Apoptotic blocks and chemotherapy resistance: strategies to identify Bcl-2 protein signatures

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Abstract
Acquired or innate resistance to chemotherapy is a major drawback of cancer therapeutics, which is frequently seen in epithelial cancers. However, the molecular mechanisms underlying chemotherapy resistance remain poorly understood. The mitochondrial pathway is a critical death pathway common to many different types of chemotherapy. Aberrations in this pathway can result in resistance to chemotherapy. The Bcl-2 family of proteins control commitment to programmed cell death by mitochondrial apoptosis. In this review, we will summarize the strategies in determining the components of apoptotic defects responsible for chemotherapy resistance, mainly focused on Bcl-2 protein network.

Keywords: apoptosis; Bcl-2 protein family; chemotherapy resistance; BH3 profiling; cancer proteomics; antibody microarray

INTRODUCTION
Chemotherapy is a widely used treatment modality in both solid tumours and haematological malignancies. Acquired or innate resistance to chemotherapy is a crucial determinant of the outcome of cancer treatment [1, 2]. The evasion of apoptosis is a critical component of oncogenic transformation and chemotherapy resistance. Cancer cells may escape from apoptosis in response to various stimuli, such as chemotherapy and radiotherapy, by increasing anti-apoptotic proteins of Bcl-2 protein family, including Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and Bfl-1. When cells are exposed to an apoptotic insult such as UV radiation, growth factor withdrawal, DNA damage or chemotherapy treatment, this insult is sensed by molecular sensors of the cell, the BH3-only proteins (BOPs), and conveyed into intrinsic apoptotic pathway [3–5]. Following mitochondrial outer membrane permeabilization (MOMP), pro-apoptotic molecules such as cytochrome c, SMAC/DIABLO and Omi/HtrA2 are released from mitochondria to cytosol. Cytosolic cytochrome c initiates the formation of an apoptosome complex with Apaf-1 and caspase-9, which in turn activates executioner caspases, such as caspase-3 and caspase-7.

Bcl-2 protein family members eventually govern MOMP and cellular fate: apoptosis or survival. Anti-apoptotic Bcl-2 proteins act as gatekeepers of MOMP through binding and sequestering pro-apoptotic Bcl-2 protein family members. These pro-apoptotic members include BH3-only Bcl-2 proteins (Bid, Bim, Bad, Noxa, Puma, Bmf, Bik, Hrk) and multi-domain pro-apoptotic proteins Bak and Bax. Upon apoptotic stimuli, Bax and Bak are activated, inserted into outer mitochondrial membrane, oligomerized and eventually they facilitate MOMP. Anti-apoptotic Bcl-2 protein family members inhibit MOMP either by directly sequestering Bax and Bak or by sequestering activator BOPs, such as Bid and Bim, and preventing Bax and Bak activation [3, 4, 6]. In this model of activators/sensitizers, sensitizer BOPs, such as Bad and Noxa,

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neutralize the anti-apoptotic functionality of anti-apoptotic Bcl-2 protein family members by competitive displacement of activator BOPs, which in turn mediate Bax/Bak activation and mitochondrial permeabilization. Therefore, deciphering the interaction patterns of Bcl-2 protein family members, in addition to expression levels and post-translational modifications, is required for an entire profile of mitochondrial apoptotic regulation.

In this review, we will discuss some of the perspectives and strategies in understanding the components of core apoptotic machinery governing chemotherapeutic response, mainly focused on the Bcl-2 protein network.

**APOPTOSIS SIGNALLING AND CHEMOTHERAPY RESISTANCE**

Many chemotherapeutic drugs target cancer cells at multiple molecular and cellular levels. Resistance to chemotherapy emerges as an important factor that negatively influences the efficiency of cancer treatment [1, 2, 7, 8]. This is especially seen in epithelial tumours including breast and lung carcinoma despite utilization of multi-drug regiments [9, 10]. There are various mechanisms responsible for cancer cell chemoresistance, such as modification of drug-target interactions, decreased uptake or increased elimination of active molecule and apoptosis defects. Thereby, developing rational interventions to reverse chemotherapy resistance and to eradicate cancer cells can be possible by understanding the defects in apoptosis signaling machineries in chemoresistant cancer cells. This will enable us to identify novel molecular targets responsible for chemotherapy resistance more accurately and efficiently.

Apoptosis is a programmed cell death mechanism, which is composed of molecular machineries integrated into each other through protein–protein, protein–organelle and organelle–organelle interactions. Mitochondria lie in the core of the intrinsic apoptotic machineries. The regulation of intrinsic apoptosis pathway by Bcl-2 family proteins is summarized in Figure 1. Additionally, the extrinsic apoptotic pathway mediates caspase activation signaled by cell surface cell death receptors, which may not necessarily operate through mitochondria. However, in many cell types death receptor-mediated apoptotic signaling induces a mitochondrial death amplification loop via proteolytic activation of the BH3-only protein Bid [11]. Of note, overexpression of anti-apoptotic Bcl-2 protein family members or loss of pro-apoptotic Bcl-2 protein family members has been shown to negatively affect the outcome of chemotherapy in various tumour types [12–16]. Since defective apoptosis signaling has been suggested to be necessary for tumourgenesis, tumour maintenance and resistance to chemotherapy, elucidating protein signatures of Bcl-2 protein family members including expression levels, posttranslational modifications and protein–protein interactions is required to understand the altered mechanisms of apoptosis signaling in chemotherapy-resistant cancer cells.

Oncoproteomics is a powerful and efficient approach to study protein signatures in cancer cells using biochemical tools such as affinity-based protein purification and enrichment, two-dimensional (2D) electrophoresis, mass spectroscopy-based peptide analysis and protein/antibody microarray platforms [17]. Using combination of these techniques provides a robust and high-throughput analysis opportunity with fast data handling and interpretation. In the following sections, we will discuss three of these strategies to elucidate Bcl-2 protein signatures in cancer cells.

**IMMUNOPRECIPITATION AND MASS SPECTROSCOPY**

The Bcl-2 protein was first identified to be over-expressed in low-grade human follicular B-cell lymphoma and further genetic and cellular studies led to the identification and characterization of other members of Bcl-2 protein family based on conserved Bcl-2 homology domains (BH1-4) [18]. Anti-apoptotic Bcl-2 proteins exhibit all the four BH domains and a transmembrane domain at their C-terminal. The α-helices of BH1, BH2 and BH3 domains of anti-apoptotic Bcl-2 proteins form a hydrophobic pocket, through which they interact with pro-apoptotic Bcl-2 proteins [19, 20]. Indeed, the presence of BH3 domain is essential for death promoting activity of pro-apoptotic Bcl-2 proteins and different anti-apoptotic Bcl-2 proteins have distinct binding affinities for unique BH3 domains and these differential binding properties define their anti-apoptotic potencies [21]. The identification of Bcl-2 interaction network (interactome) is needed for defining protein–protein interaction patterns in chemotherapy-resistant and chemotherapy-sensitive states of cancer cells. This strategy will help to clarify...
the apoptotic blocks responsible for development of chemotherapy resistance and to target these mechanisms feasibly to increase the efficiency of chemotherapy. Furthermore, it may enable to identify proteins with unknown functions involved in the emergence of chemotherapy resistance.

The protein networks in cellular systems exert their functions as molecular machineries with individual components interacting with each other in a dynamic environment. The organization of these machineries is strictly controlled by a fine tuning system of extracellular and intracellular signaling nodes. As described in Figure 1, apoptosis signaling through mitochondria and the Bcl-2 protein family network is an excellent example of these machineries. To understand the dynamic nature of these protein–protein interactions governing the most critical decisions at cellular level, we are in need of refinement of strategies and techniques to identify these signatures. Mass spectroscopy-based (MS-based) techniques are routinely used to analyse and identify proteins in cancer cells [22–24]. The ability to identify hundreds of proteins by MS allows high-throughput comparative analysis of different cellular phenotypes, including chemoresistant and chemosensitive states of cancer cells. The initial step of this research strategy is isolation and purification of high quality Bcl-2 family protein complexes. This is a mandatory step for identification of interacting proteins by the following MS analyses.

We utilize two main approaches to isolate Bcl-2 family protein complexes. The first strategy involves coimmunoprecipitation of endogenously interacting Bcl-2 proteins by utilizing antibodies against Bcl-2 proteins. The co-immunoprecipitated complex is then affinity purified from the protein mixture by incubating with Protein Sepharose A/G beads. Following this purification step, coimmunoprecipitated Bcl-2 protein complex can be resolved on a gel-based separation system, in-gel digested and
subjected to MS-based peptide analysis for identification of interacting proteins. Alternatively, the proteins can be subjected to in-solution gel digestion followed by MS analysis. The major drawback of this approach is the possibility of antibody cross-reaction with other proteins, which may lead to artifacts in analysis and identification of the protein signatures. The second approach involves an epitope-tagging of Bcl-2 family proteins at their N- or C-terminal and affinity-based purification of protein complexes through their tagged fusion protein domains. A basic scheme of this strategy is summarized in Figure 2. The most commonly used fusion tags in this approach are FLAG, c-Myc, Haemagglutinin (HA) and (6 x) His tags. Similar to co-immunoprecipitation approach, Bcl-2 protein complexes can be affinity purified and either in-gel or in-solution digested before MS analysis. The efficiency of affinity-based purification should always be checked by immunoblot analysis using anti-tag antibodies. Furthermore, utilization of chemical crosslinkers may increase the efficiency of detection of interactions, especially when the interactions are transient or weak. Separation of Bcl-2 protein complexes on either 1D or 2D-gel electrophoresis followed by silver staining of gel samples may foster the identification of differentially enriched spots in chemoresistant and chemosensitive states of cancer cells. These novel interactors can be identified by MS-based peptide mapping following in-gel digestion. For example, Lo and Hannink successfully identified Bcl-xL-interacting protein PGAM-5 as a substrate for Keap-1-dependent protein ligase complex by expressing CBD-tagged (chitin binding domain-tagged) Keap-1, chitin pull-down of Keap-1 binding proteins, 1D electrophoresis and silver staining [25]. Following tryptic digestion and desalting of tryptic peptides, they identified PGAM-5 as a Keap-1 binding protein by means of MALDI-TOF mass spectrometry. In a similar approach, Chipuk et al. [26] identified p53 and Puma as Bcl-xL-interacting proteins by using agarose-conjugated anti-Bcl-xL antibody for immunoprecipitation. After immunoprecipitation of total cell lysates of untreated and UV-treated MEFs by agarose-conjugated anti-Bcl-xL antibody, they eluted the immunoprecipitates, subjected to SDS-PAGE and visualized by silver staining. The bands were excised and analysed by liquid chromatography and tandem MS following tryptic digestion. These two research applications underscore the potential use of this strategy to identify novel 

Figure 2: Identification of Bcl-2 protein signatures in chemotherapy resistant and chemotherapy sensitive cancer cells by immunoprecipitation- and MS-based approaches. The Bcl-2 protein complex is isolated and purified from total cell lysates by means of co-immunoprecipitation or fusion-tag affinity. The purified protein complex is separated on either 1D SDS-PAGE or 2D-gel electrophoresis and visualized by Coomassie or silver staining. The bands of target proteins are in-gel trypsin digested and analysed by MS. The identification of proteins is processed by using database search with the obtained peptide mass fingerprint. Chemotherapy sensitivity and chemotherapy resistance patterns of Bcl-2 protein signatures are defined by bioinformatics approaches.
protein–protein interactions and to identify Bcl-2 protein signatures in cancer cells with different phenotypes.

**BH3 PROFILING: DIAGNOSING BCL-2 ADDICTION**

It is a fundamental issue to develop methods to decipher alterations in apoptosis pathways to identify, target and overcome chemotherapy resistance mechanisms in cancer cells. As determined by fluorescence polarization assays, anti-apoptotic Bcl-2 protein family members exert different binding affinities for specific BH3 domains [27]. Thereby, it is possible to distinguish the dependence of cancer cells on different anti-apoptotic Bcl-2 proteins for survival. The Letai laboratory has developed a powerful research tool, designated as BH3 profiling, to map the dependence of cancer cells on anti-apoptotic Bcl-2 proteins [6, 27, 28]. A basic description of this technique is summarized in Figure 3. Briefly, mitochondria are isolated from cancer cells and incubated with peptides corresponding to the BH3 domains of BOPs. The permeabilization of mitochondria by these peptides is assessed by the release of cytochrome c by means of an ELISA-based cytochrome c assay. The ability of BH3 domains of activator BOPs to induce cytochrome c release indicates the existence of Bax and Bak proteins, which are capable of MOMP when activated by activator BOPs. The ability of BH3 domains of sensitizer BOPs to induce cytochrome c release indicates the presence of anti-apoptotic Bcl-2 proteins primed with activator BOPs. The sensitizer BH3 peptides displace these activators from their corresponding anti-apoptotic partners, which are then free to activate Bax and Bak and to induce MOMP. The type of apoptotic block can be identified by comparing BH3 profiling pattern to fluorescence polarization interaction map of BH3 domains. This approach has been successfully utilized in chronic lymphocytic leukemia (CLL) to demonstrate the Bcl-2 dependence of CLL samples [6]. Moreover, Deng et al. [28] recently showed that BH3 profiling could be used

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**Figure 3:** The strategy of BH3 profiling involves isolation of mitochondria and incubation with BH3 peptides of BOPs. The anti-apoptotic potential or addiction of cancer cells to anti-apoptotic proteins can be evaluated by this novel technique.
to differentiate distinct classes of apoptotic blocks in lymphoma cells and its correlation with sensitivity to different types of chemotherapeutic drugs. All these promising results imply the potential of BH3 profiling as a high-throughput analysis tool for identifying Bcl-2 protein family signatures in cancer cells.

ANTIBODY MICROARRAYS
In addition to separation-based methods and MS, antibody microarray platforms are promising experimental tools for protein expression profiling and classification, especially for cancer research [29, 30]. Briefly, antibodies against known antigens are spotted onto modified solid support such as glass slide [31]. Either labeled protein sample (direct labelling) or unprocessed form (label-free sandwich format) is incubated with microarray to allow the antigens in the mixture bind to relative immobilized antibodies. The bound antigens are detected either by using labels on them like DNA microarrays, or by using detection antibodies that recognize preferable different epitope other than capture antibodies like in ELISA. In both cases, fluorescent and chemiluminescent methods can be used for qualitative or quantitative measurements. Proteins in complex mixtures can be quantified by using sandwich format antibody arrays, which utilize two different epitope binding monoclonal antibodies, one as a capture and the other as a detection molecule [32, 33]. Another format of protein microarrays is reverse-phase protein lysate microarray, where replicated and serially diluted homogenized samples are robotically spotted onto solid support [34]. Arrays are then hybridized with specific primary antibodies that can be recognized by labelled secondary antibodies. In general, biotinylated secondary antibodies are used with signal generating streptavidin molecules. Fluorescence and chemiluminescence agents are the most widely used detection methods [35, 36].

Detection mechanisms for direct labeling, label-free sandwich format and lysate array methods can be visualized in Figure 4. The main drawback for direct labeling method is the difficulty of labeling all antigens of interest homogenously among samples. Additionally, antigen–antibody interaction can be lost significantly after labeling procedure. On the other hand, using one antibody as opposed to sandwich method, which requires two different antibodies for each antigen, is the main advantage. Unavailability of matched pair of antibodies and the cross-reactions between antibodies are major difficulties for label-free sandwich method. Compatibility of western blotting antibodies with lysate array methodology makes this type of microarrays as valuable alternatives. Although lysate arrays lack a separation step, in contrast to gel electrophoresis before blotting, these difficulties can be handled by carefully designing experiments and analysing data [37, 38]. In a recent study, an antibody microarray constructed on a nitrocellulose film bound to a glass slide was successfully utilized for immunophenotyping of leukemias [39]. Furthermore, a high-throughput protein microarray platform was employed to compare the gene expression pattern of normal and malignant breast tissues and increased expression of a number of proteins including p53, cdc25c and MAP kinase 7 was detected [40]. Similarly, Ghobrial et al. used a protein microarray platform to compare the protein expression profiles of lymphocytes isolated from normal tonsil and mantle-cell lymphoma [41]. They detected 77 over-expressed proteins including cell cycle regulators and chaperone proteins in lymphocytes isolated from mantle-cell lymphoma samples.

In summary, the design and development of antibody arrays as a tool for identifying protein signatures is potentially valuable for cancer research. Of note, we are currently trying to utilize this methodology to monitor the expression levels and posttranslational modifications of Bcl-2 proteins in chemotherapy resistant and chemotherapy sensitive cancer cells using a fluorescent-based, sensitive and high-throughput analysis system.

SUMMARY
The protein expression profiling is essential but not enough to understand the molecular machineries governing chemotherapy response in cancer cells.
The protein–protein interaction patterns and posttranslational modifications of the Bcl-2 proteins are also critical determinants of the choice between cell death and survival. The construction of the Bcl-2 protein signatures, including protein expression levels, protein–protein interaction patterns and posttranslational modifications, in chemotherapy-sensitive and chemotherapy-resistant cancer cells is a difficult challenge. Moreover, designing a proper experimental and technical workflow of oncoproteomics application, which is compatible with in vivo physiological conditions is mandatory for the reliability of the constructed signatures. The need for the integration of multiple experimental strategies is evident.

Using affinity purification and MS-based proteomics, BH3 profiling and antibody microarray platforms concurrently may provide a capacity to identify the Bcl-2 protein signatures and facilitate the identification of chemotherapy resistance mechanisms in cancer cells.

Key Points
- Bcl-2 protein family members govern the cellular fate of cancer cells in response to chemotherapy: apoptosis or survival.
- Mapping the Bcl-2 protein–protein interaction patterns is important to decipher apoptotic blocks in cancer cells.
- Affinity-based protein isolation and mass spectrometry, BH3 profiling and antibody microarrays are potential powerful tools to construct the Bcl-2 protein signatures in cancer cells with different phenotypes.

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