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DISSECTION OF THE BCL-2 FAMILY SIGNALING NETWORK WITH STABILIZED α -HELICES OF BCL-2 DOMAINS

Kenneth Pitter, Federico Bernal, James LaBelle, and Loren D. Walensky

Department of Pediatric Oncology and the Program in Cancer Chemical Biology, Dana-Farber Cancer Institute, and the Division of Hematology/Oncology, Children's Hospital Boston, Harvard Medical School, Boston, Massachusetts

Abstract

The BCL-2 family of apoptotic proteins regulates the critical balance between cellular life and death and, thus, has become the focus of intensive basic science inquiry and a fundamental target for therapeutic development in oncology and other diseases. Classified based on the presence of conserved α -helical motifs and pro- and anti-apoptotic functionalities, BCL-2 proteins participate in a complex interaction network that determines cellular fate. The identification of BCL-2 homology domain 3 (BH3) as a critical death helix that engages and regulates BCL-2 family proteins has inspired the development of molecular tools to decode and drug the interaction network. Stabilized α -Helices of BCL-2 domains (SAHBs) are structurally reinforced, protease-resistant, and cell-permeable compounds that retain the specificity of native BH3 death ligands and, therefore, serve as ideal reagents to dissect BCL-2 family interactions *in vitro* and *in vivo*. Here, we describe the *in vitro* and cell-based methods that exploit SAHB compounds to determine the functional consequences of BH3 interactions in regulating apoptosis.

1. INTRODUCTION

The discovery of BCL-2 at the chromosomal breakpoint of t(14;18) (q32;q21) lymphomas (Bakhshi et al., 1985; Cleary and Sklar, 1985; Tsujimoto et al., 1985) led to a paradigm shift in our understanding of the origins of cancer. BCL-2 was initially defined as a survival protein capable of prolonging cellular life by evading programmed cell death or apoptosis (Hockenbery et al., 1990; Nunez et al., 1990; Vaux et al., 1988). In follicular lymphoma, the aberrant subjugation of BCL-2 to the transcriptional control of the immunoglobulin heavy chain locus leads to BCL-2 overexpression, a primary oncogenic event responsible for pathologic B-cell survival (McDonnell et al., 1989; Seto et al., 1988). Since this seminal discovery, a growing family of BCL-2-like proteins has been identified and subclassified based on the presence of conserved helical motifs and pro- and anti-apoptotic functionalities (Danial and Korsmeyer, 2004; Youle and Strasser, 2008) (Figure. 23.1). What has emerged is a complex protein-interaction network of guardians and executioners that determine cellular fate. Structural and biochemical studies identified the BCL-2 homology domain 3 (BH3) as a critical α -helical motif that engages BCL-2 family targets to regulate their activities (Cheng et al., 2001; Sattler et al., 1997; Zha et al., 1996). Thus, understanding the selectivities and functional activities of discrete BH3 domains remains essential to decoding the BCL-2 family network. To that end, we have developed a chemical toolbox of Stabilized α -Helices of BCL-2 domains (SAHBs) that preserve the primary and secondary

structural fidelity of native BH3 domains for biologic study *in vitro* and *in vivo* (Walensky et al., 2004; 2006). Here we describe the application of SAHB compounds to cell death studies focused on discerning the functional consequences of selective BH3 domain interactions.

2. BCL-2 FAMILY BINDING MEASUREMENTS BY FLUORESCENCE POLARIZATION ASSAY

Despite the major advances stemming from apoptosis studies, many critical facets of BCL-2 family death signaling—including the regulation of essential cell death executioners such as BAX—remain mechanistic mysteries. *In vitro* binding assays serve as a starting point for defining BH3 domain interactions. The resultant quantitative affinity data provide a framework for hierarchical ranking of interaction pairs, which in turn forms the basis for hypotheses regarding potential functions of BH3 interactions *in situ*.

The quality and reproducibility of *in vitro* binding assays explicitly depend on the design and purity of expressed proteins. For example, the potential impact of fusion proteins and tags should be considered and evaluated independently in negative control binding studies; ideally, tagless proteins should be used, although this may not be feasible in all circumstances because of protein solubility, stability, or recovery challenges. In the case of BCL-2 family proteins, the carboxy termini of proteins are typically removed (C) to facilitate solubilization and purification; however, results with such constructs must be interpreted with care, because these sequences could participate in the regulation of native protein interactions. Whenever comparing binding affinities of a particular BH3 domain across distinct BCL-2 family proteins, ideally, truncated constructs should be compared with truncated constructs, and full-length proteins compared with full-length proteins. It is also worth noting that different types of binding assays can produce quantitatively distinct data sets. For example, surface plasmon resonance (SPR), which involves immobilization of either ligand or target, typically yields lower K_d values than fluorescence polarization assays (FPA), which are performed in solution. By orienting immobilized ligand or protein target on a surface, binding interactions may be facilitated compared with FPA, in which there is a greater entropic cost to aligning ligand and target in solution.

For C anti-apoptotic proteins, we use pGEX vectors to express GST-BCL-2, BCL-X_L, BCL-w, MCL-1, and BFL1/A1, followed by thrombin cleavage and FPLC-based gel filtration chromatography. For full-length anti-apoptotic proteins, pET22 vectors are constructed and expressed protein purified by Ni²⁺ column chromatography and gel filtration. For pro-apoptotic BAX, we use the pTYB1 vector to generate C and full-length BAX chitin-binding fusion proteins, followed by chitin affinity chromatography, DTT-based chitin cleavage, and FPLC purification (Suzuki et al., 2000).

The fluorescence polarization binding assay uses N-terminal fluoresceinated SAHBs to determine the binding affinities of SAHBs for multi-BH domain BCL-2 family member proteins (Figure. 23.2). The technique measures the change in polarization of light that results from a freely mobile and tumbling fluoresceinated molecule engaging a larger protein, which then slows and orients the tumbling of the fluoresceinated moiety in solution. When comparing the affinity of a ligand to a panel of proteins, it is important to note that larger proteins will yield greater absolute changes in polarization than smaller proteins, but

this does not affect affinity calculations, unless the target protein is too small to generate a change in polarization when bound. Serial dilutions of protein in 50 mM Tris pH 8, 100 nM NaCl are instilled in 96-well black Costar plates (Costar #3915) to assay ligand binding over a broad range of protein concentrations. FITC-SAHB stocks are stored as a lyophilized powder at -20°C in tinted containers, and aliquots reconstituted in 100% DMSO to yield a 1 mM stock. For a final peptide concentration of 25 nM, stock FITC-SAHB compound is diluted stepwise into water to yield a 0.5 μM solution (0.1% DMSO) and then 10 μl is repeat-pipetted into each well for a final assay volume of 200 μl . Depending on the brightness of the FITC-ligand, lower or higher concentrations may be used, but the typical range is 10 to 50 nM. Each condition is run in at least triplicate, including replicates that contain no protein so that the fluorimeter can be calibrated against the FITC-ligand alone. Once the FITC-SAHB is added, the plates are incubated in the dark at room temperature until equilibrium is reached. The time to equilibrium is initially determined by monitoring the binding isotherms over time to assess stabilization of binding activity. Fluorescence polarization (mP units) is measured on a Perkin-Elmer LS50B luminescence spectrophotometer equipped with cuvette containing a stir bar, a Spectramax M5 Microplate Reader (Molecular Devices), a BMG POLARstar Optima, or similar device. EC_{50} and K_d values are calculated by nonlinear regression analysis of dose-response curves with Prism software 4.0 (Graphpad). Care must be taken to provide sufficient data points to clearly define both the baseline and maximal mP values, so that the curve fit is statistically meaningful. When the total concentration of fluorescent ligand, L_T , is less than K_d and the assumption $L_T \ll L_{\text{free}}$ applied, binding isotherms are fitted to Eq. (23.1):

$$P = P_f + \left[(P_b - P_f) \times \frac{R_T}{K_D + R_T} \right] \quad (23.1)$$

where P is the measured polarization value, P_f is the polarization of free fluorescent ligand, P_b is the polarization of bound ligand, and R_T is the receptor/protein concentration. However, when $L_T > K_d$, the assumption that $L_T \ll L_{\text{free}}$ does not hold because of ligand depletion. As such, binding isotherms are fitted to the more explicit Eq. (23.2):

$$P = P_f + (P_b - P_f) \left[\frac{(L_T + K_D + R_T) - \sqrt{(L_T + K_D + R_T)^2 - 4L_T R_T}}{2L_T} \right] \quad (23.2)$$

where P is the measured polarization value, P_f is the polarization of free fluorescent ligand, P_b is the polarization of bound ligand, L_T is the total concentration of fluorescent ligand, and R_T is the receptor/protein concentration (Copeland, 2000). Each data point represents the average of an experimental condition performed in at least triplicate.

3. *IN VITRO* RELEASE ASSAYS AS A MEASURE OF PRO-APOPTOTIC ACTIVITY

Mitochondrial apoptosis is induced by the oligomerization of BAX and BAK, which are believed to form a yet uncharacterized pore that enables release of apoptogenic

mitochondrial contents (Annis et al., 2005; Goping et al., 1998; Gross et al., 1998). How BAX and BAK are activated, either directly by selective BH3 engagement (“direct activation”) or indirectly by BH3-mediated inhibition of anti-apoptotics (“derepression”), or both, remains actively debated in the apoptosis field (Kim et al., 2006; Kuwana et al., 2005; Letai et al., 2002; Walensky et al., 2006; Willis et al., 2007). Thus, release assays that probe the biophysical and biochemical characteristics of BAX/BAK are important tools for apoptosis researchers. Like any *in vitro* assay, the release assays presented here have benefits and limitations, yet provide a practical means for probing the capacity of select BCL-2 family proteins to activate or inhibit “release” by directly or indirectly regulating BAX/BAK.

3.1. Liposomal release assay

The purpose of the liposomal release assay is to simulate mitochondrial release but only with the minimal essential components for pro-apoptotic activation: lipid, release agent, and BCL-2 family protein/ligand of interest (Figure. 23.3A). The clear benefit of this reductionist assay is the ability to monitor membrane pore formation by a singular protein in response to a stimulus and in the absence of potentially confounding cellular factors, known or unknown. Despite the clarity of interpretation that this assay provides, it is certainly a giant step away from an isolated mitochondria, which in turn is many more giant steps away from an intracellular mitochondria. Nevertheless, the *in vitro* liposomal release assay has provided important insights into BAX/BAK physiology (Kuwana et al., 2002; 2005; Oh et al., 2006; Terrones et al., 2004; 2008; Walensky et al., 2006; Yethon et al., 2003), just as *in vitro* structural biology studies have shed enormous light on how BH3 helices engage multi-BH anti-apoptotic grooves (Sattler et al., 1997).

Liposomes are prepared from a mixture of lipids that approximates the lipid content of the outer mitochondrial membrane (Ardail et al., 1990; Kuwana et al., 2002; Lutter et al., 2000; Oh et al., 2006) as indicated in Table 23.1. Solubilized lipids (e.g., chloroform) are combined in glass test tubes as 50-mg aliquots and then vortexed thoroughly (10 to 15 times) to ensure complete mixing. Subsequently, the organic solvent is evaporated by a stream of nitrogen, and the tubes are placed in a vacuum desiccator overnight, resulting in a visible lipidic film on the bottom of the glass tube by morning. Tubes containing the dried lipid mixtures can be placed in Kapak pouches, flushed with nitrogen, sealed, and then stored at -80°C .

To produce FITC-dextran encapsulated liposomes, an aliquot of mixed lipids is thawed and resuspended in 1 ml of HK buffer (20 mM HEPES, 150 mM KCl, pH 7) with 50 to 100 mg of FITC-dextran (Molecular Probes #D-1821). The resulting slurry is vortexed until the lipid film has completely dissolved and then freeze-thawed 15 to 20 times between liquid nitrogen and a 37°C waterbath. A portion of the slurry can be preserved for later use (up to a month) by storing at -80°C in a pouch flushed with nitrogen. To generate large unilamellar vesicles (LUVs), the slurry is passed through an Avanti Mini-Extruder Set (#610000) equipped with a 100-nm filter. LUVs are purified away from residual unencapsulated FITC-dextran by gel filtration with a Sephacryl S-300 HR column (GE Healthcare) at a flow rate of 1 ml/min, with liposomes typically emerging at the ~12 ml fraction. Once purified, the lipid

concentration of the solution is assessed by a colorimetric phosphate assay described in detail elsewhere (Böttcher et al., 1961) and performed in duplicate (see Table 23.1 for phosphate content of the lipid mixture). A liposomal sample of 10 μ l typically yields a value within the standard curve, which ranges from 0 to 100 nmol/ μ L. FITC-dextran encapsulated LUVs are stored in foil-wrapped tubes at 4 °C and can be reliably used for 10 to 12 days.

In an experiment that monitors BAX-induced FITC-dextran release, for example, 2 ml of HK buffer containing 10 μ g/ml lipid is added to a quartz cuvette under constant stirring at 37 °C and fluorescence monitored over time in a fluorimeter (excitation 488 nm, emission 525 nm). Once the baseline fluorescence measurement stabilizes (typically 15 min), recombinant monomeric BAX (freshly gel filtration purified, final concentration 15 to 50 nM) is added and fluorescence monitored for several minutes to ensure a stable baseline. Subsequently, a dose of SAHB compound is added (typical test range, 0 to 250 nM) and the fluorescence measurements recorded every 2 sec until a stable plateau is reached, at which time the liposomes are quenched with 1% Triton X-100. The data are presented as percent FITC-dextran release, with the average of three replicate Triton values set to 100%, and the average starting baseline value set to 0% (Figure. 23.3B). Of note, a baseline measurement of liposome mixture with SAHB alone is recorded for each dosing level to ensure that the compound itself does not disrupt the liposomes. In general, neutral to negatively charged SAHBs are well tolerated by the liposomes. The liposomal assay is reliable and versatile and can be modified to test inhibition of BH3-induced BAX activation by adding recombinant anti-apoptotic proteins or by preincubating BAX with test ligands or proteins before exposure to liposomes. We have also adapted the assay to generate liposomes containing Ni²⁺-NTA-lipids (5% DOGS-NTA, Avanti #790404C), which can be used to localize histidine-tagged BH3 peptides or BCL-2 family proteins to the membrane surface to assess the impact of membrane-targeted regulators on BAX release activity.

3.2. Mitochondrial cytochrome c release assay

The purpose of this assay is to monitor the effect of ligand- or recombinant protein-induced activation or inhibition of BAX/BAK-mediated cytochrome *c* release in the context of isolated mitochondria (Ellerby et al., 1997; Luo et al., 1998; Scorrano et al., 2002) (Fig. 23.4A). The clear benefit of this approach derives from analysis of the intact organelle, which contains the natural lipid membrane composition and topography, including the cohort of proteins endogenously embedded in the various mitochondrial compartments. Of course, the isolated mitochondria, out of context from the intact cell, are lacking the multitude of factors from other cellular compartments (e.g., endoplasmic reticulum, cytosol) that certainly impact constitutive mitochondrial physiology and signal transduction. Thus, conclusions and generalizations based on such studies must be made with care. Furthermore, meticulous and timely handling of the mitochondria throughout the isolation and experimental procedure is essential to the preparation of suitable mitochondria for meaningful and reproducible assays.

An important feature of this assay is the ability to study and compare the responses of genetically distinct mitochondria that lack one or more of the regulatory BCL-2 family proteins of interest. Because the overall character of genetically modified mitochondria may

be quite different from wild-type mitochondria, as recently demonstrated for *Bax*^{-/-} *Bak*^{-/-} mitochondria that exhibit altered architecture (Karbowski et al., 2006), data must be interpreted with this caveat in mind. To study endogenous BAX activation, we use wild-type mouse liver mitochondria that naturally lack BAX, which is constitutively cytosolic (Letai et al., 2002). To study recombinant BAX activation, we use either *Bak*^{-/-} or Alb-cre^{pos} *Bax*^{flox/-} *Bak*^{-/-} mouse liver mitochondria, which have yielded essentially identical results (Walensky et al., 2006). Theoretically, if BAX is activated during mitochondrial preparation, it is plausible that endogenous BAX could be present and, therefore, contribute to the measured cytochrome *c* release; however, we do not detect BAX by Western analysis of isolated wild-type or *Bak*^{-/-} mitochondria (Letai et al., 2002; Walensky et al., 2006). Finally, it is worth mentioning that conflicting results have been reported with regard to the activity of select BH3-only proteins and peptides on mitochondrial activation based on this assay. The finding that select BH3-containing proteins and peptides, such as BID and BIM, activate BAX/BAK-mediated cytochrome *c* release, but others, such as BAD and NOXA do not, led to a subclassification of BH3-only proteins as “activators” (i.e., direct activation of BAX/BAK by BH3) or “sensitizers” (i.e., indirect activation of BAX/BAK by inhibition of anti-apoptotic proteins) of mitochondrial apoptosis (Cheng et al., 2001; Kuwana et al., 2002; Letai et al., 2002). Whereas this model has been supported by subsequent work (Certo et al., 2006; Kim et al., 2006; Kuwana et al., 2005; Walensky et al., 2006), other mitochondrial studies demonstrate activation in response to “sensitizers” such as BAD, especially when used in combination with other BH3 peptides that expand anti-apoptotic blockade (e.g., NOXA) (Uren et al., 2007). Such studies, support a model in which BAX and BAK are activated only once BH3-only proteins or peptides target the broad spectrum of anti-apoptotic proteins, thereby eliminating the anti-apoptotic blockade of BAX/BAK (Willis et al., 2007). Indeed, results may vary on the basis of the experimental conditions and the cellular origin of the mitochondria tested (Uren et al., 2007). Ultimately, both mechanisms may contribute, singly or in combination, to the regulation of BAX/BAK activation *in vivo*, depending on the cellular context and apoptotic stimulus.

3.2.1. Buffer preparation—Mitochondrial isolation buffer is composed of 250 mM sucrose (85.6 g/L), 10 mM Tris-HCl (1.57 g/L), and 100 μM MEGTA (0.046 g/L), titrated to pH 7.4, sterile filtered, and stored at 4 °C.

The mitochondrial experimental buffer (pH 7.4) contains 125 mM KCl, 10 mM Tris-MOPS, 5 mM glutamic acid, 2.5 mM malic acid, 1 mM K-phos, and 10 μM MEGTA-Tris. This buffer is prepared fresh for each experiment from the following stock solutions: 1 M KCl (37.3 g/500 ml); 1 M Tris-MOPS (for 100 ml add 12.1 g of Tris-base, titrate to pH 11, and slowly add 20.9 g of MOPS); 1 M glutamic acid (20.9 g/100 ml of the potassium salt); 1 M malic acid (6.7 g/50 ml); K-phos solution, made by combining 2 ml of 1 M monobasic potassium phosphate with 8 ml of 1 M dibasic potassium phosphate; and 1 M MEGTA-Tris (for 5 ml add 0.6 g of Tris-base, titrate to pH 11, and add 2.34 g of tetrasodium-EGTA). Stocks are stored at 4 °C for no longer than 1 month. A 100-ml working buffer is made by combining 12.5 ml of 1 M KCl, 1 ml of 1 M Tris-MOPS, 500 μl of 1 M glutamic acid, 250 μl of 1 M malic acid, 100 μl of 1 M K-phos, 100 μl of 1 M MEGTA-Tris, diluted to 100 ml in deionized water,

titrated to pH 7.4, and sterile filtered. The buffer should be brought to room temperature before use.

3.2.2. Mitochondrial isolation—A mouse of the desired phenotype is sacrificed and the liver promptly removed and immersed in chilled isolation buffer. The liver is scissorminced in several changes of isolation buffer, with this repeat rinsing performed to eliminate trace blood. The liver fragments are then dounce homogenized in ~25 ml of chilled isolation buffer until a homogeneous suspension is achieved (typically 3 to 4 dounce cycles at high speed). The liver suspension is maintained on ice and quickly transferred for centrifugation at 800*g* for 10 min at 4 °C. The supernatant is collected (leaving gross cellular debris behind) and centrifuged at 7000*g* for 10 min at 4 °C. The supernatant is decanted, and the pellet containing the isolated mitochondria is gently resuspended by pipette trituration in 1 ml of chilled isolation buffer. Enlarging the orifice of the pipette tip by cutting with a sterile razor helps avoid shear forces that could disrupt the mitochondria and cause background cytochrome *c* release. The resuspended pellet is diluted in 30 to 40 ml of chilled isolation buffer and centrifuged again at 7000*g* for 10 min at 4 °C. After resuspending the pellet in 1 ml isolation buffer, the protein concentration of the mitochondrial suspension is promptly determined (Bio-Rad Protein Assay #500-0006). The liver from a single mouse typically yields ~5 to 10 mg of mitochondrial pellet. Once the protein concentration is determined, the suspension is diluted to a final concentration of 0.5 mg/ml in experimental buffer and promptly added to an already prepared 96-well plate (or Eppendorf tubes) containing the treatment protein(s) and ligand(s) at the desired concentrations (see 3.2.3). Of note, once the mitochondria are immersed in experimental buffer, prolonged storage, even at 4 °C, can lead to background cytochrome *c* release, and therefore, prompt use of the mitochondria is mandatory.

3.2.3. Plate design and assay—We have adapted the mitochondrial assay to a 96-well format for screening purposes. In this setup, control wells containing vehicle (e.g., 1% DMSO) or 1% Triton X-100 alone represent baseline and maximal cytochrome *c* release, respectively. The amount of cytochrome *c* quantitated in the supernatant by ELISA is inserted into Eq. (23.3) to determine percent cytochrome *c* release:

$$\% \text{ Release} = (\text{Abs}_x - \text{Abs}_{\text{veh}}) \times 100 / (\text{Abs}_{\text{Tr}} - \text{Abs}_{\text{veh}}) \quad (23.3)$$

where Abs_x , Abs_{veh} , and Abs_{Tr} are the absorbances of the sample, vehicle control, and Triton X-100 control. If fewer samples are run, an Eppendorf tube-based setup can also be used. In this case, the supernatant is separated from the mitochondrial pellet, the pellet is resuspended in 1% Triton X-100, the supernatant of the extracted pellet isolated, and percent cytochrome *c* release determined by Eq. (23.4):

$$\% \text{ Release} = \text{Abs}_s \times 100 / (\text{Abs}_s + \text{Abs}_p) \quad (23.4)$$

where Abs_s and Abs_p represent the absorbances of the original supernatant and the supernatant from the detergent-extracted pellet, respectively.

To prepare a plate (clear U-bottom) for recombinant BAX activation analysis, for example, a serial dilution of test SAHB is performed at 4× the desired final concentrations in 25 μ l experimental buffer. Recombinant BAX at 4× the desired final concentration is then added by repeat pipette, delivering an additional 25 μ l to each well. It is critically important that the recombinant BAX is freshly gel filtration purified to ensure a monomeric solution, thereby avoiding nonspecific BAX-induced release resulting from oligomer-triggered BAX activation and cytochrome *c* release. The test components for the assay can be modified to accommodate other analyses (e.g., combination SAHB treatments, investigation of endogenous BAK activation). SAHB dilutions are typically prepared before or simultaneous with mitochondrial isolation, and then BAX and mitochondria are added sequentially by repeat pipette once the protein concentration of the mitochondrial preparation is determined. It is critically important that the mitochondrial preparation be homogenously resuspended immediately before repeat pipetting to avoid variability of the suspension that arises from the settling of mitochondria in the stock tube. Experimental conditions are typically run in quadruplicate and important controls include vehicle + mitochondria, BAX + mitochondria, and SAHB + mitochondria to monitor for background and/or nonspecific release. Additional controls include blockade of cytochrome *c* release by added anti-apoptotic proteins and the use of point mutant SAHBs that exhibit impaired BCL-2 family protein interactions (Walensky et al., 2006).

Once the mitochondria have been added, the plate is incubated at room temperature for 40 min. A time course is also beneficial to monitor for changes in release kinetics (Walensky et al., 2006). After the desired incubation time, the plate is centrifuged at 3000 rpm for 10 min at 4 °C and then ~30 μ l of each supernatant is carefully removed to another 96-well plate, taking care not to disrupt the pellet, which can be seen at the very bottom of the U-shaped well. For the Eppendorf setup, tubes are spun at 14,000 rpm in a refrigerated table top centrifuge for 10 min, followed by transfer of the total supernatant (a gel loading pipette tip is helpful to avoid disrupting and removing any pellet) to another set of Eppendorf tubes. The pellets are then resuspended (by flicking and vortexing) in 100 μ l of 1% Triton X-100, spun at 14,000 rpm in a refrigerated table top centrifuge, and the supernatants transferred to another set of Eppendorf tubes. Samples of supernatant (typically 4 μ l) are then added to the wells of an ELISA plate for cytochrome *c* detection according to the manufacturer's protocol (R&D Systems #MCTC0). Of note, residual supernatants can be stored at -20 °C for repeat or deferred analysis. The absorbance data are processed as described at the beginning of this section to determine the percent cytochrome *c* released by the experimental condition (Figure. 23.4B).

4. MEASUREMENT OF CELLULAR APOPTOSIS INDUCTION

As described in our preceding article, a key benefit of SAHBs as tool compounds for dissecting apoptotic pathways is their cell penetrability. Thus, in addition to serving as potential therapeutics for reactivating apoptosis in cancer, the wide variety of SAHBs generated based on the many discrete BH3 sequences can be used to test cancer cell

susceptibilities to individual pro-apoptotic death domains. This cell-based survey, combined with identification of the corresponding intracellular SAHB targets, provides a protein interaction-based mechanism for apoptosis induction in a particular cell or tissue.

Because individual cell types (e.g., adherent vs nonadherent, transformed vs nontransformed) may exhibit differential rates and capacities for uptake of cell-penetrating peptides such as SAHBs, an assessment of cellular permeability is first performed for the cell under investigation, as described (Bird et al., 2006). Culture conditions will vary according to the cell type used. For the Jurkat studies described here, we use $1 \times$ RPMI-1600, 200 units/ml penicillin/streptomycin, 2 mM L-glutamine, 50 mM HEPES, 50 μ M 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (FBS). Serum-free media can be used to wash cells before SAHB treatment and during initial exposure to SAHB compounds (typically 2 to 4 h) to maximize cell surface contact and avoid any decrement in activity from serum binding.

A variety of kit-based assays are available to monitor cell viability (e.g., MTT assay [Roche], Cell Titer-Glo™ [Promega]), and apoptosis induction (e.g., caspase-3 activation [Oncogene], annexin-V binding [BD Biosciences]) in reasonably high throughput. Whereas viability assays are useful for screening purposes, active compounds are then assessed in more specific assays for apoptosis induction (Walensky et al., 2004). For example, the annexin-V-binding assay detects the externalization of phosphatidylserine (PS, the binding substrate for annexin V), which is a characteristic event of apoptosis induction (Figure. 23.5). SAHB stocks (1 mM, 100% DMSO) are diluted stepwise into water, and then serially diluted into serum-free media (final volume 50 μ l) to achieve the desired dose range (typically 0.15 to 10 μ M). Jurkat cells are resuspended and centrifuged twice in serum-free media, diluted to a concentration of 1×10^6 cells/ml in serum-free media, and then 50,000 cells (50 μ l) are added to the SAHB solution. After an initial incubation period (typically 2 to 4 h), an additional 100 μ l of Jurkat medium containing 20% FBS is added to restore 10% serum conditions and the cells monitored for apoptosis induction over time (e.g., 6, 12, 24, and 48 h). For annexin-V-binding analysis, the cells are pelleted, washed in PBS, repelleted, and then resuspended in 200 μ l of $1 \times$ annexin-V binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) containing a 1:500 dilution of fluoresceinated annexin-V according to the manufacturer's protocol (e.g., BD Biosciences). Cellular fluorescence is then detected and quantitated by FACS analysis. Vital dyes such as propidium iodide (PI) or 7-amino-actinomycin (7-AAD) can also be added (1 μ g/ml final concentration) to monitor the progression from early apoptosis, at which point cells display externalized PS but retain membrane integrity (annexin-V positive, PI/7-AAD negative), to end-stage apoptosis by which point the dead cells are dye-permeable (annexin-V positive, PI/7-AAD positive) (Figure. 23.5). Each experimental condition is performed in at least triplicate, and controls include vehicle (e.g., corresponding serial dilution of DMSO/water), a standard pro-apoptotic stimuli (e.g., staurosporine, 1 μ M), and negative control point mutant SAHBs.

5. IDENTIFICATION OF *IN SITU* MECHANISTIC TARGETS OF SAHBs

A major advantage of peptidic compounds is that they are readily derivatized for a host of research applications. For example, the capacity to incorporate a fluorescent label allows for intracellular tracking of SAHBs, which facilitated their visualization in pinocytotic vesicles during uptake and their ultimate localization to the outer mitochondrial membrane in live cells (Walensky et al., 2004). Whereas such FITC-labeled SAHB compounds contributed to our understanding of the import mechanism and organelle targeting, anti-FITC immunoprecipitation from extracts of FITC-SAHB-treated cells enables the identification of intracellular high-affinity SAHB targets and thus provides insight into the mechanism of SAHB-induced activation of apoptosis (Figure. 23.6).

For anti-FITC co-immunoprecipitation experiments, cells (1×10^7) are treated with SAHB peptides (10 to 20 μ M) in serum-free media for up to 4 h, followed by serum replacement and timed incubation. Cells are collected by centrifugation and subjected to lysis in 50 mM Tris pH 7.6, 150 mM NaCl, 1% CHAPS, and protease inhibitor cocktail (Sigma). A variety of detergents may be used, but when assaying for BAX/BAK interactions, it is important to use detergent conditions that do not induce BAX/BAK oligomerization (Antonsson et al., 2000). Cellular fractionation protocols that examine specific cellular compartments may also be used. Cellular lysis and all subsequent steps are conducted at 4 °C. Extracts are subjected to centrifugation and supernatants isolated and then incubated with protein A/G-sepharose (Santa Cruz) (50 μ l 50% bead slurry/0.5 ml lysate). The precleared supernatants (0.5 ml) are collected after centrifugation, incubated with 10 μ l goat-anti-FITC antibody (Abcam) for 1.5 h, and then protein A/G-sepharose (50 μ l 50% bead slurry/0.5 ml lysate) added for an additional 1.5-h incubation. After 2-min tabletop centrifugation, the pellets are isolated and then washed three times in lysis buffer containing increasing salt concentrations (150, 300, 500 mM). To liberate precipitated protein, the beads are suspended in SDS-containing sample buffer, boiled, and supernatants collected after centrifugation. Samples are electrophoresed (e.g., 4 to 12% gradient Bis-Tris gels [Invitrogen]), and proteins transferred to Immobilon-P membranes (Millipore). After blocking, blots are incubated with the specified BCL-2 family protein antibody (e.g., BAX-N20 antibody [Santa Cruz]) or rabbit anti-FITC antibody (Santa Cruz) at 1:500 dilutions in blocking buffer, followed by treatment with secondary antibody for visualization with a chemiluminescent reagent (e.g., Western Lightning™ [Perkin Elmer]). Alternate retrieval strategies, such as streptavidin-based capture of biotinylated SAHBs, are also feasible.

It is important to emphasize that *in situ* target identification studies based on noncovalent interactions between ligand and protein are limited by affinity, which dictates the capacity of the association to withstand cellular lysis and retrieval processing. Whereas high-affinity 1:1 interactions are best suited for this type of analysis, transient interactions, such as enzymatic processes or catalytic “hit and run” phenomena as has been hypothesized for BID-BAX interactions (Eskes et al., 2000; Wei et al., 2000), may require alternate strategies, such as derivitization of SAHBs with covalent trapping moieties (Danial et al., 2008).

6. SUMMARY

SAHBs are cell-permeable compounds that retain the structural and biochemical functionalities of native BH3 domains and, therefore, serve as useful tools to chemically dissect the BCL-2 family interaction network *in vitro* and *in vivo*. Through substituting only two non-natural residues at positions on the noninteracting surface of the BH3 motif, SAHBs preserve the differential binding specificities of discrete BH3 motifs and facilitate the identification of binding preferences among BCL-2 family proteins by use of *in vitro* binding assays and *in situ* co-immunoprecipitation strategies. Mitochondrial and liposomal release assays serve as robust *in vitro* studies to probe the functional impact of BH3-based interactions on activation or inhibition of mitochondrial apoptosis. Functional interaction studies can then be extended to a cellular context owing to the cell permeability of SAHB compounds. Thus, SAHBs are new chemical tools that directly and specifically alter BCL-2 family protein function, enabling the apoptotic network to be investigated, and ultimately manipulated, on a conditional basis in real time.

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Figure 23.1.

The BCL-2 family of proteins is composed of anti-apoptotic and pro-apoptotic members that share conserved BCL-2 homology (BH) domains. The pro-apoptotic BH3 domains serve as sequence templates for the generation of SAHB compounds.

Figure 23.2.

Fluorescence polarization binding assay. (A) The interaction between a FITC-ligand at fixed concentration with increasing doses of target protein results in dose-dependent polarization of light, which is measured by the detector. (B) FPA studies demonstrate the high affinity of BIM and BAD BH3 peptides for BCL-X_L, with SAHB derivatives exhibiting enhanced binding activity compared to the corresponding unmodified peptides (Walensky et al., 2006). The selectivity of BH3 interactions is highlighted by BAX binding studies. Whereas BAD BH3 and BAD SAHB peptides display no interactions with BAX even at μ M doses of protein, the α -helical BIM SAHB, but not the unmodified (and structurally unfolded) BIM BH3 peptide, binds BAX with nanomolar affinity.

Figure 23.3.

Liposomal release assay. (A) Recombinant BAX and SAHB ligand are added to liposomes containing entrapped FITC-dextran. Direct activation and oligomerization of BAX leads to release of liposomal FITC-dextran, which is detected by a fluorimeter. (B) BID SAHB_A induces dose-responsive BAX-mediated liposomal release of FITC-dextran (Walensky et al., 2006). BID SAHB_A or BAX alone have no effect; the combination of unmodified BID BH3 peptide and BAX likewise has no effect in this dose range.

Figure 23.4.

Mitochondrial cytochrome *c* release assay. (A) The molecular interactions that regulate mitochondrial apoptosis are evaluated *in vitro* by treating isolated mitochondria with BH3 ligands and/or recombinant BCL-2 family proteins followed by ELISA-based quantitation of released cytochrome *c*. (B) BID SAHB_A induced dose-responsive BAX-mediated cytochrome *c* release from Bak^{-/-} mitochondria (Walensky et al., 2006). Mitochondrial treatment with BAX or BID SAHB_A alone had no such effect. The specificity of BID SAHB_A activity was confirmed by the inability of mutant BID SAHB_{A(L,D→A)} or BAD

SAHB_A to activate BAX-induced cytochrome c release, and by abrogation of BID SAHB_A activity with BCL-X_L C co-treatment.

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Figure 23.5.

Annexin-V apoptosis induction assay. (A) Cells exposed to a pro-apoptotic stimulus and then evaluated by FITC-annexin-V and 7-AAD staining exhibit a characteristic apoptotic progression as detected by FACS analysis. Whereas healthy cells are annexin-V and 7-AAD negative, cells undergoing apoptosis externalize phosphatidylserine and become annexin-V positive. During the early stage of apoptosis induction, membrane integrity is preserved and cells are annexin-V positive but 7-AAD negative. By late-stage apoptosis, membrane integrity is lost, and cells become positive for 7-AAD as well. (B) Cultured leukemia cells

exposed to serial dilutions of pro-apoptotic SAHBs can be evaluated by FACS analysis over time to identify and quantify the stages of apoptosis induction. Whereas the cells are initially annexin-V and 7-AAD negative (left panel, 90% live), apoptosis induction (right panel) is reflected by a predominant population that is annexin-V positive and 7-AAD negative (bottom right, 53% early apoptosis), and a second population that is both annexin-V and 7-AAD positive (top right, 35% late apoptosis).

Figure 23.6.

In situ target identification by SAHB retrieval. (A) Cells treated with FITC-SAHB compounds are lysed and extracts subjected to anti-FITC co-immunoprecipitation with FITC antibody and protein A/G-sepharose pull down. After washing, SAHB-bound protein targets are eluted and identified by BCL-2 family Western analysis. (B) Jurkat T cells were incubated with FITC-BID SAHB_A and a mutant derivative for 18 h, followed by cellular lysis in a 1% CHAPS-containing buffer. Anti-FITC pull down co-immunoprecipitated native BAX with FITC-BID SAHB_A but not with the mutant BID SAHB_{A(L,D→A)} (Walensky et al., 2006).

Table 23.1
Composition of large unilamellar vesicles that simulate the lipid content of mitochondrial outer membrane contact sites

Lipid	Stock (mg/ml)	Mol wt	Wt%	Wt lipid (mg)	Vol lipid (ml)	Moles Lipid	Mole %	P atom	Moles P
POPC	10	760.1	41	20.5	2.05	.0270	42.7	1	.027
POPE	10	770.99	22	11	1.1	.0143	22.6	1	.0143
PI	25	909.12	9	4.5	0.18	.0049	7.83	1	.0049
CHOL	10	386.66	8	4	0.4	.0103	16.4	0	0
CL	10	1493.91	20	10	1	.0067	10.6	2	.0134
TOTAL			100%	50 mg	4.73 mL	.0632			0.0596

The indicated values represent the amounts of lipid combined to yield a 50 mg mixture of POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine), PI (beef liver phosphatidylinositol), CHOL (cholesterol), and CL (beef heart cardiolipin).