf, and Hrk, can also form a helix enabling them to displace activator proteins from the binding pocket of multi-domain anti-apoptotic proteins. (Petros et. al, 2000) This allows for the freedom of activator proteins to induce a

Bax/Bak conformational change which would then initiate pore formation in the outer membrane of the mitochondria. The interactions leading to the prevention and activation of apoptosis are further detailed in figures 2 and 3.

The BH3 only class of Bcl-2 proteins is also particularly interesting because of some proteins' preference in binding partners. (Figure 4) For example, Puma is quite promiscuous in that it will bind all multi-domain anti-apoptotic proteins, although all interactions are not equal in strength. Other BH3 only proteins such as Noxa are very selective, with its only binding partner being Mcl-1. (Chen et. al., 2005) The distinct binding profiles of the BH3 only proteins are essential to BH3 profiling's ability to diagnose where a block in apoptosis may occur, allowing the technique to not only identify what class of proteins is causing the block, but also what specific member of the class is responsible. This aspect of the technique will be discussed in further detail below.

Post-Translational Modifications affect BH3 only Activity

The BH3 only class of proteins exhibits further complexity through post translational modification and processing which can result in the modulation of activity for both activator and sensitizer proteins. Proteins such as Bim can exist in several isoforms with the most common splice variants being BimL, BimS, and BimEl. These different splicing patterns result in differences in activity with the most potent isoform being BimS. BimL is the least able to induce apoptosis, and BimEL has intermediate activity. (Marani et. al., 2002) Other isoforms of Bim have been discovered, but the aforementioned remain to be the most commonly acknowledged.

Bid is a unique BH3 only protein which also exists in two forms in the cell. As a part of the activator class, this molecule is able to antagonize the prosurvival function of some multidomain anti-apoptotic members, or heteordimerize with the pro-apoptotic effector Bax, enhancing its function. Bid's role in the induction of apoptosis is also unique in that it can undergo post-translational modification through cleavage by caspase 8. (Li et. al., 1998) Caspase 8 is an initiator caspase that strongly associated with the extrinsic apoptotic pathway. This pathway is activated when the cell receives external death signals in the form of the Fas Ligand, Tumor Necrosis Factor alpha, and other death signals. (Wajant, 2002) Caspase 8's ability to cleave Bid and subsequently activate intrinsic apoptosis, represents an important mechanism of crosstalk between the extrinsic and intrinsic apoptotic pathways.

Further post-translational modification of BH3 only proteins can occur by covalent additions such as phosphorylation and glycosylation. The sensitizer BH3 only protein, Bad, can be phosphorylated at Serine 155 of its BH3 domain by PKA. This modification results in Bad's reduced affinity for multi-domain anti-apoptotic proteins. (Virdee et. al., 2000) Although the protein has multiple phosphorylation sites, position 155 is particularly interesting in the context of this discussion because it lies within the BH3 domain and therefore becomes a part of the alpha helix formed by the domain. This fact may be important for the design of peptides that replicate this domain within a certain biological context. This idea is supported by serine 155's proximity to aspartic acid 156, a residue identified as essential to heterodimer formation between Bad and Bcl-XL. (Petros et. al., 2003) Because the residues are next to each other, it is likely that the addition of a chemical group to one would influence the local electrochemical

environment of the other, possibly resulting in a change in heterodimer formation kinetics. Post-translational modification also plays a role in influencing the localization of Bid. Caspase 8 cleavage reveals a site on TBid which is able to undergo N-myristoylation. This glycosylation allows for targeting of TBid to the mitochondria. (Zha et. al., 2000) As with Bad, this type of modification might prove to be important for the technique of BH3 profiling because an addition such as a myristoylation may affect the binding pattern of the protein to which the group has been added.

Bcl-2 Family Proteins Can Give Rise to Disease States

Apoptosis is acknowledged as a crucial process to cellular biology. Since the Bcl-2 family has much control over the decision to commit programmed cell death, changes in the expression or activity of many of its members can often result in pathogenesis, including many types of cancers and degenerative diseases.

Translocation t(14;18) of the Bcl2 gene (18) puts it under control of the immunoglobulin heavy chain promoter (14), leading to constitutive expression. (Tsujimoto et. al., 1984) This mutation is found in 85% of human follicular lymphomas , results in the upregulation of the pro-survival protein Bcl-2 conferring protection to the cell, against programmed cell death. (Yasukawa et. al, 2001) This ability to evade death is one of the hallmarks of cancer. (Hannahan and Weinberg, 2000) Other multidomain anti-apoptotic proteins are involved in lymphomagenesis as well. It has been shown that Mcl-1 is widely expressed in B-cell lymphoma, because of the protection it provides to these cells. This has been supported by the induction apoptosis when cells are treated with Mcl-1 antisense oligonucleotide, proving its pro-survival effects. (Michels et. al.,

2004) (Interestingly, the same study showed that Mcl-1 can be converted into a pro-death molecule by caspase cleavage, adding another layer of complexity to the regulatory mechanisms of the Bcl-2 family.)

While the increased presence of multi-domain pro-survival proteins can lead to the development of cancers, loss of these proteins, such as Bcl2 and Bcl-XL can lead to degenerative diseases. The absence of these anti-apoptotic members results in over activation of the death pathway. (Veis et. al., 1993) This state can be rescued with simultaneous loss of pro-apoptotic Bim, suggesting that both Bcl-2 and Bcl-XL interact with Bim to attenuate its pro-apoptotic function. (Bouillet et. al, 2001) This observation also highlights the importance of the ratios between pro- and anti- apoptotic molecules.

Changes in the expression of other classes of the Bcl-2 family are also involved in pathogenesis. Loss of proteins from both the BH3 only class (i.e. Bim) and the effector class (i.e. Bax) have been demonstrated to contribute to tumor formation in different study models of cancer. (Heiser et. al., 2004) Although most of the members of the Bcl-2 family have been implicated in disease formation, serious therapeutic targets have mostly derived from the multi-domain anti-apoptotic sub class, where development in lymphoma is now in late stage clinical trials. (Adams and Cory, 2007)

BH3 PROFILING

In its simplest form, BH3 profiling is essentially a technique which uses the isolated BH3 domains of BH3 only proteins and administers them in prescribed concentrations to induce a calculated change in the mitochondrial membrane potential. This change is engendered by the different peptides' abilities to limit the survival capabilities which the multi-domain anti-apoptotic proteins confer upon cancer cells. Although the technique is in fact very simple, it allows for a great deal of insight into the nature of the cancer cell.

BH3 profiling takes advantage of the fact that mitochondrial mediated apoptosis is a cellular process which can be stimulated by a limited number of inputs (BH3 only peptides) and will result in only a couple of different outputs (a change or lack thereof in mitochondrial membrane potential). By manipulating this complex pathway at a point where the user has only a few options to stimulate the system and where the output is essentially binary, the technique allows the user to avoid the excess interpretation which might be encountered if the points at which we interrogate and receive answers from the system were located at more dynamic positions in the pathway. This allows the user to somewhat avoid subtle nuances in how proteins interact that might have large consequences for the interpretation of the experiment. This is especially true for the Bcl-2 family of proteins, where there are many pathways feeding into this regulation step and the interactions between these regulators themselves are anything but exclusive.

The defined inputs of this technique come in the form of BH3 domain peptides which represent each of the known BH3 only proteins of the Bcl-2 family. Essential to using these peptides as a stimulus is the fact that even in isolation, these 20-25mers are

able to assume an active conformation which will interact with the correct BH3 domain with the only requirements for activation of apoptosis being the presence of Bax and/or Bak along with a complete BH3 domain from a BH3 only protein. (Letai et. al., 2002) All of the peptides are derived from two classes of BH3 only proteins, either activators or sensitizers. Activator peptides are functionally more effective at inducing apoptosis because they possess the ability to directly induce Bax and Bak to undergo conformational change which allows for homo/hetero oligomerization and therefore mitochondrial outer membrane permeabilization. Sensitizer peptides however, are not able to act in this way and must instead occupy multi-domain proapoptotic proteins in order to induce oligomerization of Bax and Bak. (Ryan and Letai, 2013) By doing so, sensitizers peptides almost act as competitive inhibitors of the pro-survival proteins, allowing direct binders (activators) to interact with Bax and Bak. Negative control peptides can be synthesized by mutating specific residues within the conserved core sequence. For example, the Bid BH3 domain has been used as a negative control by substituting two alanines for lysine and aspartic acid in the highly conserved sequence LXXXXD. (Certo et. al., 2006) Positive controls have been designed by using TBid, which is expected to strongly induce apoptosis as compared mutated Bid negative controls. (Goldsmith et. al, 2009)

The original iteration of the technique utilized isolated mitochondria where peptides were able to pass the membrane without any further manipulation of the system. (Certo et. al., 2006) An important consideration for this version of the technique is the localization pattern of Bcl-2 family proteins, which may potentially affect results if proteins belonging to this family are present outside of the mitochondria. (Figures 5 and

6) The entry of isolated BH3 domains into the mitochondria occurs in intact cells as evidenced by TBid's relocation to the mitochondria after its cleavage by Caspase 8. (Lutter et. al., 2000) This is especially important to note, not only because less work is required to get the peptides inside the mitochondria, but also because permeabilization of the mitochondrial membranes would result in the artificial change of mitochondrial membrane potential and release of apoptotic factors SMAC, DIABLO, and Cytochrome C, which would render the assay useless. Peptides of BH3 domains cannot however cross the plasma membrane thus requiring permeabilization of this barrier in the more evolved assay using whole cells. (Ryan and Letai, 2013)

Permeabilizing the Plasma Membrane

Using whole cells when executing the assay is quite obviously advantageous because it provides a more biologically relevant environment. The fact that the BH3 peptides, along with the succinate used to help maintain the mitochondrial membrane potential, cannot cross the plasma membrane requires that this barrier be permeabilized. Membrane pore forming toxins such as alphatoxin and SLO were considered, but their abilities to selectively permeabilize the plasma were inadequate at concentrations which would not affect the mitochondrial membrane. The size of the pores formed by these toxins were also too small to allow for efficient peptide diffusion across the plasma membrane. (Ryan and Letai, 2013) Instead weak detergents such as saponin and digitonin provided more attractive alternatives for the permeabilization of the plasma membrane. While these agents are able to produce pores useful for the passive diffusion of BH3 profiling components across the plasma membrane, they also preferentially act on this membrane because of their affinity to cholesterol (since the plasma membrane

contains a greater amount of cholesterol compared to the mitochondrial membrane). (Gottlieb and Granville, 2002) Additionally, although the pores created by treatment with detergent may allow some soluble proteins to leave the mitochondria, it is reasoned that since many members of the Bcl-2 family are either associated with intracellular membranes or with cytoskeletal elements, that permeabilization will not affect cellular localization. (Ryan and Letai, 2013) In the further development of the technique, digitonin was used as the detergent of choice, although saponin may have proved to be just as able to perform. Ryan and Letai also attempted the technique using cell permeant peptides modified with specific sequences (TAT, antennepedia, and poly-arginine) to facilitate peptide entry into the cell, but this method is seen as less favorable because of the resulting toxicity and cell death which is caused by mechanisms which are independent from intrinsic apoptotic pathway. (2013)

Buffer Considerations

Optimization of the buffer was necessary when designing this technique. The buffer provides a stable environment that allows for maintenance of the mitochondrial membrane potential in the absence of a depolarizing agent. Using isolated mitochondria normally requires use of mannitol/sucrose buffer for the organelle to retain functionality but for whole cells, trehalose buffer is able to stabilize the mitochondrial membrane potential over a dynamic range of voltages and provide protection to mitochondria through freeze/thaw cycles which is not the case with the sucrose/manitol solution. (Yamaguchi et. al., 2007) Having a sufficient amount of KCl is also critical to the success of the assay and the concentrations of these ions must be maintained by the buffer. The levels of KCl can have significant impacts on the ability of the mitochondria

to release cytochrome C and to depolarize completely. If KCl concentration is too low, the result is the retention of cytochrome C within the intermembrane space, even after outer membrane permeabilization. Conversely, if KCl levels are too high, mitochondria will undergo incomplete depolarization despite the strength of the stimulus. (Ryan and Letai, 2013) In addition to maintaining appropriate ion concentrations for the preservation and stabilization of the mitochondrial membrane potential, the buffer used must also protect mitochondria from excessive release of calcium ions by neighboring organelles such as the endoplasmic reticulum. Calcium exposure to the mitochondria can result in mitochondrial outer membrane permeabilization by Bax and Bak, which could potentially contribute to the occurrence of a false positive result, a concern for the validity of the results, both when using isolated mitochondria and permeabilized cells. To achieve this protection, calcium chelators such as EDTA or EGTA are used to sequester any calcium that may be present. Levels of these chelators may need to be modulated for use with different types tissues as natural calcium levels may vary between the different cell types. (Ryan and Letai, 2013)

Additional considerations for buffer composition include the presence of succinate which provides the carbon for the electron transport chain, as well as BSA, which contributes to the stability of the mitochondrial membrane potential. Maintaining pH 7.2 also helps to provide a stable environment allowing for a proper response of mitochondria to various stimuli.

Sensing a Change in the Membrane Potential

Measuring the change in the mitochondrial membrane potential is achieved by utilizing the electrically sensitive dye JC-1. JC-1 is a small molecule which exists as a

monomer, or when in high enough concentration as a J aggregate. (Smiley et. al., 1991) When excited at certain wavelengths these two states produce different emission spectra. This molecule can be described electrochemically as a small positive charge which is delocalized over a large conjugated system. This positive charge allows the molecule to associate with negatively charged membranes such as the mitochondria when its electrochemical potential is properly established. (Ryan and Letai, 2013) Association with this membrane results in high enough local concentrations of the dye so that it exists in mainly in the aggregate state. Permeabilization of the plasma membrane (and dissipation of the potential over this membrane) prevents non-specific localization and dissociation of the J-aggregates.

As stated above, JC-1 aggregates fluoresce at a distinct wavelength (590 nm) when excited with wave in the range of 540 to 560 nm. The emission of these aggregates is proportional to the mitochondrial membrane potential. Thus, because of the specific localization and because of their distinct emission spectra, they are able to able to accurately inform the observer of a change in potential at the mitochondrial membrane.

High-throughput Forms of Profiling

Whole cell profiling has evolved to increase the volume of data that can be obtained from a single experiment. High throughput versions of the whole cell method include plate based profiling and FACS based profiling. Plate based profiling is advantageous because it allows for the highest volume of data at the least cost. In addition, this version of the technique also allows for the collection data over time, which gives a more accurate portrayal of the extent of depolarization of the mitochondrial membrane. (Ryan and Letai, 2013) An original display of this type of data collection is

illustrated in figure 7. Plate based profiling is also very useful for assaying homogenous cell populations or when using samples that do not require differentiation of the responses by different cell types. It is also important to note that, should a selection process (via column or by physical attributes) be employed before profiling, significant cost may be incurred. When these types of distinctions need to be made, the FACS based profiling method should be employed. Discrimination of cell types within a sample may be accomplished using the FACS method by using the desired markers to discriminate a specific sub-population of cells from the rest of the sample. This alternative could be especially useful to implement if no purification methods existed for the type of cell that was to be assayed. Additionally this method confers an advantage upon the investigator in that several populations within a heterogeneous sample can be interrogated at the exact same time, under the exact same conditions, with discrimination of cell types and responses occurring after treatment with the peptides. Additional dyes can also be added in this version of the technique to discriminate dead cells from the live population. The ability to use additional markers in the technique has proven to be especially useful in sorting cells derived from heterogenous cell populations from patient tissue in clinical applications of BH3 profiling. (Pierceall et. al., 2013)

DEMONSTRATED APPLICATIONS OF BH3 PROFILING

Translational Applications

The main application of BH3 profiling is to provide an assessment of how close a cell is to death. Deng et. al. introduce a concept describing how "primed for death" cells are at the their steady state, in which the anti-apoptotic proteins (Bcl-2, Mcl-1, Bcl-XL...) within the cell are largely occupied by BH3 activator proteins. (2007) Interestingly, malignant cells seem to be "primed for death" more often than non-malignant cells. This is presumably due to the presence of one or multiple phenotypes (genomic instability, oncogene activation, cell cycle checkpoint violation, and loss of pro-survival signaling) that bring on apoptosis by the intrinsic pathway. The increased propensity of malignant cells to commit apoptosis means that these cells must employ protective counter measures towards the activation of programmed cell death. According to Deng et.al, cancer cells could theoretically block apoptosis through manipulation of the Bcl-2 family by three distinct mechanisms. The first, designated as a Class A block, could arise by failure to activate the BH3 only proteins which would then be unable to inhibit multidomain anti-apoptotic proteins. A Class B block would entail the loss of Bax and Bak, rendering the cell unable to form the pore required for the release of apoptotic factors. Finally, Class C blocks involve preventing the activation of Bax and Bak through the increased expression of multidomain pro-apoptotic proteins such as Bcl-2 , Mcl-1, etc... (2007)

BH3 profiling has the ability to determine which type of block is used by the cell to evade apoptosis. This can be inferred from the identities of peptides able to stimulate cytochrome C release along with their already known binding patterns. As discussed

previously, activator peptides are able to directly induce conformational change in Bax and Bak which results in oligomerization and pore formation. If an activator peptide such as Bid or Bim is able to cause a release of cytochrome C, then this implies that Bax and/or Bak are present and the cell is not evading apoptosis through a Class B block. The resistance to death may instead be caused a pool of unprimed anti-apoptotic proteins, although there are multiple members which may be responsible for this method of death evasion. To discern which specific anti-apoptotic member is unprimed, and representing the block in apoptosis, it is important to recognize that BH3 only proteins of the sensitizer class bind anti-apoptotic proteins in a preferential manner at physiologically relevant concentrations. Puma is the most promiscuous sensitizer peptide, binding all anti-apoptotic members, albeit with differing affinities. If treatment with the Puma peptide elicits a strong response, then this can provide the experimenter with a useful gauge of whether the cell is primed at all. (Certo et. al., 2006)

In order to identify the anti-apoptotic protein(s) that may be responsible for the block, it is helpful to look at the response to the full range of sensitizer peptides. While Puma is the most social of the sensitizer peptides, Bmf and Bik interact with all anti-apoptotic proteins with the exception of Bfl-1, and while Bmf has strong interactions with all of the anti-apoptotic proteins, Bik only strongly interacts with Bcl-XL and Bcl-W. Bik moderately associates with Mcl-1 and Bcl-2 and these interactions are not as strong as with Bcl-XL and Bcl-W. Treatment with Bik and Bmf is helpful to identify blocks caused by Bcl-2 and Mcl-1 given that it would be expected to result in strong depolarization and cytochrome C release from exposure to Bmf while Bik treatment might give rise to a response, but one that is somewhat attenuated compared to the Bmf

peptide. To determine the contribution of Mcl-1 or Bcl-2, the cells or mitochondria would be exposed to the Noxa peptide which strongly interacts only with Mcl-1. One would expect that if the cell was reliant on Bcl-2 for survival then, in our scenario, treatment with Noxa would yield no response because Bcl-2 would remain free to inhibit apoptosis. On the other hand, one could predict that the cell is dependent upon Mcl-1 if treatment with Noxa results in the release of Cytochrome C. Combined dependence on Mcl-1 and Bcl-2 would be suggested by response to treatment with Noxa and Bik. This could then be confirmed by response to administration with the Bad peptide. Letai and his group were able to make predictions in this manner in a panel of lymphoma cell lines (SU-DHL-4, SU-DHL6, SU-DHL-8, and SU-DHL10). Their results indicated that SU-DHL-4 and SU-DHL-6 had class C blocks in which dependence was placed on Bcl-2 and a combination of Bcl-2 and Mcl-1 respectively. Treatment of the SU-DHL-8 cell line with the Puma peptide showed very limited response, but was able to respond to administration of the activator peptides Bim and Bid. This result suggested that Bax and Bak were present and that the block was due to a failure inactivating the BH3 only proteins (Class A Block). SU-DHL-10 was unable to respond to the full panel of both activator and Sensitizer BH3 peptides, suggesting that the Bax and Bak effector arm was non-functional and that the cell line was dependent upon a Class B block for survival. (Deng et. al., 2007)

The ability to diagnose the mechanism of resistance to apoptosis activation was also demonstrated to be interesting from a clinical standpoint because it was shown to predict the response of this selection of lymphoma cell lines to ABT-737, a BH3 mimetic drug which inhibits Bcl-2 and Bcl-XL. (Van Delft et. al., 2006) Inhibition of each

protein by binding of the mimetic to the hydrophobic pocket allows for BH3 only proteins to be released where some members can activate Bax and Bak. So, while inhibition of Bcl-2 and Bcl-XL does not initiate pore formation directly, it allows for pore formation by releasing key players that are instrumental to the process. As mentioned above, it was predicted that SUDHL-4 was dependent on Bcl-2 while SU-DHL-6 was dependent upon both Bcl-2 and Mcl-1. Furthermore these two cell lines were predicted to be sensitive because of their mutual dependence on Bcl-2 for survival. SUDHL-8 and SUDHL-10 did not exhibit a Class C Block and thus were not expected to respond to ABT-737. As predicted, SU-DHL-4 and SU-DHL-6 were affected by ABT-737 (EC50 140 nM and 250 nM respectively) while the other two cell lines were unaffected by the drug, even at concentrations of greater than 10,000 nM. (Deng et. al., 2007) The relatively similar response of the sensitive cell lines despite SU-DHL-6's co-dependence on Mcl-1 might be explained by the fact that activator and sensitizer BH3 peptides displaced from Bcl-2 would have then been free to interact with Mcl-1, thus dealing with SU-DHL-6's simultaneous dependence on Mcl-1 without small molecule inhibition.

BH3 profiling has also been shown to be easily translated to preclinical studies of other diseases. Its application towards neuroblastoma, an aggressive pediatric cancer, allowed for the prediction of the mechanism by which three cell lines were able to evade apoptosis. These predictions were again confirmed by treatment of the cell lines with antagonists of different anti-apoptotic proteins. In addition to testing the ability of the technique to be applied to diseases other than lymphoma, these findings were also important because it showed that the technique was able to be easily translated to a solid tumor application. The assayed tumors were derived from xenograft models of the same

three cell lines which the authors initially profiled. The patterns observed in the mitochondria derived from a solid tumor environment were identical to the results when using the cultured versions of cell lines, providing precedence for expanding the clinical application of BH3 profiling beyond hematological malignancies. (Goldsmith et. al., 2009)

Clinical Applications

BH3 profiling's success in preclinical contexts has paved the way for the technique to be translated for clinical implementation. Response to cytarabine treatment in acute myeloid leukemia has been studied through the dynamic lens that BH3 profiling provides. This study revealed that strong response of patient samples to BH3 peptides Puma and Bim correlated with response to cytarabine based therapy. This correlation was strengthened by performing a multivariate analysis of different clinical factors including response to BH3 peptides, patient age, and cytogenetic status. Interestingly, mitochondrial depolarization by Bim and Puma, two peptides that function as a part of the activator class of BH3 probes, were correlated to patient response, whereas peptides classified as sensitizers did not cause this depolarization in samples from responsive patients. (Pierceall et. al., 2013) This observation provides evidence in support of the fact that those patients that were able to respond to cytarabine based treatment, had an intact Bax/Bak effector arm, ruling out this mechanism for malignant cell resistance to apoptosis. (Gallene et. al., 2009; Dai et. al., 2014)

BH3 profiling is also currently involved in a clinical trial which will aim to use the technique to predict response to ABT-199 in acute myeloid leukemia. Other studies of the method in hematologic malignancies include predicting the response of patients to

allogenic stem cell transplants in order to exclude patients which might develop graft versus host disease. (A. Letai, personal communication)

POTENTIAL APPLICATIONS OF BH3 PROFILING

Basic Applications

Although BH3 profiling is now being developed as a clinical tool, it is possible that the technique could be applied in many different basic and translational settings. Despite the fact that the intrinsic pathway of apoptosis and the Bcl-2 family of proteins is relatively well characterized (compared to death processes such as autophagy, or necrosis), many aspects of the process remain unclear to researchers. It is possible that there are undiscovered BH3 proteins within the family. (Letai et. al., 2008) The low number of conserved residues within the BH3 domains of these proteins makes finding potential members quite difficult to achieve by genetic or bioinformatic means. BH3 profiling however, might present a useful approach to this problem by providing a high throughput phenotypic assay to test the ability of each candidate gene to induce apoptosis. Potential hits to be screened by this method could be identified by looking for tentative BH3 domains within the human genome. This approach would result in a very large number of peptides but, imposing restrictions on the allowed characteristics of the domains would allow for the number of candidates to be winnowed down. In this case restriction of the core composition, by including mostly basic amino acids (as found in the cores of most BH3 only proteins) (Figure) as well as allowing only the amino acids with the highest propensity for alpha helix formation to be included in the regions flanking the core. Any predicted proteins that share sequence homology with a peptide that is able to change the mitochondrial membrane potential could be further verified through a yeast two hybrid system to detect their interactions with multidomain proteins.

Thus proteins that make it through these two screens would very likely be new members of the BH3 only class of proteins.

In addition to verifying potential members of the Bcl-2 family, the technique might also be used to study subtleties in how Bcl-2 family members function. For example it has been suggested that differences in specific regions of the Bax and Bak proteins may translate into differences in ion selectivity of the pores formed by dimers of the channels. (Petros et. al., 2003) This question might be probed by using cells with knock downs of either protein and assessing the ability of either homo-oligomer to dissipate the mitchondrial membrane potential. With this technique we could not only see how strongly the ion gradient is dissipated but also how quickly it is dissipated over time, using the plate based profiling method. Assessment of the characteristics of the pore formed by hetero-oligomers of Bax and Bak might be more complicated to achieve, but may be possible through a dual knockdown of the two native proteins followed by a reintroduction of a Bax/Bak fusion protein. This would ensure that the only pores formed would be composed of hetero-oligomers, assuming that diffusion after activation is the only force driving oligomer formation.

Additionally, mutational studies of the pore channel could be carried out in order to assess for possible mutations that significantly decrease the permeability of the pore, negatively affecting its ability to dissipate the mitochondrial membrane potential, and to release apoptotic factors. Changes in pore conductance could be reflected in the measurement of the mitochondrial membrane potential. This type of mutation may represent an alternative route to pathogenesis as it would create an inability to initiate apoptosis.

BH3 profiling might also prove to be a useful tool in the further elucidation of pathways which lead lead to apoptosis. The Unfolded Protein Response is also an especially good candidate pathway to be studied via BH3 profiling because of the pre-apoptotic localization of some Bcl-2 family members to the ER. In addition to this, the ER has been suggested in several studies to be a critical checkpoint leading to the initiation of the intrinsic apoptotic pathway. (Szegezdi et. al., 2009) Systems in which mouse embryonic fibroblasts have been modified to only express Bak localized to the endoplasmic reticulum, without endogenous Bax/Bak expression at the mitochondria, have been used to study the role of BH3 proteins in the activation of apoptosis via signals from the ER. (Klee et. al., 2009) This model system demonstrated that activation of Bak at the ER was sufficient for the release of cytochrome C from the mitochondria, demonstrating an alternative mode of death. With this fact in mind, we might expect that blocks in apoptosis could occur at the endoplasmic reticulum and give rise to malignant cells. This question might be probed with BH3 peptides which are modified with ER localization signals, so that apoptosis is not induced directly at the mitochondria. Specifically we could utilize a modified version of the Bim BH3 peptide which, as an activator, can directly interact with Bax and Bak to promote apoptosis. Failure of this peptide to induce apoptosis might mean that the cells we are probing are experiencing a Class C block (Loss of the Effector Arm) at the level of the Endoplasmic Reticulum. One could carry out this type of study in cells which have not been genetically manipulated because ER localization signals on the BH3 peptides would prevent the initiation of apoptotic mechanisms at the mitochondrial level.

Translational Applications

BH3 profiling might also prove to be a useful tool to study therapeutic approaches to hematological malignancies. In the context of lymphoma, a relatively new therapeutic approach is the activation of the unfolded protein response via significant ER stress, followed by transition to apoptotic pathways due to the inability to relieve that stress. This method of treatment is currently under study in hematological malignancies including multiple myeloma and diffuse large B-cell lymphoma. (Santos et. al., 2012, Amengual et. al., 2013) In these two diseases drug combinations which effectively inhibit two important protein degradation pathways, the proteasome and the aggresome, serve to overload the cell with mis-folded proteins. Inhibition of the aggresome is accomplished by administration of the HDAC6 inhibitor ACY-1215 and results in the increased presence of CHOP in models representing diffuse large B-cell lymphoma, presumably due to activated PERK, a transmembrane protein which provides upstream regulation for the transcription of this protein. (Amengual et. al., 2013) CHOP is a transcription factor with many roles in the unfolded protein response, one of which is to induce death via the down regulation of the Bcl-2 protein. (McCullough et. al., 2001)

Inhibition of the proteasome is caused by the presence of bortezomib which has the ability to disable the UPR by inhibition at multiple points in the pathway including destabilization of the pro-survival XBP-1s mRNA transcript (the active splice variant of this transcription factor), in addition to its diminishing the activity of its upstream regulator, IRE-1, which can then activate the JNK signaling cascade. (Lee et. al., 2003) The JNK pathway can also lead to cell death, through p53 mediated induction of BAX. (Zhang and Kaufman, 2005) The actions of both drugs induce death via the manipulation

of UPR machinery, although the connection of how this is achieved might be made clearer. A similar approach to BH3 profiling might be utilized to test the hypothesis that the JNK pathway represents a link between the ER stress caused by the combination therapy and apoptosis.

The Bcl-2 family is not only present in the mitochondria, but also acts in the ER and may serve to regulate members of the UPR. (Szegezdi et. al, 2009) Of particular interest in the context of the ACY-1215 and bortezomib story is the fact that the reticular BH3 only protein Bim activates IRE-1 mediated JNK-P signaling. (Klee et. al., 2009) It may therefore be possible to test for the role of JNK signaling in bortezomib/ACY-1215 induced apoptosis. Treatment of cells with a combination of ACY-1215 and a reticular Bim BH3 peptide could be assayed via change in mitochondrial membrane potential. This could be compared to ACY-1215 alone and ACY-1215 with bortezomib, to see if treatment with the Bim peptide induced a larger change in the potential or a similar change in the potential, respectively. After Bim induced activation of JNK signaling (and subsequent apoptosis) was demonstrated, the effect of JNK induction could be confirmed through inhibition or knockdown of JNK in the presence of the reticular Bim BH3 peptide and bortezomib, both as single agents, and in combination with ACY-1215. This type of experiment would provide mechanistic insight into the actions of currently used therapeutics through phenotypically relevant assay.

BH3 profiling might also be utilized to quickly validate newly proposed drug combinations using either of these therapeutic candidates. New candidates to be administered in combination with ACY-1215 could be suggested based on the BH3 profiles of cell models/patient samples which have been previously treated with the drug.

BH3 peptides with the highest efficacy in combination with ACY-1215 would reveal appropriate therapeutic targets to be combined with the inhibition of HDAC6. For example, ACY-1215's ability to induce CHOP and concomitantly decrease Bcl-2 expression would lead one to believe that BH3 peptides that act on Bcl-2 would be the most effective in pre-treated cells dependent upon this survival protein. If this were the case, further studies might be done with ACY-1215 in combination with a Bcl-2 inhibitor like ABT-199 because both agents attenuate the actions of pro-survival Bcl-2. (Souers et. al., 2013) For this combination, we may have been able to hypothesize that the two drugs would have been effective without the use of BH3 profiling, but the information which led us to this conclusion would not be available for most drugs. Studying the combination of ACY-1215 and ABT-199 would be important because of its ability to serve as a model system to validate BH3 profiling's ability to predict efficacious drug combinations. The approach could then be extended to other therapeutics such as bortezomib and beyond.

BH3 profiling could potentially have further application as a translational research tool which could drastically improve the efficiency of preclinical and phase 1 clinical studies by serving as a biomarker for response. One important use of the method might be to assess the validity of different disease models. For example, in lymphoma many different cell lines exist which are meant to represent different forms, subtypes, and variants of lymphoma. (Deng et. al., 2007) If these in vitro model systems responded to a panel of effector peptides in manner similar to tissue samples from current patients, their validity as a model system would be verified. This same approach could be extended to in vivo models such as xenograft mouse models. By having more effective study models,

it is reasonable to assume that the effects of preclinical drugs assayed in these models would have biological effects that are closer representations of their activity in humans. This would potentially help to ease the high cost of drug development. In the same manner, it would also be possible to use BH3 profiling as a method to determine the therapeutic index for tumor tissue and normal tissue. To be able to contrast the response of each of these types of tissue to the same drug would be invaluable in guiding the selection of an appropriate therapeutic dose which could maximize the effect of the drug on tumor tissue, while minimizing side effects on normal tissue.

BH3 profiling might also be able to perform an important role in rational drug design. As previously mentioned therapeutics such as ABT-737, mimic specific BH3 domains in order to inhibit multidomain apoptotic proteins such as Bcl2. Petros et. al have made and verified predictions as to how they can make interactions between the BH3 domain of BH3 only proteins and the multidomain apoptotic proteins have a higher affinity. (2003) This was accomplished with the Bcl-XL protein and Bad peptide. Structural analysis of the interaction helix formed by the Bad peptide revealed two negatively charged residues whose proximity was predicted to cause a repulsion (due to charge) that disfavored helix formation. This hypothesis was tested through mutational studies by removing each of the negatively charged residues, resulting in a 100 fold increase in the affinity of the Bad peptide for the Bcl-XL protein. These types of studies might further enhance drug design of BH3 mimetic small molecules, by mimicking the optimized peptide as opposed to the natural peptide. BH3 profiling would play an important role in these types of studies because it would assay the ability of the optimized peptide to be able to induce apoptosis through interaction with a multidomain anti-

apoptotic protein as this ability may have been compromised during the optimization process.

BH3 profiling may also be modified so that post translational modifications of BH3 domains might be represented. To some extent this has already been done with the BH3 only protein Bid, whose cleaved TBid form was used as a positive control as previously discussed in this review. (Goldsmith et. al, 2009) Additionally, in clinical applications, this truncated form might be administered to cells or isolated mitochondria of patient samples to provide information as to whether the cells are more susceptible to therapeutics which work exclusively via the intrinsic pathway, or whether activation of the pathway through the crosstalk of caspase 8 via death receptor activation would be a better course of action. N-Myristoylation of this peptide might also provide more physiologically relevant results.(Puthalakath and Strasser, 2002)

Phosphorylation of BH3 peptides could be another modifiable parameter of the technique. As mentioned previously, Serine 155 is a residue within the BH3 region of the Bad protein and thus is present within the effector peptide used in the assay. This serine residue is a site of phosphorylation by PKA. It has been shown that phosphorylation of this residue contributes to the abrogation of Bad's pro-apoptotic activity. (Virdee et. al, 2000) The modulation of Bad's activity in this manner might have important implications in the interpretation of the response to administration of the Bad effector peptide to patient samples. At the very least, the phosphorylation state of the effector peptide should match the state of the tumor's phosphorylation site within the BH3 domain.

Bim is another BH3 only protein which is regulated by phosphorylation. In its phosphorylated state, Bim is unstable and is susceptible to degradation. However Bim can be stabilized when dephosphorylated by PP2A in response to endoplasmic reticulum stress. This results in Bim being able to more efficiently induce death. Although the phosphorylation site may not exist naturally within the BH3 domain of the protein, it might be added to tyrosines which exist within this domain. This modification would give experimenters the ability to manipulate the half-life of this peptide within a whole cell system, which could provide insights into the nuances of Bim presence in terms of time and levels achieved. (Puthalakath et. al., 2007)

CONCLUSION

BH3 profiling is a rapid and easy technique which is significant to clinical application and is also a technique which continues to evolve to be applied to new contexts. This ability to easily probe a complex set of inter-family protein interactions is a remarkable accomplishment and provides an efficient companion diagnostic to inform treatment options in different hematological malignancies, with the potential for being developed for solid tumor applications for the clinic.

The need for reliable diagnostics has been acknowledged, especially in the arena of malignant lymphomas. The disease states of this particular hematopathy are difficult to diagnose and subsequently difficult to treat. While some diagnostics have been developed using PCR based methods to detect chromosomal translocations, these genetic characteristics may not be the best method to identify treatment options because genetic changes do not necessarily reveal themselves as an expected phenotype. (Van Kreiken et. al., 2007) BH3 profiling offers an advantage over genetically based diagnostic techniques in that it provides both, a physiologic representation of how close malignant cells are to the apoptotic cliff, as well as answers in regards to the sources of the intrinsic/acquired resistance to cell death mechanisms. It is also faster and cheaper.

BH3 profiling's ability to provide physiologic feedback in response to the inhibition of potential drug targets has allowed for this diagnostic to fill a market niche as outlined in the FDA's 2011 roadmap announcement. In this request, the government agency made a call "for companion tests that will improve treatment efficacies by selecting appropriate use of targeted oncology therapies based on predictive molecular discriminators".(Eutropics Pharmaceuticals, 2012) The desire for this type of diagnostic

has allowed Eutropics pharmaceuticals, a company founded based on the technology of BH3 profiling, to partner with various academic and government institutions to validate the method in the clinical context. Having already demonstrated the ability to live up to its expectations in clinical trials for acute myeloid leukemia, along with the likelihood that it will do the same in future clinical opportunities, BH3 profiling has emerged as a dynamic technique which is able to provide a simple physiologic endpoint in the analysis of a complicated biological network.

References

Adams JM and Cory S (1998). The Bcl-2 protein family: arbiters of cell survival. Science 281, 1322-6

Adams JM and Cory S (2007). The Bcl-2 apoptotic switch in cancer development and therapy. Oncogene 26,1324-37.

Borner C, Martinou I, Mattmann C, Irmler M, Schaerer E, Martinou JC, and Tschopp J (1994). The protein bcl-2 alpha does not require membrane attachment, but two conserved domains to suppress apoptosis. J Cell Biol. 126, 1059-68.

Bouillet P, Cory S, Zhang LC, Strasser A, and Adams JM (2001). Degenerative disorders caused by Bcl-2 deficiency prevented by loss of its BH3-only antagonist Bim. Dev. Cell,1, 645-53.

Certo M, Del Gaizo Moore V, Nishino M, Wei G, Korsmeyer S, Armstrong SA, and Letai A (2006). Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. Cancer Cell 9,351-65.

Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, Colman PM, Day CL, Adams JM, and Huang DC (2005). Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell 17, 393-403.

Coultas L and Strasser A (2003). The role of the Bcl-2 protein family in cancer. Semin. Cancer Biol. 13,115-23.

Dai H, Pang YP, Ramirez-Alvarado M, and Kaufmann SH (2014). Evaluation of the BH3-only Protein Puma as a Direct Bak Activator. J Biol Chem. 289, 89-99.

Del Gaizo Moore V and Letai A (2013). BH3 profiling--measuring integrated function of the mitochondrial apoptotic pathway to predict cell fate decisions. Cancer Lett. 332, 202-5.

Deng J, Carlson N, Takeyama K, Dal Cin P, Shipp M, and Letai A (2007). BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. Cancer Cell. 12, 171-85.

Eutropics Pharmaceuticals (2012). Eutropics Pharmaceuticals signs $1.5M NCI/SBIR contract to develop companion diagnostic for guiding Multiple Myeloma treatments. Retrieved March 21, 2014, from http://www.prnewswire.com/news-releases/eutropics-pharmaceuticals-signs-15m-ncisbir-contract-to-develop-companion-diagnostic-for-guiding-multiple-myeloma-treatments-170525636.html

Gallenne T, Gautier F, Oliver L, Hervouet E, Noël B, Hickman JA, Geneste O, Cartron PF, Vallette FM, Manon S, et. al. (2009). Bax activation by the BH3-only protein Puma promotes cell dependence on antiapoptotic Bcl-2 family members. J Cell Biol. 185, 279-90.

Goldsmith KC, Lestini BJ, Gross M, Ip L, Bhumbla A, Zhang X, Zhao H, Liu X, and Hogarty MD (2010). BH3 response profiles from neuroblastoma mitochondria predict activity of small molecule Bcl-2 family antagonists. Cell Death Differ. 17, 872-82.

Gottlieb RA and Granville DJ (2002). Analyzing mitochondrial changes during apoptosis. Methods. 26, :341-7.

Hanahan D and Weinberg RA (2000). The hallmarks of cancer. Cell. 100, 57-70.

Heiser D, Labi V, Erlacher M, and Villunger A (2004). The Bcl-2 protein family and its role in the development of neoplastic disease. Exp Gerontol. 39, 1125-35. Hsu SY, Kaipia A, McGee E, Lomeli M, and Hsueh AJ (1997). Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. Proc Natl Acad Sci U S A. 94, 12401-6.

Klee M, Pallauf K, Alcalá S, Fleischer A, and Pimentel-Muiños FX (2009). Mitochondrial apoptosis induced by BH3-only molecules in the exclusive presence of endoplasmic reticular Bak. EMBO J. 281, 757-68.

Krajewski S, Tanaka S, Takayama S, Schibler MJ, and Fenton W, Reed JC (1993). Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. Cancer Res. 53, 4701-14.

Lee AH, Iwakoshi NN, Anderson KC, and Glimcher LH (2003). Proteasome inhibitors disrupt the unfolded protein response in myeloma cells. Proc Natl Acad Sci U S A. 100, 9946-51.

Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, and Korsmeyer SJ (2002). Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2, 183-92.

Letai AG (2008). Diagnosing and exploiting cancer's addiction to blocks in apoptosis. Nat Rev Cancer 8, 121-32.

Li H, Zhu H, Xu CJ, and Yuan J (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 94, 491-501.

Lutter M, Fang M, Luo X, Nishijima M, Xie X, and Wang X (2000). Cardiolipin provides specificity for targeting of tBid to mitochondria. Nat Cell Biol. 2, 754-61

Marani M, Tenev T, Hancock D, Downward J, and Lemoine NR (2002). Identification of novel isoforms of the BH3 domain protein Bim which directly activate Bax to trigger apoptosis. Mol Cell Biol. 22, 3577-89.

Mattson MP (2000). Apoptosis in neurodegenerative disorders. Nat Rev Mol Cell Biol. 1, 120-9.

McCullough KD, Martindale JL, Klotz LO, Aw TY, and Holbrook NJ (2001). Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. Mol Cell Biol. 21, 1249-59.

Michels J, O'Neill JW, Dallman CL, Mouzakiti A, Habens F, Brimmell M, Zhang KY, Craig RW, Marcusson EG, Johnson PW, et. al. (2004). Mcl-1 is required for Akata6 B-lymphoma cell survival and is converted to a cell death molecule by efficient caspase-mediated cleavage. Oncogene 23, 4818-27.

Petros AM, Nettesheim DG, Wang Y, Olejniczak ET, Meadows RP, Mack J, Swift K, Matayoshi ED, Zhang H, Thompson CB, et. al. (2000).Rationale for Bcl-xL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies. Protein Sci. 9, 2528-34.

Petros AM, Olejniczak ET, and Fesik SW (2004). Structural biology of the Bcl-2 family of proteins. Biochim Biophys Acta. 1644, 83-94.

Pierceall WE, Kornblau SM, Carlson NE, Huang X, Blake N, Lena R, Elashoff M, Konopleva M, Cardone MH, and Andreeff M (2013). BH3 profiling

discriminates response to cytarabine-based treatment of acute myelogenous leukemia. Mol Cancer Ther. 12, 2940-9.

Puthalakath H and Strasser A (2002). Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ. 9, 505-12.

Puthalakath H, O'Reilly LA, Gunn P, Lee L, Kelly PN, Huntington ND, Hughes PD, Michalak EM, McKimm-Breschkin J, Motoyama N, et. al. (2007). ER stress triggers apoptosis by activating BH3-only protein Bim. Cell 129, 1337-49.

Reed JC (1999). Dysregulation of apoptosis in cancer. J Clin Oncol. 17, 2941-53.

Ryan J and Letai A (2013). BH3 profiling in whole cells by fluorimeter or FACS. Methods 61, 156-64.

Santo L, Hideshima T, Kung AL, Tseng JC, Tamang D, Yang M, Jarpe M, van Duzer JH, Mazitschek R, Ogier WC, et. al. (2012). Preclinical activity, pharmacodynamic, and pharmacokinetic properties of a selective HDAC6 inhibitor, ACY-1215, in combination with bortezomib in multiple myeloma. Blood 119, 2579-89.

Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, et. al. (1997). Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science 275, 983-6.

Smiley ST, Reers M Mottola-Hartshorn C, Chen A, Lin M, and Chen LB (1995). Mitochondrial membrane potential monitored by JC-1 dye.Methods Enzymol. 260, 406-17.

Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, Dayton BD, Ding H, Enschede SH, Fairbrother WJ, et. al. (2013). ABT-199, a potent and selective

BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med. 19, 202-8.

Szegezdi E, Macdonald DC, Ní Chonghaile T, Gupta S, and Samali A (2009). Bcl-2 family on guard at the ER. Am J Physiol Cell Physiol. 296, 941-53.

Tsujimoto Y, Finger LR, Yunis J, Nowell PC, and Croce CM (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. Science 226, 1097-9.

Van Delft MF, Wei AH, Mason KD, Vandenberg CJ, Chen L, Czabotar PE, Willis SN, Scott CL, Day CL, Cory S, et. al. (2006). The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. Cancer Cell 10, 389-99.

Van Krieken JH, Langerak AW, Macintyre EA, Kneba M, Hodges E, Sanz RG, Morgan GJ, Parreira A, Molina TJ, Cabeçadas J, et. al. (2007). Improved reliability of lymphoma diagnostics via PCR-based clonality testing: report of the BIOMED-2 Concerted Action BHM4-CT98-3936. Leukemia 21, 201-6.

Veis DJ, Sorenson CM, Shutter JR, and Korsmeyer SJ (1993). Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. Cell 75, 229-40.

Virdee K, Parone PA, and Tolkovsky AM (2000). Phosphorylation of the pro-apoptotic protein BAD on serine 155, a novel site, contributes to cell survival. Curr Biol. 10, 1151-4.

Wajant H (2002). The Fas signaling pathway: more than a paradigm. Science 296, 1635-6.

Walczak H and Krammer PH (2000). The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. Exp Cell Res. 256, 58-66.

Wang K, Yin XM, Chao DT, Milliman CL, and Korsmeyer SJ (1996). BID: a novel BH3 domain-only death agonist. Genes Dev. 10, 2859-69.

Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, and Youle RJ (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol. 139, 1281-92.

Yamaguchi R, Andreyev A, Murphy AN, Perkins GA, Ellisman MH, and Newmeyer DD (2007). Mitochondria frozen with trehalose retain a number of biological functions and preserve outer membrane integrity. Cell Death Differ. 14, 616-24.

Yasukawa M, Bando S, Dölken G, Sada E, Yakushijin Y, Fujita S, and Makino H (2001). Low frequency of BCL-2/J(H) translocation in peripheral blood lymphocytes of healthy Japanese individuals. Blood 98, 486-8.

Zha J, Weiler S, Oh KJ, Wei MC, and Korsmeyer SJ (2000). Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. Science 290, 1761-5.

Zhang K and Kaufman RJ (2006). The unfolded protein response: a stress signaling pathway critical for health and disease. Neurology 66, 102-9.

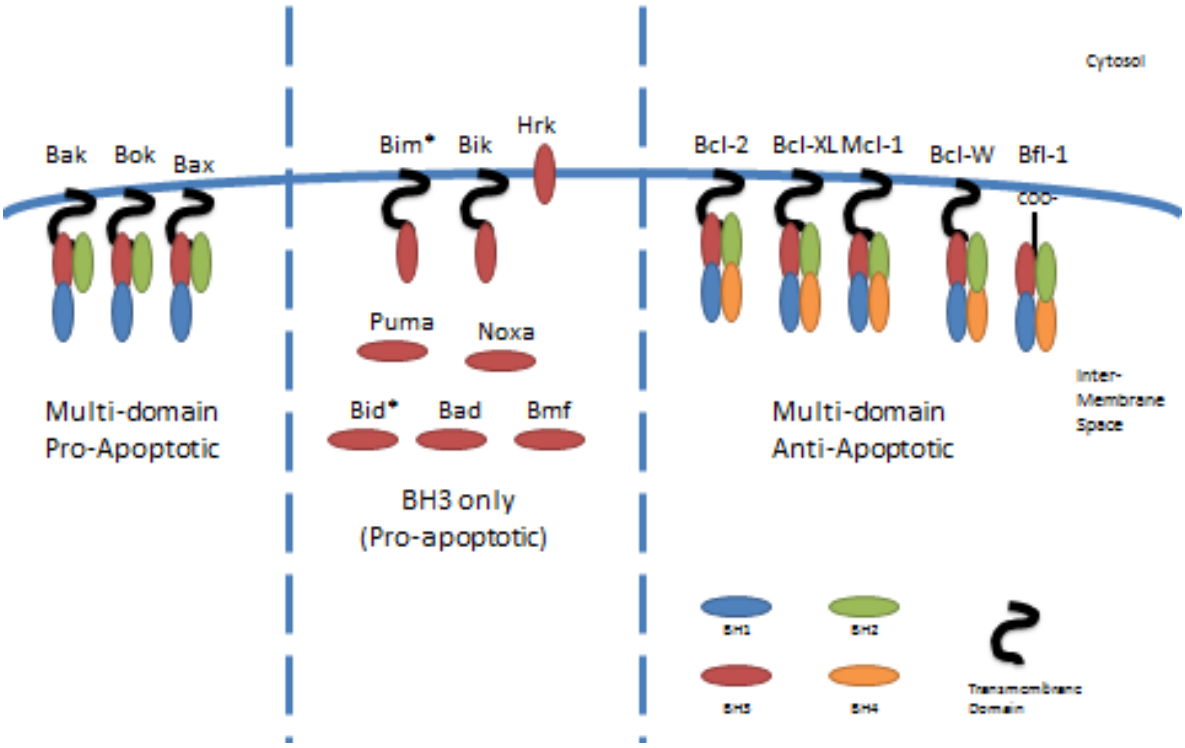

Figure 1. Classes of the Bcl-2 family. The Bcl-2 family of proteins contains three main classes of proteins. These classes are defined based on each protein's contribution towards preventing or supporting the activation of apoptosis. Proteins belonging to this family are identified by the presence of the BH3 domain, whose structural conformation allows for both multi-domain proteins and BH3 only proteins to interact with each other.

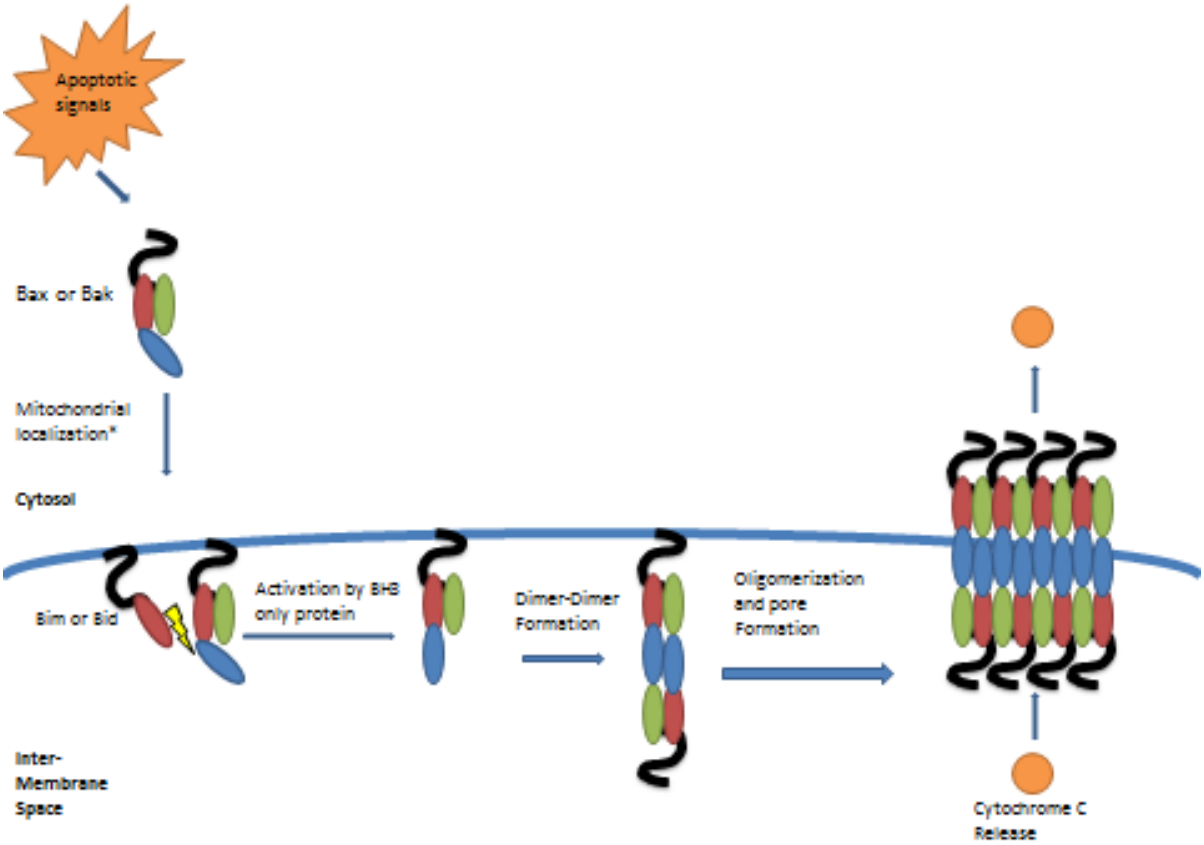

Figure 2. Activation of Apoptosis. The intrinsic pathway of apoptosis can be activated by cellular stress signals such as the unfolded protein response or by pharmacologic means. These upstream signals can result in the mitochondrial localization of Bax (Bak is constitutively located at the mitochondria) and activation of these proteins by Bim or Bid. Activated Bax and Bak molecules form homo- or hetero-dimers and these dimers eventually oligomerize, forming a pore in the outer mitochondrial membrane and allowing for the release of cytochrome C, SMAC, and DIABLO. Release of these factors is considered to be the point at which the cell has committed to apoptosis.

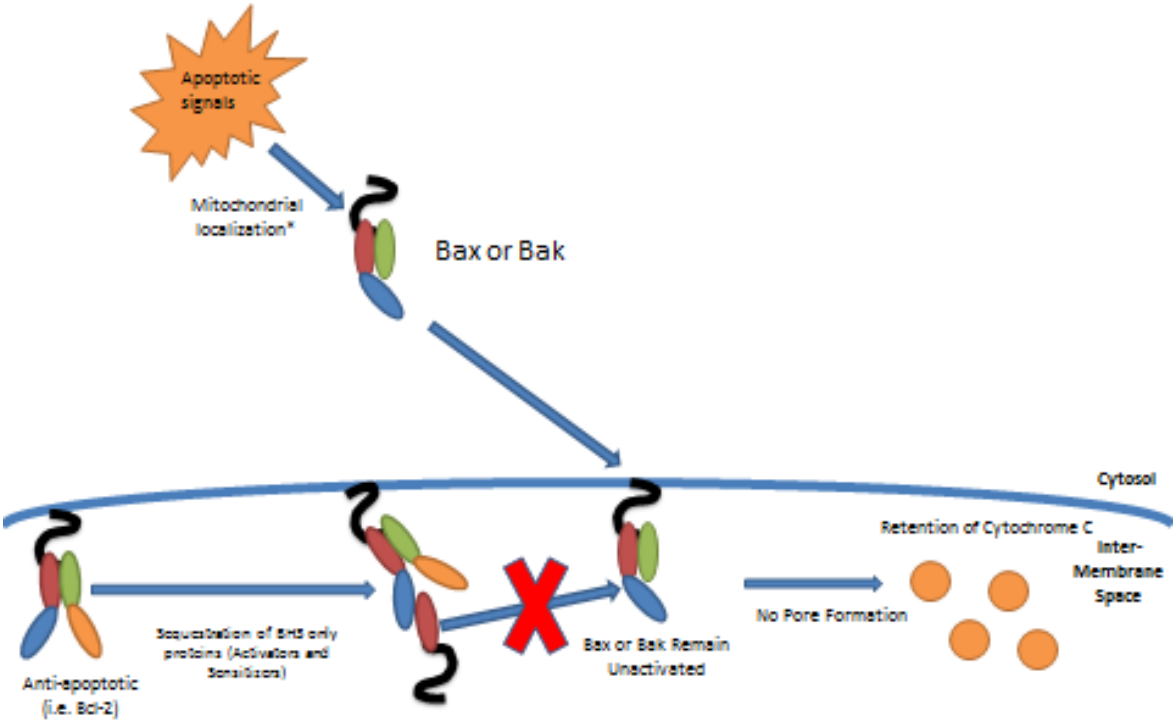

Figure 3. Preventing the activation of apoptosis. Regulation of apoptosis activation is crucial to ensure that cells do not die prematurely. Multi-domain anti-apoptotic proteins such as Bcl-2 sequester proteins Bim and Bid, and prevent the activation of mitochondrially localized Bax and Bak, thereby preventing apoptosis. Sequestration of Bim and Bid can be overcome by the presence of decoys termed sensitizers (not shown) which can occupy multi-domain anti-apoptotic proteins, freeing Bim and Bid, thus allowing for apoptosis to occur.

	BIM*	BID*	PUMA	NOXA	BMF	BIK	BAD	HRK
BCL-2	<10	66+/-6	18+/-1	-	24+/-1	151+/-2	11+/-3	-
BCL-XL	<10	12+/-9	<10	-	<10	10+/-2	<10	92+/-11
BCL-W	38+/-7	<10	25+/-12	-	11+/-3	17+/-12	60+/-19	-
MCL-1	<10	<10	<10	19+/-2	23+/-2	109+/-33	-	-
BFL-1	73+/-3	53+/-3	59+/-11	-	-	-	-	-

Figure 4. Binding affinities between activators/sensitizers and anti-apoptotic multidomain proteins. BH3 only peptides run along the top row and grouped as activators and sensitizers. * denotes the activator state of the peptide (PUMA's status as an activator is curently under debate) Kd of interactions is displayed in uM concentrations as reported by Certo et. Al., 2006. Red Boxes denote strong interactions, yellow denotes medium interactions, and green shows that there is no association between those two proteins at physiologic concentrations.

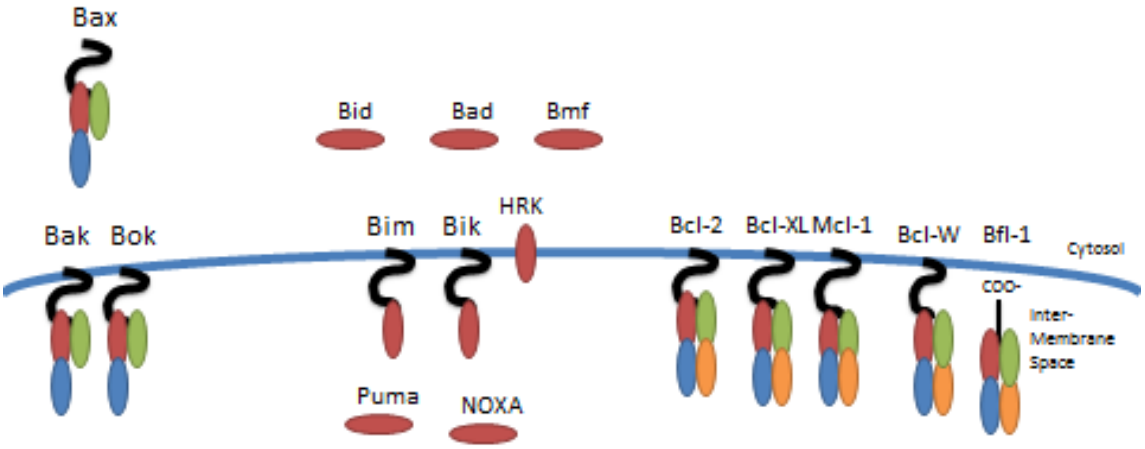

Figure 5. Localizations of the Bcl-2 family of proteins in healthy cells. While many members of the Bcl-2 family can be found in the mitochondria of the cell, some pools of these proteins may also be found in the cytosol where they are mostly associated with cytoskeleton elements. Additionally, some family members such as Bcl-2 and Bim can be found at the Endoplasmic Reticulum where they have been implicated in allowing for the transition of activation of the unfolded protein response to the activation of the intrinsic apoptotic pathway.

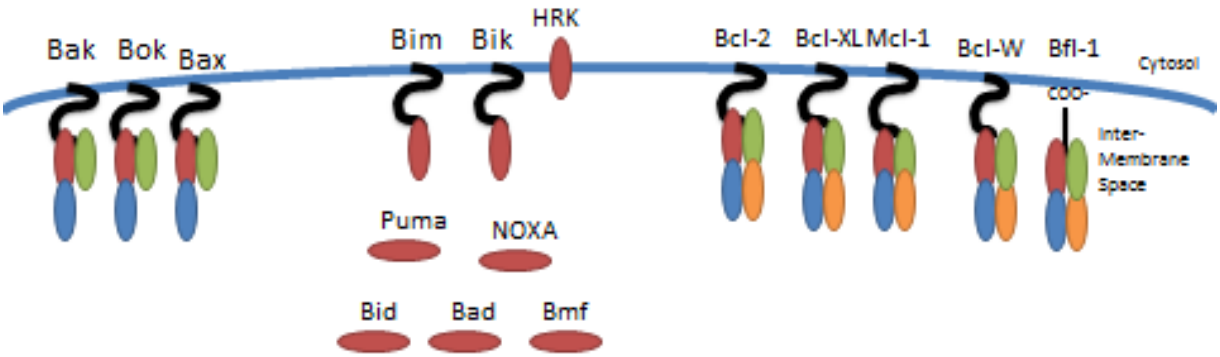

Figure 6. Localizations of the Bcl-2 family of proteins in primed cells. Upon activation of the intrinsic apoptotic pathway Bcl-2 protein family members localize to the inter-membrane space (if they are not already located there). From here proteins can interact as determined by their affinities and either commit to apoptosis or become more primed for apoptosis.

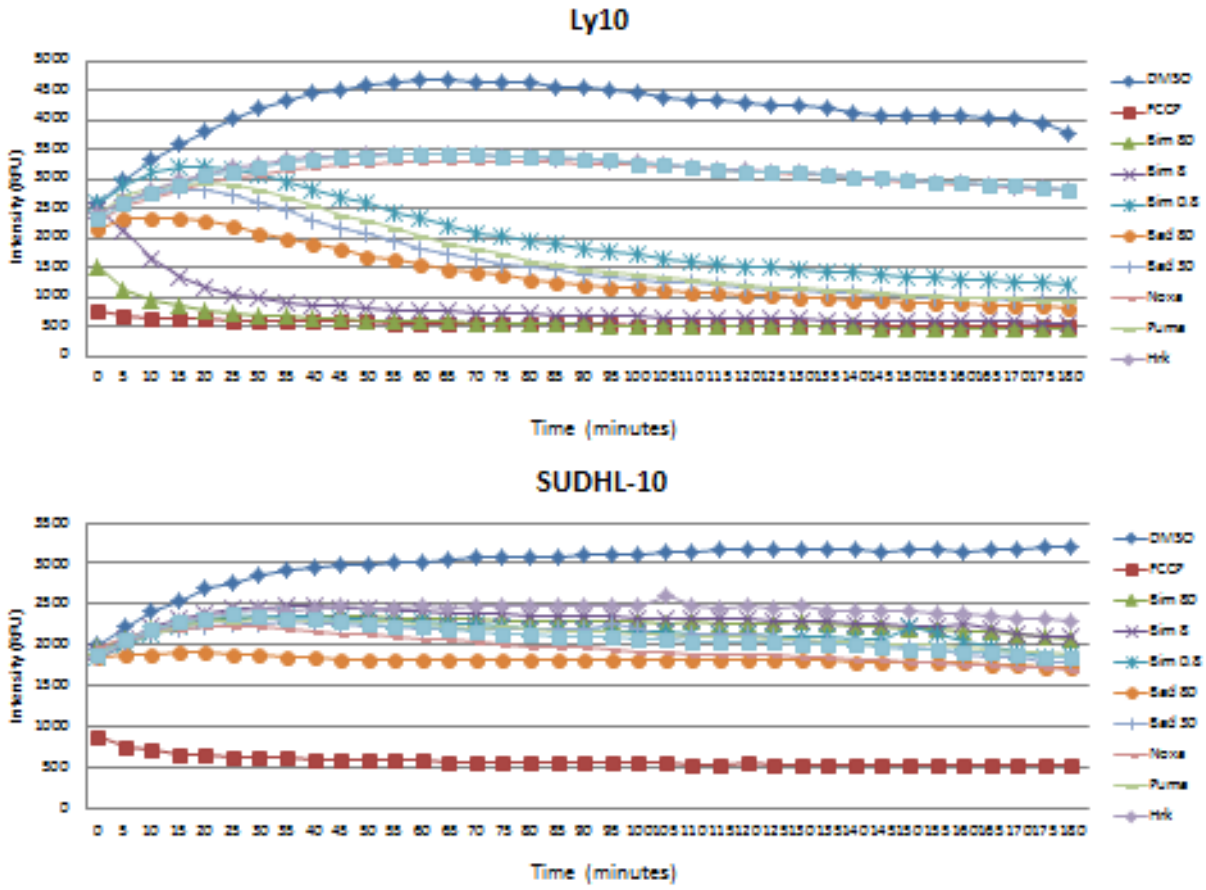

Figure 7. Kinetic trace of cellular response to BH3 only peptides. Response curves show the effect of peptide administration on mitochondrial membrane potential as measured by fluorescence intensity over time. Ly10 cell lines demonstrate a combined dependence on Bcl-2 and Bcl-W based on their response to the BAD peptide and lack response to the NOXA and HRK peptides. SUDHL-10 represents a cell line which is lacking BAX and BAK by virtue of the fact that the cell line does not depolarize in response to administration of any of the peptides. All peptide concentrations are administrated at 80 uM concentrations unless otherwise state