Flexibility and Specificity of the Interaction of MCL-1 with BIM BH3

by

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Abstract

Interactions among proteins of the BCL-2 family regulate apoptosis – the process of programmed cell death. This thesis focuses on interactions between anti-apoptotic BCL-2 proteins and BH3 peptides derived from pro-apoptotic BCL-2 proteins, observed in earlier studies to occur with significant selectivity. In order to better understand determinants of specificity in anti-apoptotic/BH3 interactions, I have employed structural and binding studies of BIM BH3 point mutants with anti-apoptotic proteins.

I report X-ray crystal structures of MCL-1 in complex with peptides of wild-type and three point mutant sequences of BIM BH3. Together, these structures exhibit a range of conformations within the complex. Structural change with respect to wild-type is most dramatic for an isoleucine-to-tyrosine mutant of BIM (I2dY). The larger residue is accommodated by helix α3 of MCL-1 and the BIM BH3 peptide helix shifting away from one another. In a phenylalanine-to-glutamate mutation (F4aE), the altered side chain is accommodated by a simple rotation of the side chain out of the hydrophobic pocket, a more modest structural change overall.

I have also adapted SPOT array technology to qualitatively and simultaneously measure the interactions of anti-apoptotic BCL-XL and MCL-1 with a large number of surface-bound BH3 peptides. The best results were obtained when (i) longer BH3 peptides (26-mer vs. 20-mer) were employed, and (ii) antibodies were used to detect the binding of anti-apoptotic proteins. The SPOT technique was used to perform substitution analyses, wherein each position of interest in a BH3 peptide is mutated to all possible amino acids (or a subset thereof). The results of these experiments identify several sites that, in the context of BIM, play a role in discriminating between MCL-1 and BCL-XL. Two of these sites are always occupied by small amino acids in known native BH3s, while two others are typically hydrophobic positions that become buried upon interaction with anti-apoptotic BCL-2 proteins.

Together, these studies illustrate that the MCL-1 interaction with BIM BH3 is flexible enough to accommodate numerous point mutations, and that certain sites within BIM can be used to encode interaction specificity for both MCL-1 and BCL-XL.

Thesis Supervisor: Amy E. Keating
Title: Associate Professor
Dedication

For my parents
Acknowledgements

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BCL-2 proteins are regulators of cell death. Members of this family interact with one another to regulate apoptosis. BCL-2 family interactions are structurally conserved and are mediated by an α-helical region of a pro-apoptotic BCL-2 protein binding into a hydrophobic groove on the surface of anti-apoptotic BCL-2 proteins, as observed in numerous high-resolution structures. In this thesis, I examine structure and specificity of the interaction of MCL-1, an anti-apoptotic protein, with the BH3 region of BIM, a pro-apoptotic protein. Therefore, in this chapter, I will summarize what is known about:

(i) how the BCL-2 proteins are central to cell death,
(ii) biochemical and structural studies that have been performed to characterize BCL-2 family interactions, and
(iii) how BCL-2 family interactions can be inhibited by small molecules.
1.1 Cell death pathways

Healthy multi-cellular organisms require homeostasis: too many or too few of any one type of cell can be detrimental. Thus, individual cells need the ability to respond to cues that indicate damage or stress, along with the ability to act upon those cues to commit cellular suicide for the benefit of the organism as a whole. This process of programmed cell death is called apoptosis. Loss of regulation in apoptosis can result in a variety of problems, including cancer, autoimmunity, and neurodegenerative disease [12]. BCL-2 proteins are central to regulation of apoptosis, and are the focus of this study.

Once a cell commits to apoptosis, e.g. after receiving a pro-death signal, the actual act of killing is taken on by caspases, a family of cysteine proteases that cleave after aspartic acid residues [13]. Caspases are synthesized as inactive enzyme precursors known as zymogens in normal, healthy cells, but become activated through auto-proteolysis in response to a variety of death-inducing stimuli [14]. Caspases come in two varieties: initiators and effectors. The initiator caspases, such as caspase-8 and caspase-9, function early in the apoptotic process. After these initiators are activated, they cleave and activate effector caspases - the “workhorses of apoptosis”. These proceed to dismantle the cell by acting upon substrates, including laminin, poly (ADP-ribose) polymerase (PARP), and cell cycle proteins [12].

In C. elegans, the mechanism of caspase activation (summarized in Figure 1.1) was elucidated through genetic and biochemical studies of programmed cell death. CED-3 is the caspase responsible for apoptosis and is the only caspase expressed in C. elegans [15] (Figure 1.1). In cells that are fated to die, CED-3 becomes active when tetrameric CED-4 binds CED-3 zymogens. The close proximity of CED-3 molecules promotes autocatalysis, forming the active CED-3 dimer [12]. This caspase activation is normally regulated by CED-9, which forms a complex with dimeric CED-4, thus preventing CED-4 from binding and activating CED-3 [16]. Upon receiving death stimuli, EGL-1 binds CED-9, releasing CED-4 to form a complex with CED-3, leading to auto-activation and eventual cell death [17].

In mammals, there are two caspase-dependent apoptosis pathways (Figure 1.1). In the extrinsic death receptor pathway, tumor necrosis factor ligands bind death receptors, leading to the recruitment and activation of caspase-8 through adaptor proteins via the death-inducing signaling complex (DISC). Active caspase-8 cleaves other caspases, including the effector
caspase-3. The intrinsic apoptosis pathway, which looks similar to the *C. elegans* programmed cell death pathway, is regulated by a family of CED-9 homologues known as the BCL-2 proteins. When activate, pro-apoptotic BCL-2 protein, BAX and BAK, are capable of causing mitochondrial outer membrane permeabilization (MOMP), releasing a variety of pro-apoptotic proteins from the mitochondria, including cytochrome c and Smac/Diablo. The now cytosolic cytochrome c forms a complex with APAF-1, a CED-4 homolog, and procaspase-9, a zymogen [18]. The resulting ‘apoptosome’ complex activates caspase-9 [19], which in turn cleaves caspase-3, a CED-3 homologue. Additionally, the extrinsic pathway can feed through the mitochondrial pathway via cleavage of a BCL-2 protein, BID. This produces a feed forward loop that has been found to be crucial for the rapid and complete execution of apoptosis by the cell [20].

**Figure 1.1  Cell death pathways**
A simplified schematic of the cell death pathways in worms (*C. elegans*) and humans, both intrinsic and extrinsic.
The BCL-2 family regulates mitochondrial permeabilization, but is also poised to regulate death-ligand induced apoptosis. Thus, these proteins are of interest for multiple reasons, including their mechanism of tight regulation and their potential as therapeutic targets. They also provide an interesting model to study the protein-protein interactions of helical proteins.

1.2 Discovery of the BCL-2 family

The first mammalian proteins shown to regulate the intrinsic mitochondrial death pathway were identified in a follicular lymphoma where a t(14;18) chromosomal translocation linked the bcl-2 (B-cell lymphoma 2) gene to an immunoglobin locus, thus leading to its constitutive expression in B cells [21-23]. This was the first proto-oncogene identified where cell survival, rather than cell proliferation, was enhanced [24]. Evasion of apoptosis has since been established as a hallmark of cancer [25].

Identification of BCL-2 as an inhibitor of programmed cell death prompted discovery of several proteins related to BCL-2 in sequence and function. In 1993, the homologous proteins were classified as the BCL-2 family. Three members with protective function were identified: bcl-x was cloned on the basis of its similarity to bcl-2 [26], mcl-1 was isolated from the ML-1 myeloid leukemia cell line [27], and a1 was found to be induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) [28]. In addition to these anti-apoptotic proteins, BAX – a protein that interacts with BCL-2 – was identified. While this protein shares significant sequence similarity with BCL-2 (21% identity), it promotes apoptosis rather than protecting against it [29]. In the following years, even more examples of both anti- and pro-apoptotic BCL-2 homologues were identified. Two additional cellular anti-apoptotic proteins identified were BCL-W [30] and BCL-B [31, 32]. Pro-apoptotic BCL-2 proteins have been found to include BAK [33], BIK [34], BAD [35], BID [36], BOK/Mtd [37], Hrk [38], BIM [39], Noxa [40], PUMA [41], and BMF [42].

In addition to mammalian members of the BCL-2 family identified over the last couple of decades, several BCL-2 homologues in viruses have also been discovered. A viral BCL-2 homologue – BHRF1 – was identified within the Epstein-Barr virus, and was found to protect human B cells from apoptosis despite very low similarity to the mammalian sequences [43]. In adenovirus, the E1B 19K protein has been found to prevent apoptosis by inhibiting pro-apoptotic
Similarly, from Kaposi’s sarcoma-associated herpesvirus, a BCL-2 homolog was identified that inhibits BAX and interacts with cellular BCL-2 family members [45]. Anti-apoptotic BCL-2 homologues have also been identified in vaccinia virus [46-48].

1.3 Sequences and structures of the BCL-2 family

All members of the BCL-2 family regulate apoptosis – either promoting or protecting against it. Family members also share sequence similarity and can include one or more of four BCL-2 homology (BH) regions (Figure 1.2) [49]. Though typically referred to as BH “domains,” these regions of homology correspond to α helices and loop regions, not autonomously folding substructures [50-52]. BCL-2 homologues have been classified into three subfamilies: the anti-apoptotic BCL-2 subfamily, the pro-apoptotic multi-BH region BAX-like subfamily, and the pro-apoptotic BH3-only subfamily (Figure 1.2). It is now well established that protein-protein interactions between BCL-2 proteins, particularly between subfamilies, regulate apoptosis.

<table>
<thead>
<tr>
<th>Anti-apoptotic BCL-2 subfamily</th>
<th>Family members</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH4</td>
<td>BCL-2, BCL-XL, BCL-W, MCL-1, A1/BFL-1, BCL-B</td>
</tr>
<tr>
<td>BH3</td>
<td></td>
</tr>
<tr>
<td>BH1</td>
<td></td>
</tr>
<tr>
<td>BH2</td>
<td></td>
</tr>
<tr>
<td>TM</td>
<td></td>
</tr>
</tbody>
</table>

Pro-apoptotic subfamilies

- multi-BH

| BH3 | BH1 | BH2 | TM |
|--------------------------------|
| BAX, BAK, BOK |

- BH3-only

| BH3 | TM |
|--------------------------------|
| BAD, BID, BIK, BIM, BMF, Hrk, Noxa, PUMA |

Figure 1.2 BCL-2 proteins

BH regions generally present in each subfamily are shown in a schematic representation of the protein sequences.

Mammalian members of the anti-apoptotic subfamily include BCL-2, BCL-XL, BCL-W, MCL-1, A1, and BCL-B. These anti-apoptotic proteins usually include all four BH regions, as well as a membrane-spanning anchor (Figure 1.2). The first anti-apoptotic BCL-2 family structure to be solved was that of BCL-XL [51]. NMR and X-ray crystal structures of BCL-XL show an α-helical protein, with two predominantly hydrophobic core helices surrounded by six smaller helices (Figure 1.3). The closest known structural homologues were bacterial toxin proteins, particularly diphtheria toxin and colicin, in which core helices insert into membranes to
Figure 1.3 Structures of BCL-2 proteins
The structures of BCL-2 proteins are shown, rainbow colored from the N-terminus in blue to the C-terminus in red. The top row (BCL-2, BCL-XL, BCL-W, and MCL-1) are mammalian anti-apoptotic proteins. The second row (BAX, BAK, and BID) are mammalian pro-apoptotic proteins. The proteins in the last two rows are structural homologues of the BCL-2 family from *C. elegans* (CED-9) or viruses. These proteins share a similar overall eight α-helix fold. BAX and BCL-W have an additional helix at the C-terminus that binds across the groove (shown in red, discussed in text).
form pores [51]. The surface of BCL-X\textsubscript{L} reveals a hydrophobic groove partially formed by BH regions 1-3, with which pro-apoptotic proteins are proposed to interact (Figure 1.4). This was subsequently shown to be the case in structures of BCL-X\textsubscript{L} in complex with BH3 peptides of BAK, BAD, BIM, and Beclin [4, 53-55].

The structures of most of the known mammalian anti-apoptotic receptors have now been solved and show similar overall structures (Figure 1.3). BCL-2 was solved by NMR as a chimera, in which the flexible loop between \( \alpha1 \) and \( \alpha2 \) was replaced with BCL-X\textsubscript{L} sequence, due to insolubility of the BCL-2 protein [56]. BCL-2, BCL-X\textsubscript{L}, and MCL-1 all have eight \( \alpha \) helices: two central hydrophobic helices surrounded by amphipathic helices. The backbone RMSD between the structures is less than 2\text{Å}, not including the variable, flexible loop between helix 1 and helix 2 [57, 58]. The structure of BCL-W is similar to its anti-apoptotic family members, but it has an additional helix (formed by residues 157-173), C-terminal to the core BCL-2 fold and that binds into the hydrophobic groove formed by BH1-3 [59, 60]. This helix, immediately N-terminal to the transmembrane region, can be displaced by the binding of BH3 peptides [58, 61]. These anti-apoptotic BCL-2 proteins are also referred to as ‘receptors’, due to the hydrophobic groove capable of binding BH3 peptides.
The multi-BH-region, pro-apoptotic BCL-2 subfamily is similar in sequence to the anti-apoptotic subfamily, including three of the four BH regions (BH1-3) and a transmembrane domain. This family includes BAX, BAK, and BOK; the NMR structure of BAX and the X-ray crystal structure of dimeric BAK have been solved. They have the same fold as their anti-apoptotic counterparts. The backbone RMSD between BAX and BCL-XL is 3.2 Å [62]. Though these pro-apoptotic proteins have no region with sequence similarity to the BH4 domain (Figure 1.2), there is an α helix in the same position as the BH4 α helix observed in the structures of the anti-apoptotic proteins. BAX also has a C-terminal helix that binds the hydrophobic cleft in very much the same manner as BCL-W [63, 64]. The hydrophobic groove of BAK is short and narrow in comparison to the groove on the anti-apoptotic homologues [65], making it less likely to bind a BH3-like helix at this site.

Members of the BH3-only subfamily share sequence homology with other subfamilies solely in the BH3 region. Despite low sequence similarity and lack of BH1, 2, and 4, the structure of BH3-only protein BID is similar to that of BCL-XL [66, 67]. However, due to extreme variations in sequence length and the presence of multiple BH3 regions in some sequences, it is unlikely that all BH3-only proteins are structurally similar to the multi-BH region BCL-2 proteins. There is evidence that some of the BH3 proteins, including BIM, BAD, and BMF, may be intrinsically unstructured and change conformation locally in the BH3 region upon binding to BCL-2 receptors [68].

There are a growing number of viral BCL-2 homologues, as discussed above. Most appear to be protective, but sequence similarity to cellular BCL-2 proteins varies greatly [69]. Structures of many of these proteins have been solved showing the same overall fold as the cellular proteins (Figure 1.3). Viral BCL-2 homologues are capable of binding pro-apoptotic proteins via BH3 regions, like their cellular counterparts [46, 70, 71]. Structures of several herpesvirus BCL-2 homologues have been solved: BHRF1 from Epstein-Barr virus, ksBCL-2 from Kaposi Sarcoma-associated herpes virus, and γHV68-BCL-2 from γ-herpesvirus 68 [71-73]. These viral proteins have the BCL-2 fold (Figure 1.3), and have RMSD less than 3Å with cellular BCL-2 family members. Additionally, BCL-2 homologues have been identified in vaccinia and myxoma viruses, N1L and M11L, respectively. While these proteins have very weak sequence similarity to the mammalian BCL-2 proteins, they are structurally homologous (Figure 1.3) and can also bind mammalian pro-apoptotic family members [46, 74, 75].
1.4 BCL-2 protein-protein interactions

The BCL-2 family of proteins regulates apoptosis through protein-protein interactions. The pro-apoptotic, multi-BH-region proteins, including BAX and BAK are thought to promote apoptosis by homo-oligomerization, compromising the integrity of the outer mitochondrial membrane and allowing the release of cytochrome c and other proteins. Cytochrome c and APAF-1 activate initiator caspase-9 via the apoptosome complex, leading to eventual cell death [19]. The anti-apoptotic BCL-2 subfamily promotes survival by preventing this BAX/BAK-dependent mitochondrial dysfunction, almost certainly via their direct interaction with these pro-apoptotic proteins [12, 53, 76, 77]. The BH3-only proteins promote apoptosis by multiple mechanisms: inhibition of the anti-apoptotic BCL-2 proteins and activation of the BAX and BAK proteins (Figure 1.1). Computational modeling of the death receptor pathway with ordinary differential equations (ODEs) has shown that BCL-2 protein interactions play an important role in extrinsic apoptosis, even when only three proteins (BCL-2, BAX, and BID) are included in the model [1].

BH3-only proteins bind to anti-apoptotic BCL-2 proteins, thereby inhibiting their protective function of sequestering pro-apoptotic BAX and BAK. The more BCL-2 proteins a BH3-only protein can bind, the greater its effectiveness at inducing apoptosis [7]. Some BH3-only proteins, like BID, are also thought to promote apoptosis directly by activating the BAX/BAK proteins, leading to the oligomerization of these multi-BH region pro-apoptotic proteins and the compromise of the mitochondrial membrane [11, 78]. Recent studies suggest that these ‘activator’ BH3s directly bind the first α helix of BAX, although this interaction may be transient [79, 80]. If enough anti-apoptotic proteins are present, activators may be sequestered, preventing their pro-apoptotic function. Competitive binding of BH3-only peptides can release activators from this inhibition. BH3-only proteins are considered to be transmitters of death signals, being activated by various stresses or cues and relaying the death signal to the rest of the BCL-2 pathway [81].

Protein-protein interactions among BCL-2 family members are responsible for regulating apoptosis, as described above, and nearly all BCL-2 members interact with another BCL-2 protein [13]. A number of interactions, both homodimeric and heterodimeric, have been observed via yeast two-hybrid screens or co-immunoprecipitation experiments [82, 83]. Recently, considerable attention has been focused on the interactions of anti-apoptotic BCL-2
proteins with peptides corresponding to the BH3 region of pro-apoptotic proteins. In several extensive studies, the interactions between eight BH3 regions and five anti-apoptotic BCL-2 protein have been measured using SPR and fluorescence polarization [7, 10]. These two methodologies gave similar binding results (Figure 1.5) and have established that the strength of different interactions varies greatly depending on the BH3 and the BCL-2 protein involved. There are, however, some discrepancies in the relative affinities that have been reported, possibly due to differences in the BH3 peptide lengths and measurement techniques used.

Many structures of anti-apoptotic proteins in complex with BH3 peptides have been solved (Figure 1.6) [16, 46, 53-55, 84-88]. These uniformly show the BH3 peptide binding to a groove formed by BH1-3 of the anti-apoptotic protein. While there is some shifting and bending of the BH3 and receptor helices, the overall structures of the complexes are highly similar. BH3 peptides bind BCL-2 receptors as amphipathic α-helices, burying a hydrophobic face into the groove formed by BH1-3. This hydrophobic face of BH3s is comprised of four residues that follow a heptad repeat, reminiscent of that in coiled coils (Figure 1.7). The 2d, 3a, 3d, and 4a positions are generally hydrophobic and pack into the groove on the receptor surface. Various positions within the BH3 have been determined to be important binding BCL-2 receptors.

Examination of the various binding affinities (Figure 1.5) reveals the specificity of some BH3 peptides for certain BCL-2 receptors. Some BH3-only peptides, such as BIM and PUMA, bind all anti-apoptotic BCL-2 proteins with low nanomolar affinity. However, others exhibit more selective binding. For example, Noxa binds tightly to MCL-1 and A1, but not to BCL-2, BCL-X_L, or BCL-W. Conversely, BAD binds the latter three anti-apoptotic proteins with high affinity, but has little or no affinity for MCL-1 or A1.
Figure 1.6 Structures of BCL-2/BH3 complexes
The name of the BCL-2 protein and the protein from which the BH3 region is derived are indicated below each structure, along with the PDB ID. The bound BH3 peptide is shown in cyan while the BCL-2 receptor is shown in green. The dark blue helix in BCL-XL/Beclin is the first helix from a domain-swap dimer that is likely non-physiological. The first row shows the BCL-XL complexes. The second row shows MCL-1 complexes. The third row shows A1/BFL-1 complexes. The last row shows complexes of BCL-W with BID, *C. elegans* CED-9 with EGL-1, and vaccinia virus M11L in complex with human BAK BH3 peptide (left-to-right).
## Table 1.7 Native and mutant BH3 sequences

BH3 regions of a selection of human BH3-only proteins shown with the heptad repeat on top [2] and binding preference for MCL-1 or BCL-X\(_L\). The four hydrophobic positions are in gray. Mutants of BIM and Noxa, referred to in the text, are shown.

<table>
<thead>
<tr>
<th>BH3 peptide</th>
<th>sequence</th>
<th>anti-apoptotic preference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAD</td>
<td>NLWAAQRYGRELRRMSDEFVDSFKKG</td>
<td>BCL-X(_L)</td>
</tr>
<tr>
<td>BID</td>
<td>QEDIIRNIARHLAQVGDSDRISPPG</td>
<td>BCL-X(_L), MCL-1</td>
</tr>
<tr>
<td>BIM</td>
<td>DMRPEIWAQEELRRGDEFNAYYARR</td>
<td>BCL-X(_L), MCL-1</td>
</tr>
<tr>
<td>Noxa</td>
<td>PAELEVECATQELRRFGDKINFRQKLL</td>
<td>MCL-1</td>
</tr>
<tr>
<td>PUMA</td>
<td>EEQWAREIGAQLRMADDLEAQQYERR</td>
<td>BCL-X(_L), MCL-1</td>
</tr>
<tr>
<td>BIM 2A</td>
<td>DMRPEIWAQEELRRGDEFNAYYARR</td>
<td>MCL-1</td>
</tr>
<tr>
<td>BIM 1</td>
<td>DMRPEIWAQEELRRGDEFNAYYARR</td>
<td>MCL-1</td>
</tr>
<tr>
<td>BIM 2</td>
<td>DMRPEIWAQEELRRGDEFNAYKARR</td>
<td>BCL-X(_L)</td>
</tr>
<tr>
<td>Noxa m3</td>
<td>PAELEVECATQELRRGDKINFRQKLL</td>
<td>BCL-X(_L), MCL-1</td>
</tr>
</tbody>
</table>

Several studies have examined the role of certain BH3 residues in determining interaction specificity. Noxa has been shown to be less specific when bearing two point mutations (Figure 1.7): Phenylalanine at position 3d and lysine at 3g were mutated to isoleucine and glutamate, respectively; both changes correspond to replacing the Noxa residue with the amino acid present in the promiscuous BH3-peptide BIM. These mutations enable the Noxa mutant to bind BCL-X\(_L\) and BCL-W, in addition to MCL-1 [89]. BIM, normally capable of binding all anti-apoptotic BCL-2 proteins, has also been engineered to bind MCL-1 over BCL-X\(_L\) (like Noxa). Alanine scanning mutagenesis led Lee et al. to make a double mutant of BIM in which both leucine at 3a and phenylalanine at 4a were mutated to alanine. This BIM variant (BIM 2A) has a >1000-fold preference for MCL-1 over BCL-X\(_L\) [5]. Boersma et al. similarly generated a specific BIM variant, with approximately 1000-fold preference for MCL-1, by mutating three sites to glutamate (BIM 1 in Figure 1.7). In addition, a BCL-X\(_L\)-specific BIM variant was generated, containing two mutations (BIM 2 in Figure 1.7). This peptide had an ~100-fold preference for BCL-X\(_L\) over MCL-1 [6]. Further, computational design of BCL-X\(_L\)-binding peptides generated peptides that bound BCL-X\(_L\) with nanomolar affinity but showed no appreciable binding of MCL-1 [8]. These BH3-derived peptides present an important starting point for exploring the space of sequences selective for BCL-X\(_L\) or MCL-1.
1.5 Regulation of BCL-2 in cells

The studies mentioned above examine interactions among the BCL-2 proteins in vitro. While the interactions observed are likely relevant, it is important to remember that many other factors may be regulating these interactions in the context of the cell. For example, most in vitro studies have used purified BCL-2 proteins that lack the C-terminal transmembrane domain. While this keeps the proteins soluble in these biochemical studies, in the cell, most of these proteins reside at mitochondrial membranes, and to some extent the ER membrane [90, 91].

Additionally, most of the studies above use peptides based on the BH3 region of BH3-only proteins. While studies have shown that these peptides are competent for inducing apoptotic hallmarks, including mitochondrial permeabilization [11, 78], much of the protein that is missing may play a role in modulating their ability to interact. Indeed, many of the BH3-only proteins are physically prevented from interacting with the BCL-2 proteins in non-apoptotic cells via other non-BH3 parts of the protein. For example, BAD is phosphorylated by growth factor signaling at sites outside of the BH3 region [92, 93]. Phosphorylated BAD then interacts with 14-3-3, keeping it sequestered in the cytosol, away from the anti-apoptotic proteins at the mitochondrial membrane [94]. BIM and BMF are both sequestered away from the mitochondria by association with motor protein complexes: dynein light chain 1 (DLC1) at microtubules [95] and DLC2 in myosin V at actin-cytoskeleton [42], respectively. BID, on the other hand, is normally in the cytosol, folded such that the interacting face of its BH3 region is inaccessible (Figure 1.3). Upon signaling via the death receptor pathway, BID is cleaved by caspase-8, exposing a glycine that is N-myristoylated, allowing for localization to the mitochondrial membrane, where it can interact with other BCL-2 proteins via its exposed BH3 region [96-98].

Cellular localization and the role of other regions of the protein are thus important considerations when evaluating the binding affinity of these proteins, since they may affect the in vivo interaction specificity. Nevertheless, interaction specificity does seem to be encoded in the BH3 region itself, as seen when the BH3 region of BIM is replaced by the BH3 regions of PUMA, Noxa, and BAD [7]. Thus, the studies of anti-apoptotic BCL-2 interactions with BH3 peptides remain pertinent to the BCL-2 field.
1.6 BCL-2 inhibitors as anti-cancer drugs

The discovery of bcl-2 as a proto-oncogene, followed by elucidation of the biochemistry of the proteins of this family, has led to many studies targeting these proteins for the development of anti-cancer therapeutics. The Gellman group has developed α/β-peptides (using a combination of α and β amino acids) that bind BCL-XL. These peptides have versatility of sequence space, like normal α peptides, but are resistant to degradation in vivo due to the non-native β amino acids [99]. The development of stapled peptides has also generated peptides that are both more stable than traditional peptides and allow for large sequence diversity [100, 101]. These stabilized peptides have allowed for the identification of the site of interaction between BIM BH3 (as a stapled peptide) with BAX [80]. Additionally, high-throughput experimental and computational screening has led to the identification of several lead compounds. For example, HA14-1, antimycin A, and chelerythrine are capable of displacing BAX or BAK from anti-apoptotic proteins, and therefore induce apoptosis [102-105]. However, these compounds, along with many others found from screens, have micromolar affinity and have not progressed to clinical trials [106].

Abbott Laboratories generated the first low nanomolar affinity inhibitor of BCL-2 proteins. ABT-737 was made by linking two small molecule leads identified by NMR to bind adjacent pockets in the hydrophobic groove [107]. The crystal structure of BCL-XL with ABT-737 shows that this compound binds in the hydrophobic groove, with BCL-XL in a conformation similar, but not identical, to the BH3-peptide bound state [3] (Figure 1.8). ABT-737 has a high affinity for BCL-2, BCL-XL, and BCL-W (Ki < 1 nM for all three), but does not bind MCL-1 or A1 (Ki > 1μM for both) [107]. It has been shown to dissociate BCL-2/BAX heterodimers in cells and to increase the survival of mice with Raf-induced leukemia [108]. ABT-737 was also able to increase the survival of mice with myc/BCL-2-induced lymphomas, but not of those with myc/MCL-1-induced lymphomas [104], highlighting that the interaction specificity observed in in vitro binding experiments correlates with the effectiveness against cancers induced by different BCL-2 proteins. An orally bio-available version of the molecule, ABT-263, is in clinical trials for the treatment of a variety of leukemias, lymphomas, and myelomas, as well as lung cancer [106, 109].
Gossypol is a natural compound isolated from cotton seeds and roots that is known to have anti-cancer effects. The (-) enantiomer (marketed as AT-101 by Ascenta) was found to have cytotoxic effects [110]. AT-101 is capable of binding BCL-2, BCL-X\(_L\), and MCL-1, with IC\(_{50}\) values of 320 nM, 480 nM, and 180 nM, respectively [111]. However, in multiple myeloma cell lines, the IC\(_{50}\) rises into the low micromolar range [112]. This compound induced apoptosis in CLL (chronic lymphocytic leukemia) patient cells. Gossypol has served as a starting point for the design of new molecules that bind the BCL-2 proteins with improved affinity, although these have not yet made it to clinical trials [106, 111].

Obatoclax (GX-015-070 by GeminX), a compound optimized from a natural product library screen lead, has low micromolar affinity for BCL-2, BCL-X\(_L\), BCL-W, and MCL-1 [113]. This compound disrupts MCL-1/BAK complexes, induces apoptosis in a BAX/BAK-
dependent manner, and disrupts MCL-1/BAK interaction in cells [114]. For a variety of multiple myeloma cell lines, the IC50 is generally less than 1 μM [115]. However, a possible side effect of obatoclax is neurological toxicity, which is observed with bolus injections in mice [115].

There are many compounds that induce apoptosis that are currently studied as potential BCL-2 inhibitors. The most promising, discussed above, are in clinical trials. The highest affinity BCL-2 inhibitors, the ABT-737 and ABT-263 compounds, are specific to BCL-2, BCL-XL, and BCL-W and do not bind MCL-1, A1, or BCL-B [113]. Thus, these drugs are ineffective in cancers that depend on the protection provided by these later proteins, leaving the possibility of resistance due to up-regulation of these receptors [104, 116]. However, the inhibitors capable of binding MCL-1, in addition to BCL-XL and BCL-2, though at a lower affinity, have potential side effects, such as with obatoclax. Specific inhibitors may be able to overcome some of these drawbacks. What is learned about the specificity of the BH3 interactions with the BCL-2 proteins can be applied to the generation of more specific small-molecule BCL-2 inhibitors.

1.7 Conclusions and summary of thesis

Extensive understanding of the specificity of BCL-2 protein-protein interactions is important for elucidating the regulation of apoptosis by the BCL-2 proteins. Identification of binding specificity determinants will aid in the design of peptides that specifically bind one or a number of these proteins, and this information can potentially be applied to anti-cancer therapeutic development.

In this thesis, I discuss my work on determinants of BCL-2 family specificity. In Chapter 2, I present the structures of MCL-1 in complex with BIM BH3 and BIM point mutants. Both the receptor and the peptide exhibit flexibility. Such structural plasticity likely underlies aspects of the diverse specificity profiles observed among the native BH3 sequences and anti-apoptotic BCL-2 proteins. In Chapter 3, I discuss application of SPOT-array technology to the BH3/BCL-2 interaction. BH3 peptides were synthesized on a membrane surface, which was then probed using a BCL-2 protein in solution. This assay allows for qualitative testing of many interactions between BH3 peptides and BCL-2 proteins, and has been used to test 170 different BIM point mutants. This study reveals several sites within BIM – A2e, I3d, G3e, and F4a – that may play a role in specificity. In the context of the structures, these sites may point to other regions of
flexibility. Additionally, I have begun to examine the role of multiple BCL-2 proteins, with differing interaction specificities, in death receptor-mediated (extrinsic) apoptosis using modeling with differential equations, described in the Appendix.
Chapter 2

Structures of MCL-1 bound to wild-type and mutant BIM BH3 peptides display flexibility

MCL-1 is an anti-apoptotic BCL-2 protein that protects cells against death, via interactions with pro-apoptotic BCL-2 proteins. Structures of MCL-1, and other BCL-2 proteins, reveal a surface groove into which the α-helical BH3 regions of pro-apoptotic proteins bind, burying four conserved hydrophobic residues. In this chapter, I report the crystal structure of human MCL-1 bound to a BIM BH3 peptide. Compared to the peptide-free murine MCL-1 structure, the surface groove widens to accommodate binding of the BIM BH3 peptide. This complex is similar to the murine BCL-X\textsubscript{L}/BIM BH3 structure, with differences seen in the conformation of the α3 region of the anti-apoptotic proteins. In addition, I solved the structures of three BIM BH3 point mutants, each a variation at a buried hydrophobic position, in complex with MCL-1. These structures show that MCL-1 can make small changes to fit the mutant peptides. For example, a shift in a leucine side chain fills a hole left by an isoleucine-to-alanine mutation at the first hydrophobic buried position of BIM BH3. More drastic changes are also possible, with shifting of the α3 helix to accommodate an isoleucine-to-tyrosine mutation at this same position. In this complex, the mutant BIM peptide also bends to allow room for the tyrosine. In the third mutation, a phenylalanine-to-glutamate mutant shows that while MCL-1 does not change, the glutamate rotates out of the groove to allow binding. These structures combine to show flexibility in MCL-1 and BH3 peptide that allows different BIM BH3 sequences to bind.

Collaborators:
Dr. Stefano Gulla performed the fluorescence polarization binding assays (Table 2.2). Dr. Robert Grant provided support in X-ray data collection, processing, and refinement for all of the crystal structures. DelPhi electrostatics calculation for Figure 2.4 was done Scott Chen.
2.1 Introduction

Anti-apoptotic BCL-2 proteins protect cells from programmed death. Death signals from a variety of cues, including DNA damage, growth factor withdrawal, and the presence of death ligands are transmitted through many different pro-apoptotic BH3-only BCL-2 proteins to induce apoptosis [12]. Some BH3-only proteins are able to activate pro-apoptotic BAX and BAK proteins, leading to the permeabilization of the outer mitochondrial membrane [78]. Once released from the inter-membrane space of the mitochondria, other pro-apoptotic proteins, including Smac/Diablo and cytochrome c, activate a caspase cascade, leading to the death of the cell [12]. However, if activator BH3s, or the BAX and BAK proteins, are sequestered by anti-apoptotic BCL-2 proteins, cell death is averted [7]. Thus, the interactions of these life-saving proteins with the messengers and executioners of death are crucial to the survival of the cell.

In humans, there are six protective BCL-2 proteins: BCL-2, BCL-X\textsubscript{L}, BCL-W, BCL-B, MCL-1 and A1/BFL-1. Of these proteins, BCL-X\textsubscript{L} has been most extensively studied; however, much has also been learned about the other family members. The least characterized is BCL-B, which has little in the way of comparative studies. These proteins share a structurally conserved hydrophobic groove, into which the BH3 α-helix from pro-apoptotic proteins can bind [4, 53, 54]. It has been demonstrated that peptides derived from the BH3 region of these pro-apoptotic proteins are sufficient for binding to anti-apoptotic BCL-2 proteins and promoting mitochondrial permeabilization, a hallmark of apoptosis [4, 11, 78]. The affinity of anti-apoptotic receptor proteins for various BH3 peptides has been determined by a number of groups. While differences in reagents and methods have lead to significant variability in reported results, certain trends are clear: (i) Some BH3 peptides bind with high affinity to all anti-apoptotic receptors. (ii) Some bind BCL-X\textsubscript{L}, BCL-2, and BCL-W proteins (that have comparatively high sequence identity) with high affinity, but not the more distantly related MCL-1 and A1; other BH3 peptides have the reverse specificity (Figure 1.5). Thus, BCL-X\textsubscript{L} and MCL-1 have distinct BH3 interaction profiles.

While MCL-1 has very similar structure to other BCL-2 proteins (backbone RMSD less than 2Å), it only shares ~25% identity with other family members [2] and has a significantly different BH3 binding profile (Figure 1.5). Most notably, MCL-1 binds to Noxa and not BAD, a reversal of specificity when compared to most other anti-apoptotic receptors. MCL-1 is a highly
regulated protein, both at the level of expression, degradation, and post-translational modification [117]. It also has a short half-life, due to the role of its amino-terminal PEST domain in targeting proteins for proteasomal degradation [2]. MCL-1 was isolated from human myeloid leukemia cells [27] and has been shown to be critical in immune cell development and maintenance [118].

MCL-1 plays an interesting role in the regulation of apoptosis. In response to TRAIL-induced apoptosis, MCL-1 has been observed to be cleaved by caspase-3, which was reported to endow it with a pro-apoptotic function [119]. In healthy cells, MCL-1 has been observed to interact with BAK. In this case, MCL-1 sequesters BAK, preventing its pro-apoptotic function, until competitive binding of another BH3 peptide removes it [9]. BCL-XL also appears to function to sequester BAK. Though BCL-XL and MCL-1 share a similar function of protecting the cell from death via similar structure, these two proteins have very different binding specificities, expression patterns, and regulation.

Targeting the BCL-2 family of proteins is an appealing way to treat cancer. Evading apoptosis is one of the hallmarks of cancerous cells [25]. Up-regulating the anti-apoptotic BCL-2 proteins is one way to avoid cell death; thus a small molecule capable of inhibiting these anti-apoptotic proteins may be an effective anti-cancer therapeutic.

One of the most promising BCL-2 inhibitors was developed at Abbott Laboratories. ABT-737, and its orally available counterpart, ABT-263, have low nanomolar affinity for BCL-2, BCL-XL, and BCL-W and are in Phase I/II clinical trials [107, 109]. Reminiscent of BH3 peptide binding preferences, many small-molecule inhibitors bind the highly related proteins BCL-2, BCL-XL, and BCL-w, while showing little or no binding to MCL-1 and A1. This inability to inhibit MCL-1 is significant, as up-regulation of MCL-1 appears to be a major source of resistance to the Abbott compounds [104, 120]. There are other molecules in clinical trials, Gossypol and Obatoclax, that target a broader range of BCL-2 proteins, including MCL-1 [111, 113]. However, these bind with lower affinity than the Abbott drugs. Additionally, Obatoclax may be dose limited due to neurological toxicity [115]. Thus, the ability to specifically target individual BCL-2 proteins may be beneficial to the advancement of cancer therapeutics, enabling the ability to selectively target each BCL-2 protein without the potential side effects of the pan-specific inhibitors.
The specificities of BH3 peptides for BCL-2 proteins, particularly for BCL-X\(_L\) vs. MCL-1, vary significantly. The sequences of the BH3 peptides are also highly variable (Figure 2.1). Consequently, the determinants of the specificity are currently unclear. However, analysis of structures and sequences may enable the elucidation of such determinants.

The helical nature of the BH3 sequence, when bound to the BCL-2 receptor, places one face of the amphipathic helix in the receptor hydrophobic groove. The residues buried in the interface follow a heptad repeat with “a” and “d” positions being buried—a characteristic also found in coiled coils [2] (Figure 2.1). Four hydrophobic residues, corresponding to positions 2d, 3a, 3d, and 4a, from the peptide, are buried in the interface with BCL-X\(_L\) in structures of complexes with BH3 peptides. Position 3a is absolutely conserved as leucine in the known, native, BH3-only proteins. When mutated to alanine in BAK and BID, binding to BCL-X\(_L\) is completely lost [78, 121]. However, peptides with these sites mutated to large hydrophobic amino acids still bind the receptor tightly [3, 8], and the 3a binding pocket on the BCL-X\(_L\) surface can similarly be filled by a phenyl group from the small molecule BCL-2 inhibitor ABT-737 [3].

Previous studies have begun addressing the mechanism of BH3 binding specificity. A study by Lee and co-workers performed an alanine scan on the BH3 region of BIM and tested for binding to the anti-apoptotic BCL-2 family members [5]. Boersma and co-workers expanded the scan to include glutamate and lysine, testing the effect of each residue on binding to BCL-X\(_L\) and MCL-1 [6]. Earlier studies speculated that crowding and electrostatics are important at several

<table>
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<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>defgabcdefabcdefabcdefgabcdefgabcd</td>
<td></td>
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</tr>
</tbody>
</table>

| BAD | NLWAAQRYGERLRRMSDEFDGSKKGLPR |
| BAK | PSSTMQGVRQIAIGDDINRYDSEQFT |
| BAX | QDASTKKSCEKLKRIGDELDSNMELQRTMI |
| BID | QEDIIRNIARHLAQGDSMDRSTIPGVLN |
| BIK | CMEGSDALRGLACIGDEMVDLRAPRLA |
| BTM | DRMPEINIAQRRTIGDEFNAYAYRFVL |
| BMF | QHAEVQIARKLQCIAQFPRLHVEQQHQQ |
| Hrk | RSSAQLTAAALKLGEDHLHQTMRWRRRA |
| Noxa | PAELEVECATQRRFGKDINFRQKLLNL |
| PUMA | BEQWAREIGAQLRMADLNAQYERRRE |

**Figure 2.1 BH3 sequences**
The BH3 regions of human BCL-2 proteins are shown, with the heptad notation used in the text indicated at the top. The four hydrophobic positions are in red letters, with two strictly conserved sites highlighted in yellow.
BH3 positions for determining the binding specificity for BCL-X\textsubscript{L} and MCL-1 [2, 7]. The buried 2d position was proposed as a possible site of specificity determination against MCL-1 due to crowding, and was supported by a tyrosine-to-isoleucine mutant of BAD that gained binding to MCL-1 [2]. The 3g position, on the other hand, was proposed to play a role in specificity determination against BCL-X\textsubscript{L}, based on electrostatics. This position is typically a negatively charged residue in BH3 sequences. However, in Noxa, this site is lysine, and was hypothesized to prevent binding to BCL-X\textsubscript{L} due to an arginine (R100) on the edge of the hydrophobic groove. Mutation of this lysine in Noxa to glutamate improved binding to BCL-X\textsubscript{L} [7].

Herein I examine the specificity of BCL-2 interactions with a focus on MCL-1. I solved the X-ray crystal structure of human MCL-1 in complex with four variants of BIM BH3: wildtype and three point mutants. The four MCL-1/BIM complexes resemble the structure of BCL-X\textsubscript{L} bound to BIM, but show regions of structural variation that may play a role in observed specificity differences. Structural flexibility of both the binding peptide and MCL-1 allow for binding of variable BH3 sequences.

2.2 Materials & Methods

2.2.1 BCL-2 Proteins

The cDNA for BCL-X\textsubscript{L} and MCL-1 was obtained from the Harvard Institute of Proteomics. MCL-1, residues 166-327, was cloned into pDEST17-TEV, a pDEST17 vector modified to contain a TEV cleavage site between the His-tag and the cloned sequence. MCL-1 was expressed in RP3098 \textit{E. coli}. Cultures were induced with 0.3 mM IPTG at an OD600 of 0.4-0.6. The proteins were purified from lysate by Ni-affinity chromatography followed by anion exchange chromatography using Q Sepharose resin. Additionally, BCL-X\textsubscript{L} (residues 1-209) and MCL-1 (residues 172-327) were expressed as MBP fusions from the pSV282 vector (provided by Dr. Laura Mizoue at Vanderbilt University). These proteins were expressed in BL21 pLysS and purified by Ni-affinity chromatography and cleavage with TEV protease in 50 mM TRIS, 50 mM NaCl, 0.5 mM EDTA and 1 mM DTT at pH 8.0 for 3 hours at room temperature. The cleaved MCL-1 was passed over a Ni column and the flow-through was further purified by gel filtration chromatography on S75 resin.
Selenomethionine-derivatized MCL-1, residues 172-327, was expressed using the same vector (pSV282) and cell line (BL21 pLysS) as unmodified protein. Cultures were grown in autoclaved M9 media supplemented with sterile 1 mL 1 M MgSO$_4$ (100 mM), 132 $\mu$L 0.5 M CaCl$_2$ (66 $\mu$M), 527 $\mu$L 8 g/L FeSO$_4$ (4.2 g/L), 10 mL 40% glucose (0.4%), 100 $\mu$L 0.5% thiamine (0.00005%), 1 mL 34 mg/mL kanamycin (34 $\mu$g/mL, pSV282 selection) for 1 L (final concentrations in parentheses). Media pre-warmed to 37 °C was inoculated with soft pellets of 10 mL overnight LB cultures resuspended in warm prepared media. When the culture reached OD600 of 0.5, amino-acid supplements were added: 100 mg of lysine, phenylalanine, threonine, and 50 mg of isoleucine, leucine, valine, and selenomethionine for 1 L. Thirty minutes after addition of the amino acids, protein expression was induced with 0.2 mM IPTG (200 $\mu$L 1 M IPTG for 1 L). Cultures were transferred to 30 °C and incubated overnight, for ~16 hours. Protein was purified as before, except for the addition of 10 mM $\beta$-mercaptoethanol in the first pass Ni purification buffers, and after the second Ni purification, DTT concentration was kept at 5 mM. The selenomethionine expression protocol was modified from Van Duyne et al. [122].

The TEV protease was expressed using plasmid pRK793 was obtained from the Macromolecular Crystallography Laboratory at the National Cancer Institute in a BL21(DE3)pRP strain. Cultures were grown to OD600 of ~0.5 at 37 °C, and then induced with 1 mM IPTG for 4 hours at 30 °C. The TEV was purified from cell lysate by Ni affinity chromatography followed by size-exclusion chromatography on an S200 column.

### 2.2.2 BH3 peptides

Human BIM BH3 (GGSGRPEWIAQLRRIGDEFNAYYARRV-CONH$_2$) peptide was synthesized by SynPep of Dublin, CA. BIM mutants I2dA and F4aE (CH$_3$CONH-RPEIWAAQELRRIGDEFNAYYAR-CONH$_2$ and CH$_3$CONH-RPEIWAIQELRRIGDEENAYYR-CONH$_2$) were synthesized by the MIT Biopolymers Laboratory in the Koch Institute for Integrative Cancer Research. These peptides were purified by reverse-phase HPLC using a C18 column and a linear water/acetonitrile gradient in the presence of 0.1% TFA. The I2dY peptide was encoded in a pSV282 vector as an MBP fusion. The peptide was expressed in BL21 and purified by Ni-affinity chromatography under native conditions.
Following cleavage by TEV protease, the cleaved product was passed over a Ni column and the
flow-through was further purified by reverse-phase HPLC. Mutant BIM I2dY used for
crystallography was shorter, due to degradation during native purification, as determined by
mass spectrometry (GSGGRPEIWYAQLRRIGDEFNAYYAR).

Fluoresceinated peptides were synthesized and purified by CHI Scientific of Maynard,
MA. BIM (FITC-RPEIWYAQLRRIGDEFNAYYAR-CONH₂) and mutants I2dA, I2dY, F4aE
(mutations at underlined positions) were purchased with greater than 95% purity. Additionally, a
purified fluoresceinated BAD peptide (CH₃CONH-NLWAARYGRELRRMSD₇KFVD-COOH),
in which FITC was attached to the italicized K, was purchased from Calbiochem.

2.2.3 Fluorescence Polarization Binding Assay

The concentration of the fluoresceinated peptide was at 50 nM with the MCL-1 or BCL-
Xₓ proteins at varying concentrations set by serial dilution. The buffer for these binding
reactions was 20 mM NaPO₄, 50 mM NaCl, 1mM EDTA, and 0.001% Triton X-100 at pH 7.5.
The proteins and peptides were set in 96-well plates, pre-treated with 0.02% Tween-20 in the
above buffer, and incubated for one hour at room temperature before measuring on a SpectraMax
M5 plate reader (Molecular Devices). The Kᵅₛ of the interactions were calculated from the
anisotropy data using a quadratic function in KaleidaGraph.

2.2.4 X-ray Crystallography

Crystals of the wild-type MCL-1/BIM BH3 complex were grown in hanging drops over a
reservoir containing 0.2 M zinc acetate, 0.1 M imidazole pH 7.0, 17.5% PEG 3350 at ~25 °C.
Crystals were flash frozen in liquid nitrogen directly from the mother liquor. Diffraction data for
the wild-type complexes were collected at the Advanced Photon Source at the Argonne National
Laboratory, NE-CAT beamline 24ID-C (Table 2.1). Three selenium sites were identified using
SHELX with a 2.03 Å dataset collected at the selenium absorption peak [123]. SHARP was used
to refine the selenium sites, and density modification applied to produce an initial map [124].
This map was of sufficient quality that the majority of the MCL-1 sequence could be auto-built
into it. The BIM BH3 peptide, and some loops in MCL-1, were built in using COOT [125]. The
model from the selenomethionine peak diffraction data was used to phase the diffraction data collected from the native protein at 2.00 Å. The final structure, refined using REFMAC5 and TLS refinement, has an R\textsubscript{work} of 19.4% and R\textsubscript{free} of 23.0% [126] and has been deposited in the PDB with the ID 2PQK.

The I2dY mutant complex was crystallized by hanging drop method at ~25 °C in 0.1 M TRIS pH 7.5 and 45% MPD. Crystals were flash frozen in liquid nitrogen directly from the mother liquor. Diffraction data for the P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} crystal were collected at the Advanced Photon Source, beamline 24ID-C, and scaled to 2.15 Å. Data were phased by molecular replacement using PHASER [127], with chain A of structure 2PQK (MCL-1 chain) as a model. This gave a single solution in which the density of the BIM mutant was clearly visible, though BIM was not present in the phasing model. Refinement of the I2dY mutant complex used REFMAC [126]5 and PHENIX [128]. COOT was used for model building. The structure has an R\textsubscript{work} of 18.5% and R\textsubscript{free} of 22.8%.

Both I2dA and F4aE co-crystals were grown in 0.2 M zinc acetate and 0.1 M imidazole, pH 7.0, at ~25 °C. Crystallization conditions for I2dA also contained 16% PEG 400 while F4aE had 2% PEG 3350. I2dA crystals were flash frozen with liquid nitrogen directly from the mother liquor, while F4aE crystals were cryo-protected by transferring into a solution of the crystallizing liquor mixed with PEG 400 at a final concentration of 20% prior to freezing. Diffraction data were collected on a Rigaku MicroMax007-HF rotating anode source and an RAXIS-IV detector. The 1.95 Å and 2.35 Å data sets – I2dA and F4aE, respectively – were scaled with HKL2000 [129]. The data were phased using chain A of structure 2PQK. Iterative rounds of refinement and model building were performed using PHENIX [128] and COOT [125], respectively. The I2dA structure complex has an R\textsubscript{work} of 18.7% and R\textsubscript{free} of 21.3% and the F4aE structure has an R\textsubscript{work} of 20.8% and R\textsubscript{free} of 24.7%.
### Table 2.1. Data collection and refinement statistics of MCL-1 complexes

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<th>I2dA mutant</th>
<th>F4aE mutant</th>
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<td>40.56, 53.33,</td>
<td>53.07, 72.01,</td>
<td>51.46, 71.49,</td>
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<td>118.12</td>
<td>69.73</td>
<td>117.85</td>
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#### 2.3 Results

##### 2.3.1 MCL-1 construct design

To study MCL-1 biochemically, significant amounts of stable MCL-1 protein were required. Many previous studies of MCL-1 used the murine sequence, and removed both the large, unstructured and destabilizing N-terminal PEST region and the transmembrane domain [2, 7, 10]. I generated a similar human MCL-1 construct by expressing residues 166-327. Ni purification followed by anion exchange or size exclusion chromatography was unsuccessful at removing both higher and lower molecular weight contaminants.

The smaller contaminant is likely a degradation product, because it often appears only in later purification steps. Upon incubation at ambient temperature (~25 °C), this band can be observed within an hour, becoming prominent after one day (Figure 2.2A). The degradation process is moderately slower at 4 °C, with the lower molecular weight product becoming visible after two to four hours (Figure 2.2B). Removal by TEV cleavage of a 33 amino acid N-terminal tag in the initial human MCL-1 construct helped to remove some, but not all, contaminants.
Thus, a new construct of MCL-1 in pSV282 was designed that began at residue 172, removing a series of glutamate residues from the PEST region that may have contributed to the low stability of the previous construct. The protein was made as a maltose binding protein (MBP) fusion, with the N-terminal MBP removable by TEV cleavage. Removal of MBP was confirmed by MALDI mass spectrometry. Also, since degradation had been a problem with the previous MCL-1 construct, I tested the degradation of this new MCL-1 construct at room temperature with time. As can be seen in Figure 2.2C, this form of MCL-1 is stable over the course of many days in 20 mM NaPO4, 50 mM NaCl, and 1 mM EDTA at pH 7.5.

![Figure 2.2 MCL-1 degradation with time](image)

**Figure 2.2 MCL-1 degradation with time**
Ni purified His-tagged MCL-1 (residues 166-327) was incubated at room temperature (A) or at 4 °C (B) to monitor degradation. This protein is 22 kDa, indicated by the arrow above the second standard from the bottom at 0 hr. (C) Purified tag-less MCL-1 (residues 172-327) after incubation at room temperature for 1, 4, and 9 days. The molecular weights of the markers in the ladder of the SDS-PAGE are 14.4, 21.5, 31.0, 45.0, 66.2, and 97.4 kDa from the bottom of the gel. SDS-PAGE visualized with Coomassie blue stain.

### 2.3.2 MCL-1/BIM BH3 structure

BCL-X\textsubscript{L} and MCL-1 are members of the anti-apoptotic BCL-2 family and protect the cell from death induced by a variety of signals. Although these proteins share protective function and several binding partners, they have low sequence similarity, with an identity of only 19%.
Despite this, their structures are quite similar, with an RMSD of 2.0 Å (Cα atoms, between 1PQ0 and 1WSX). A structure of murine BCL-XL in complex with BIM has been solved [54]. To compare and contrast this with MCL-1 complexes, I solved the structure of human MCL-1 bound to human BIM.

I crystallized MCL-1 in complex with a peptide derived from the BH3 region of BIM. The crystal was I-centered orthorhombic (I222). Unfortunately, molecular replacement using various models of MCL-1 (1WSX) and BCL-XL (1PQ1) did not work to phase the data. Selenomethionine-derivatized MCL-1 was made and crystallized with the BIM peptide. SAD phasing provided the final wildtype structure at a resolution of 2.0 Å, with an Rfree of 23.0 %. Residues 172-197, 203-321 of MCL-1 and 0-22 of BIM (of 25 BH3 and 4 linker residues) are present in the model.

There was only one MCL-1/BIM complex per asymmetric unit of the crystal. A disulfide bond is present, connecting two MCL-1 molecules across a two-fold crystallographic interface via the sole cysteine residue. This is likely an artifact, as only a small amount of reductant (1 mM DTT) was present in the protein stock and none was added to the crystallization conditions. Four ions, three zinc and one sodium, are present in the asymmetric unit with MCL-1 and BIM. The zines – coordinated by acidic residues, histidines, and water molecules – are essential for the crystallization of these complexes, with two zines at crystal contacts.

As expected, the structure of MCL-1 bound to wild-type BIM looks similar to other BCL-2-family complexes. The MCL-1 protein has eight α helices, with helices 2-5 and 8 forming a hydrophobic groove into which the BH3 peptide binds (Figure 2.3). The RMSD of this BIM-bound MCL-1 complex compared to a peptide-free murine MCL-1 NMR structure is 1.43 Å (Cα atoms, comparing helices). The structure of MCL-1, mostly human with some murine sequence, in complex with human BIM BH3 peptide has also been reported [85]. Despite the murine sequence in this complex, the fully human and chimeric structures are nearly identical, with an all-atom RMSD of 0.27 Å. Though these structures were solved independently, both used similar crystallization conditions, with zinc as a key factor.
2.3.3 Comparing MCL-1 and BCL-X\textsubscript{L} complexes with BIM BH3

The complexes of BIM bound to MCL-1 and BCL-X\textsubscript{L} were compared by aligning $\alpha$ helices 1, 2, 4, 5, 6, and BIM in PyMol, which gave a backbone RMSD of 1.1 Å. This alignment excluded regions that are not well-defined (loops) and regions of the groove that move upon BH3 binding. Differences between the two complexes are readily apparent. The most obvious difference is that the $\alpha$3 region of MCL-1 is longer and more helical (Figure 2.3A). The bottom of the groove, where the amino-terminal part of BIM binds, is more open in BCL-X\textsubscript{L} than in MCL-1. The pocket into which the I2d position (native isoleucine at position 2d) of BIM binds is more constrained in MCL-1 as compared to BCL-X\textsubscript{L}. Additionally, MCL-1 is more positively charged at the base of the groove, with K234 in this region (the corresponding residue in BCL-X\textsubscript{L} is Q111, Figure 2.4). Similarly, BCL-X\textsubscript{L} is much more open around the conserved L3a position of BIM. The crowding in MCL-1 is due to $\alpha$3, which is more $\alpha$-helical than the equivalent helix in BCL-X\textsubscript{L}. This helix in MCL-1 brings M231 close to the BIM peptide to cap the pocket binding the L3a position (Figure 2.5A). The $C_\beta$ of M231 is 2 Å closer to $C_\beta$ of L3a on BIM than the equivalent L108 position in BCL-X\textsubscript{L} (Figure 2.5B). In contrast, the top of the
groove in MCL-1 is more open than in BCL-X\textsubscript{L}. The upper part of the hydrophobic groove has a bridge that separates the pockets into which the third and fourth buried positions (I3d and F4a) of BIM bind. In BCL-X\textsubscript{L}, the aromatic F97 forms this bridge, while MCL-1 uses the smaller V220 at this position, creating a less tight interface (Figure 2.5C-D). Additionally, where MCL-1 has H224, BCL-X\textsubscript{L} has Y101, which creates a higher wall at the groove’s edge.

![Image](image_url)

**Figure 2.4** Electrostatic potential mapped onto a surface representation of MCL-1 and BCL-X\textsubscript{L} bound to BIM

(A) Structure of MCL-1 (2PQK) bound to BIM BH3. (B) Structure of BCL-X\textsubscript{L} (1PQ1) bound to BIM BH3. BIM BH3 peptides are shown using stick representation. Electrostatic calculation by DelPhi, mapped onto PyMOL surface representation. The arrows indicate K234 in MCL-1 (A) and Q111 in BCL-X\textsubscript{L} (B).

MCL-1 and BCL-X\textsubscript{L} also differ in the conformation changes that each make upon binding BIM BH3. When BIM BH3 binds to MCL-1, the hydrophobic groove widens, predominantly through reorientation of the carboxy-terminal end of \(\alpha4\) away from the BIM helix at the top of the groove (Figure 2.3B). Residue A227, at the amino-terminus of \(\alpha3\), and V253, at the carboxy-terminus of \(\alpha4\), move away from one another upon BIM binding. The distance between these residues increases from 10.2 Å in the unbound state to 13.9 Å in the BIM-bound state. Conversely, the bottom of the hydrophobic groove in BCL-X\textsubscript{L} shifts to accommodate the BIM BH3 helix, partially by means of the decrease in helicity of \(\alpha3\) (Figure 2.3C). L108 in \(\alpha3\)
of BCL-X\textsubscript{L} shifts 6.1 Å from the $\alpha$-helical unbound state to the bound state, where it is no longer in an $\alpha$-helix.

![Figure 2.5 Differences in the hydrophobic groove between MCL-1 and BCL-X\textsubscript{L}](image)

**Figure 2.5 Differences in the hydrophobic groove between MCL-1 and BCL-X\textsubscript{L}**

Surface representation of MCL-1 (2PQK) and BCL-X\textsubscript{L} (1PQ1) is shown in CPK coloring, unless indicated as follows. The BIM peptide backbone is shown using sticks, with Mcl-1-bound BIM in pink and BCL-X\textsubscript{L}-bound BIM in blue. The side chains of the buried hydrophobic positions are also shown using sticks. Equivalent residues at the bottom of the groove, M231 in MCL-1 (A) and L108 of BCL-X\textsubscript{L} (B), are shown in green. Equivalent residues at the top of the groove, V220 and H224 in MCL-1 (C) and F97 and Y101 of BCL-X\textsubscript{L} (D), are shown in cyan.

### 2.3.4 Binding data of point mutants

The binding of three BIM point mutants was tested by fluorescence polarization (Table 2.2). Two mutations were at the 2d site to test the size range of the amino acid capable of binding MCL-1. Both alanine and tyrosine at this position bound with nanomolar affinity. The
I2dA and I2dY mutants have Kd's of 18 and 35 nM, respectively, as compared to wild-type Kd of 13 nM. In comparison, these mutants bind BCL-XL with even higher affinity than wild-type BIM. The third mutant, glutamate at the fourth buried position (F4a), looks at an extreme mutation - a buried charge - that has been reported to maintain high-affinity binding to MCL-1 [3, 6]. MCL-1 binds this mutant with similar affinity as wild-type, while BCL-XL binds this mutant at 60 nM, a 6-fold reduction in affinity as compared to wild-type binding (Table 2.2).

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<th>BCL-XL Kd (nM)</th>
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<td>10*</td>
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</tr>
<tr>
<td>F4aE</td>
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<td>BAD</td>
<td>NLWAARYGRELRRMSDFVD</td>
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</table>

Table 2.2. Binding affinity of BIM mutant peptides for MCL-1 and BCL-XL.
Sequences of peptides are shown, with mutations in red. All BIM peptides were fluoresceinated at the N-terminus. The BAD peptide was fluoresceinated at K. Kd values are an average from two fluorescence polarization measurements, except for starred values (*), in which only one measurement was available.

2.3.5 MCL-1 complexes with mutant BIM BH3 peptides

To examine the effect of the above point mutations in BIM BH3 on binding to MCL-1, I solved X-ray crystal structures of three mutant complexes (Figure 2.6A). All diffracted to 2.35 Å or better and were solved by molecular replacement (Table 2.1). The I2dA and F4aE mutant complexes were crystallized in the presence of zinc acetate in the I222 space group, similar to wildtype. As expected, these structures had several zinc ions in the asymmetric unit, most of which are in similar locations as in the wildtype structure. Additionally, these structures also include the disulfide bond across the two-fold crystallographic interface, as did the MCL-1 complex with wildtype BIM. The I2dY mutant in complex with MCL-1 crystallized in the P2₁2₁2₁ space group in the absence of ions or disulfides.
Figure 2.6 Structures of MCL-1 in complex with BIM and BIM point mutants.

(A) Wildtype MCL-1 bound to BIM BH3 (2PQK) is shown in red, BIM I2dY in dark blue, BIM I2dA in cyan, and BIM F4aE in green are shown superimposed on one another. The vertical peptide in the front is the bound BIM BH3 peptide. (B) Superimposed are the wildtype complex and the BIM F4aE. In addition to the mutated glutamate, E3g and Y4e of BIM and H224 of MCL-1 are shown using sticks. (C) The wildtype complex and the BIM I2dY mutant complex, with the 2d positions shown using sticks, are superimposed. (D) Superimposed are the wildtype complex and the BIM I2dA mutant complex, with the mutation position and L235 of MCL-1 shown using sticks.
Figure 2.7 Electron density on F4aE BIM/MCL-1 model.
2Fo-Fc maps of electron density at 1.0σ shown over the BIM F4aE (E17) coordinated zinc, with BIM E16, MCL-1 H224, and a water molecule. Only the density around these residues and atoms is shown.

The F4aE mutant is very similar to the wild-type structure. The RMSD between the two structures is 0.32 Å. The glutamate at the 4a position (E17) rotates out of the hydrophobic groove. Along with this glutamate, the glutamate at 3g on BIM (E16) and H224 on MCL-1 coordinate a zinc ion present from the crystallization conditions (Figure 2.7). The F4aE mutation is not solely responsible for zinc coordination at this site. In a crystal structure of BIM L3aA, F4aA double alanine peptide in complex with MCL-1 (3D7V), a zinc ion is similarly coordinated, without the glutamate at 4a [130]. While this coordinating conformation is likely non-physiological, it is interesting to see the glutamate rotate out and the next hydrophobic groove position Y4e shift to partially fill the vacated space (Figure 2.6B). It is likely that even in the absence of zinc, the glutamate at this position would have to rotate out of the protein core to avoid a severe desolvation penalty. Our structure illustrates that it can readily do so, with only minor perturbation to the rest of the complex structure.

Comparison of the I2dY peptide complex with the wild-type structure shows an overall similar structure with an RMSD of 0.76 Å. The biggest differences are at the N-terminus of the peptide and in the α3 region of MCL-1. The peptide moves away from the receptor by 0.92 Å (measured at the Cα of position 2d), and the α3 helix moves away from the peptide by 0.94 Å in
order to accommodate the larger tyrosine residue (Figure 2.6C). Thus, MCL-1 is able to bind a BIM mutant with a large tyrosine at the first buried position (with an affinity similar to that of wild-type BIM), by local conformation changes of the peptide and α3.

As discussed above, MCL-1 has increased helicity in the α3 region compared to BCL-XL. In the context of backbone structure, mutating isoleucine at position 2d to tyrosine would prevent binding to MCL-1, due to a bad steric interaction. Additionally, computational modeling of the structure of BIM bound to MCL-1 using SCADS [2] indicates that this region cannot accommodate a large aromatic residue (X. Fu, personal communication). The structure of the mutant complex shows that binding to a mutant with a large residue at 2d is possible, but only with structural changes that are not accounted for in the computational modeling. Structures of additional mutants should better define the range of local rearrangements that are possible in MCL-1.

The final BIM mutant complex is an I2dA mutant. This structure is very similar to the wild-type complex, with an RMSD of 0.25 Å. The most significant difference between the wild-type and I2dA mutant complexes is a shift of L235 on MCL-1 to fill the hole left by the small alanine (Figure 2.6D). The alanine mutation, in conjunction with the tyrosine mutation, demonstrates flexibility in the α3 region of MCL-1 and the binding BH3 peptide, which allows a range of sequences to bind.

### 2.4 Discussion

I have solved four structures of MCL-1 in complex with BIM BH3 peptides that vary in affinity by a factor of three. These structures exhibit conformational differences that explain the ability of MCL-1 to bind a range of sequences, and also inform speculation about possible determinants of MCL-1 binding specificity.

BH3 peptides of diverse sequence bind the BCL-2 receptors; however MCL-1 has very different specificity from BCL-2, BCL-XL, and BCL-W. Even among the buried hydrophobic positions, many different residues are observed. For example, at the first buried position (2d), isoleucine is common among the BH3 peptides capable of binding both MCL-1 and BCL-XL. However, in BAD, which does not bind MCL-1, this residue is tyrosine. Day et al. hypothesized that particular crowding effects at this position in the peptide binding region of MCL-1 prevented
MCL-1 binding [2]. In BIM BH3 complexed with MCL-1, this residue binds into a pocket partially formed by M231, K234, and L235 on α3. In BCL-XL, the corresponding residues are L108, Q111, and L112. In MCL-1 this region is more α-helical, as compared to BCL-XL, which brings the residues in close to the first buried position of the binding BH3, creating a tighter pocket. The tighter packing and smaller pocket in this region could potentially provide some of the specificity observed for MCL-1. This may prevent the tyrosine in BAD from fitting, and possibly explain the low affinity of BAD for MCL-1. Indeed, mutation of tyrosine to isoleucine in the context of BAD improves binding to MCL-1, and retains binding to BCL-XL [5, 6].

To test the generality of this steric exclusion model, I made the I2dY mutant in BIM. This peptide bound MCL-1 with an affinity comparable to wild-type. Therefore, steric clashes at position 2d do not provide a reliable rule to govern specificity. Our structure demonstrates why this is the case. MCL-1 accommodates tyrosine at the first buried position with a shifting of helix α3, while the mutant peptide bends, to provide the necessary space for the aromatic residue. Furthermore, the I2dA mutant showed that small residues are also tolerated at this same position, where small changes in residue conformation compensate for the alanine mutation, with mutant affinities similar to wild-type (Table 2.2) [2, 7]. These mutants showed similar affinity for MCL-1 as compared to the wild-type versions of these peptides, indicating that the simple crowding model for BH3 specificity at position I2d is insufficient.

The fourth buried hydrophobic position (4a) possibly encodes specificity. Saturating mutagenesis previously indicated that any amino acid at this site maintained MCL-1 binding, but many charged or polar amino acid substitutions attenuated binding to BCL-XL [3]. Our binding studies indicate that the F4aE mutant binds MCL-1 with wild-type affinity. For BCL-XL, the affinity was reduced approximately six-fold (Table 2.2), relatively similar to a quantitative phage-ELISA assay that showed an approximately 13-fold reduction in affinity [6]. However, this F4aE mutant was observed to not bind BCL-XL in a fluorescence polarization assay using 18-mer peptides [6]. The peptide length is likely responsible for the large difference in observed affinities in the fluorescence polarization assays, as 18-mer wild-type BIM showed a 6-fold reduction in affinity as compared to the 26-mer wild-type BIM peptide [5, 6]. The rotation of the glutamate at this site out of the hydrophobic groove demonstrates how MCL-1 is likely able to maintain high affinity for peptides with this residue mutated. However, BCL-XL is more
constrained at this site (Figure 2.5D), possibly preventing similar side chain rotation observed in the F4aE/MCL-1 complex to accommodate charged and polar amino acids.

The flexibility of this family of proteins is intriguing, allowing for binding to diverse sequences, such as a tyrosine mutation at the first hydrophobic position in BIM. Single site mutations in BIM are capable of creating binding specificity for MCL-1 or BCL-XL, and combinations of sites are likely to generate even more selective peptides, as was shown with two and three site mutants of BIM [7, 9-11, 53]. However, this flexibility also makes the identification of a simple model of specificity difficult.
Chapter 3

Development of BH3 SPOT arrays

Abstract
The study of the interaction specificity of the BCL-2 receptor with respect to BH3 sequences requires binding data for many mutants. Here we apply the SPOT technique – the application of peptide synthesis on membranes – to the BH3/BCL-2 interaction. Method development testing indicated that longer 26-mer BH3 peptides (as opposed to 20-mer) are better for detecting interactions with BCL-2 proteins. Binding of BCL-2 proteins was best observed by detection with fluorescently-labeled antibodies which reduced false positive signals accompanying directly-labeled BCL-2 proteins. SPOT technique was used to perform substitution analysis of the BIM BH3 peptide to elucidate specificity determinants from single residue substitutions. The potential sites of specificity include positions A2e, I3d, G3e, and F4a. Two of these sites are generally occupied by small amino acids in native BH3 sequences. The other two are buried hydrophobic positions that allow for differential binding: most residues at I3d maintain binding to BCL-XL, yet kill binding to MCL-1, and most mutations of F4a maintain binding to MCL-1, yet destroy binding to BCL-XL. This technique provides a means of testing the binding of many unique BH3 peptides to BCL-2 proteins simultaneously.

Collaborators:
Dr. Stefano Gulla performed the substitution analyses experiments shown in Figure 3.9.
3.1 Introduction

We are interested in understanding the nature of specificity in BCL-2/BH3 interactions. Elucidating such specificity can aid in the future studies of the BCL-2 family, including protein design, inhibitor design, and the search for new BH3s in the proteome. I have been using BIM BH3 mutants to study this specificity, as shown in Chapter 2. While such a study details the structural effects of a few BIM mutants, a more high-throughput method for obtaining binding information about many more mutant peptides would aid in studying more general BH3 specificity. Specifically, we would like the ability to monitor the binding of multiple BCL-2 receptors to many BH3 mutants to gain insight into how these mutations contribute to affinity and specificity toward multiple BCL-2 receptors.

The interactions of BH3 sequences with BCL-2 proteins have been studied for well more than a decade. Initially these interactions were identified qualitatively by co-immunoprecipitation and yeast two-hybrid assays using full-length proteins [82, 83]. However, as it has become apparent that 16 to 26 residue peptides corresponding to the BH3 regions of the pro-apoptotic proteins are functional – interacting with their pro-survival counterparts and inducing apoptosis – many groups have begun to quantitatively study the interaction of BH3 peptides binding to anti-apoptotic BCL-2 proteins [11, 78]. Researchers have used fluorescence polarization, surface plasmon resonance, and isothermal titration calorimetry to determine binding affinities of BH3 peptides for BCL-2 proteins[10, 11, 84, 89].

Early quantitative characterization of BH3 interactions with BCL-XL and BCL-2 proteins employed fluorescence polarization (FP) with short BH3 peptides [53]. The peptides in these studies were 16 amino acids long, but in later studies it became evident that longer peptides usually bound to BCL-XL with higher affinity, and this often correlated with the helicity of the peptide free in solution [4, 78]. Additionally, FP competition assays have been used frequently to determine IC50s. Many groups began to use surface plasmon resonance (SPR), also in competition assays. In fact, the first “large-scale” characterization of the binding of the BCL-2 proteins to BH3 peptides was performed with a competition-based SPR assay, in which the mouse BIM peptide was competed out from five anti-apoptotic BCL-2 proteins with eight 26-mer BH3 peptides to give IC50 values [7]. Isothermal titration calorimetry (ITC) has also been recently used to determine the K_d of label-free peptides [5, 84].
The benefit of all of these binding assays is that they are quantitative. With the exception of ITC, which typically requires large amounts of protein, direct binding detection usually requires modification of each peptide being tested, either being fluorescently labeled or attached to a derivatized surface. Competition assays allow for fewer specialized peptides. The direct readout from the competition assay is an inhibitory concentration (IC50), from which a Kd can be calculated with information about the peptide being competed [8].

The downside of these quantitative assays lies in creating the reagents. Not only do the soluble, folded BCL-2 receptors need to be expressed and purified, but peptides for the BH3 sequences must also be made and purified. Peptide synthesis is expensive; thus alternatively, the peptide sequences can be cloned. For short sequences, they must be expressed as a fusion, from which they can be cleaved. Other high-throughput techniques that have yet to be employed by the BCL-2 community, such as protein microarrays, would be subject to the above constraints [131, 132].

Some methods have emerged that allow for the quantitative detection of BCL-2/BH3 peptide interactions without the need for peptide purification. These involve the expression and display of the BH3 peptides at a phage or yeast cell surface. Quantitative phage ELISA is a method in which phage-displayed peptides are competed from surface-immobilized BCL-2 receptor with free receptor. Additionally, a method has been developed that allows for the detection of binding of BCL-2 proteins to BH3 peptides expressed on yeast surfaces [133]. In this method, binding is detected by the presence of BCL-2 proteins on the surface of the yeast cell after unbound BCL-2 proteins are washed away. The remaining BCL-2 proteins are then detected with fluorescently labeled antibodies. Incubating yeast displaying these BH3 peptides with increasing concentration of BCL-2 shows increased binding, which can then be used to calculate Kd's [133, 134]. While these methods provide a means of obtaining quantitative binding information without the necessity of purifying the BH3 peptides, the different BH3 sequences must be cloned into the appropriate vectors and subsequently expressed.

These techniques work well for determination of quantitative binding constants, especially when examining the eight typically studied BH3 peptides and a handful of mutants. However, if one wishes to test the binding of hundreds of peptides – for example systematic mutagenesis of the native BH3 sequences – significant time and money would be required to synthesize, or to clone and express, the necessary sequences. A couple of groups have cloned or
synthesized on the order of fifty BIM BH3 variants and tested the binding of these mutants to MCL-1 and BCL-XL [3, 5, 6]. However, applying these methods to several hundred BH3 variants is not reasonable. Thus, a method in which hundreds of peptides can be made in a short period of time, and at a reasonable cost, would be preferable. While a quantitative method would be ideal, high-throughput binding assays are rarely quantitative. Thus, a method that could provide qualitative data for hundreds of peptides binding to BCL-2 receptors, would allow for the identification of interesting candidate peptides for further quantitative studies using synthetic or expressed peptides. This chapter demonstrates that SPOT arrays provide such a method.

SPOT arrays are a technique in which traditional solid-phase peptide synthesis is carried out on a “spot” on a membrane surface, rather than on a resin, as used in large-scale peptide synthesis. The technique was first described by Ronald Frank [135], using standard FMOC chemistry to build the peptides on the cellulose membrane. This protocol was simple enough to synthesize many short peptides in parallel, with no specialized equipment. New instruments have been developed that allow for the synthesis of peptides more than 30 residues in length at a density of six hundred spots per four by six inch membrane (Intavis). Further, the peptides need not stay on the membrane for the subsequent assays. With the incorporation of the appropriate linkers, individual spots can be punched out and cleaved from the membrane, allowing for the small-scale parallel synthesis of hundreds of peptides for solution assays as well. While these SPOT membranes are expensive (approximately $800 each), a membrane with up to six hundred unique peptides can be synthesized, and can be used to obtain qualitative binding data, for the same price as two peptides synthesized on the larger scale required for the previously described quantitative assays.

The first use of SPOT-technology was for antibody epitope mapping [135]. Use of overlapping peptide sequences and substitution analyses allow for the identification of binding sequences, and the residues critical for binding within them. Other applications now include enzyme substrate recognition. For example, with the use of radio-labeled ATP, one can determine which peptide sequences are phosphorylated by a particular kinase [136]. These studies use short peptides, often 6-15mers, which is the limit of many array-style peptide synthesizers.
This method has become a good way to study protein-protein interactions on a fairly large scale. SPOT-technology has been used to test the binding of potential SH3 and PTB binding peptides identified from the proteome (yeast for SH3 and human for PTB) [137, 138]. Modifications of the SPOT technique have made peptide inversion possible. SPOT array peptides with free C-termini allow the application of this technique to PDZ domains [139]. Also, the application of native chemical ligation allows for the application of the SPOT technique to much longer peptides and proteins, as long as the region of modification is at the C-terminus. Native chemical ligation in combination with the SPOT method was used to examine more than 6000 variants of a WW domain [140]. Newer machines, such as those manufactured by Intavis, allow for the synthesis of still longer peptides, which have been applied to WW domains. Substitution analysis of the 34 amino acid FBP28 WW domain allowed monitoring of the effect of sequence on binding to a poly-proline ligand [141].

We proposed that this SPOT technology could be applied to the interaction of BH3 peptides with BCL-2 proteins. With the proper SPOT peptide synthesizers, BH3 peptides (which are probably best to study as 26mers) are ideal for SPOT studies. Many different BH3 sequences can be simultaneously synthesized on a cellulose membrane, and each of these membranes, or pieces cut from the membrane, can be incubated with particular BCL-2 proteins to test for binding. While most studies have focused on linear peptide motifs printed on the membrane, some studies have printed sequences of WW domains, which must be in their native β-sheet fold for interactions with peptide ligands to occur, as observed in the study [141]. Thus, we postulated that it would be possible for the BH3 peptides to form α-helices upon binding to the BCL-2 proteins. The SPOT technique provides a framework in which many BH3 peptides can be tested for binding to a few BCL-2 proteins.

In SPOT arrays, the interaction is observed by detecting bound protein directly on the membrane. This can be done with radio-labeled proteins. Many methods have used antibodies to detect the presence of bound proteins [137-139]. Additionally, the binding protein or peptide can be directly conjugated to fluorescent dye [140] or an enzyme for subsequent detection [141]. While this method is not quantitative, these spots can, to some extent, provide information about the relative binding affinities. A study by Weisner et al. showed that signal intensities could be used to classify peptides into three binding affinity groups: tighter than 100nM, between 100nM and 10μM, and weaker than 10μM Kds [142].
We set out to see if the SPOT technique could be applied to the study of BH3/BCL-2 interactions. BH3 peptides, of greater than 26 amino acids in length, can be synthesized on cellulose membranes. These peptides are capable of binding BCL-2 proteins and largely exhibit the binding specificity profiles observed by other researchers. Using the SPOT technique, we performed substitution analysis on the BIM peptide, in which sites of interest are mutated to all native amino acids (or a subset thereof). The binding of 170 BIM BH3 point mutants to MCL-1 and BCL-XL, each at two concentrations, was monitored. This analysis highlighted a couple of sites within the BIM BH3 region that can encode specificity for individual BCL-2 proteins. While there is a lot of room to improve this technique, it appears to be applicable to the BH3 peptides, and can be used to gain information about the relative binding affinities for the BCL-2 interacting peptides.

3.2 Materials & Methods

3.2.1 SPOT membranes

Initial test membranes were synthesized by Intavis AG (Germany). These synthetic peptide arrays, with a variety of BH3 and control peptides, were made using FMOC chemistry on cellulose membranes [135]. For each of the BH3 peptides, four versions were printed: 26-mer with a four amino acid linker, 26-mer with a twelve amino acid linker containing FLAG, and 20-mers with both types of linkers. The BH3s printed in this fashion were BIM, BIM L3aD (negative control), BAD, Noxa, and designs N2-Y19N and X1 [8]. Additionally, non-BH3 controls were printed. These included cJUN, FLAG tag and HA tag with the two different linker lengths. Sequences of these peptides are listed in Table 3.1. Two membranes were obtained: one with peptide printed in the normal fashion and one where the loading capacity was reduced by diluting the first printed FMOC-amino acid ten-fold in the acetylated version of the amino acid.

All subsequent membranes were synthesized at Biopolymers laboratory of the Koch Institute of Integrative Cancer Research (MIT), using an Intavis arrayer. In general, these peptides were synthesized with PEG3 (three ethylene glycol units) linkers, though both four amino acid GSGS linker and no linker variants were tested. Each time a membrane was
synthesized by Biopolymers, a few spots were synthesized with Rink Linker, to allow for removal of the peptide from the cellulose for verification by mass spectrometry.

<table>
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<tr>
<th>peptide</th>
<th>region length</th>
<th>linker length</th>
<th>sequence</th>
</tr>
</thead>
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<td>4</td>
<td>RPEIWIAQELRRIGDEFNAYYARRVGGS</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>12</td>
<td>RPEIWIAQELRRIGDEFNAYYARRVGYKDDDKGGS</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>4</td>
<td>EIWIQAELRRIGDEFNAYYGGS</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>12</td>
<td>EIWIQAELRRIGDEFNAYYGDYKDDDKGGS</td>
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<tr>
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<td></td>
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<td>4</td>
<td>EIWIQAEDRRIGDEFNAYYGGS</td>
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<td>4</td>
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<td>12</td>
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<tr>
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<td>4</td>
<td>DYKDDDKGGS</td>
</tr>
<tr>
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<td>8</td>
<td>30</td>
<td>DYKDDDKGIARLEEKVLQKONSEELASTANMLRQVL</td>
</tr>
</tbody>
</table>

Table 3.1 BH3 and other SPOT test sequences
Peptides sequences for SPOTs shown in Figure 3.1. The linker to the membrane is shown in gray italics.

3.2.2 BCL-2 proteins

The majority of SPOT binding studies reported here used c-myc-tagged BCL-2 proteins. These were expressed in *E. coli* BL21(DE3) strains in a modified pSV282 vector (pSVM). This vector was generated by S. Dutta to express MCL-1 as a maltose binding protein (MBP) fusion, which upon TEV cleavage leaves an N-terminally c-myc-tagged protein. Human MCL-1, residues 172-327, was purified from cell lysate by Ni-affinity chromatography, cleavage with TEV protease, and a final Ni-affinity chromatography step, in which the c-myc-tagged MCL-1
protein (tcMH5) is in the flow-through and early washes while the MBP and TEV protease bind the Ni resin. The pSVM vector was also used for expression of c-myc tagged human BCL-X_L (tcXH8, residues 1-209, Δ38-81). These c-myc-tagged BCL-2 proteins were expressed and purified similarly to MCL-1. All proteins were determined to be pure, as seen by Coomassie-stained SDS-PAGE.

Testing also involved a couple other versions of BCL-2 proteins. Tag-less receptors of BCL-X_L (tcXH3, residues 1-209) and MCL-1 (tcMH4, residues 172-327) were generated from the pSV282 vector. Proteins were expressed as MBP fusions. The purification protocol was similar to that for the c-myc-tagged proteins, but instead the final protein has only a GS cloning artifact at the N-terminus. Some MBP fusion proteins were tested: MBP- BCL-X_L (XH3 residues 1-209) and MBP-MCL-1 (MH4 residues 172-327). These were expressed in BL21 pLysS from the pSV282 vector, producing protein with an N-terminal MBP, but without the c-myc tag. These Ni purified proteins were further purified by size exclusion chromatography, omitting TEV cleavage. Additionally, a couple BCL-X_L constructs were expressed in pDEST17-TEV, a modified pDEST17 vector. The protein was purified from lysate by Ni-affinity chromatography followed by anion exchange chromatography using Q Sepharose resin. Human BCL-X_L (XH2, residues 1-209, Δ38-81) and mouse BCL-X_L (XM1, residues 1-209) were expressed in BL21 pLysS and purified this way.

Some proteins were fluorescently labeled for direct visualization of binding on the membranes. BCL-X_L (tcXH3) and MBP- BCL-X_L (MBP-XH3) were labeled on lysines using Cy3-NHS-ester (GE Healthcare), while MCL-1(tcMH4) was labeled on a cysteine using fluorescein-maleimide (Invitrogen). For the Cy3 labeling, 50 μM protein stocks in NaCO₃ were incubated with ten-fold molar excess dye for 2.5 hours at room temperature, protected from light. The reaction was stopped by diluting the mixture two-fold into TBS. For fluorescein labeling, dye at 20 mg/ml in DMF was added to tcMH4 at 290 μM in TBS with 0.2 μM TCEP, to a final concentration of 2 mg/ml. Upon mixing, precipitation was observed, and the mixture was diluted three-fold with PBS. After 3 hours incubation at room temperature, protected from light, the reaction was stopped by the addition of 5 μL βME (0.5-1% v/v). Labeled proteins were purified using 2 mL Zeba desalting columns (Pierce) equilibrated with TBS and 1 mM DTT. Concentrations of the proteins were calculated from the absorbance at 280 nm, using corrections for the dye contributions: 8% of 552 nm for Cy3 and 20% of 490 nm for fluorescein (as
recommended by their respective companies). Additionally, a fluorescein-labeled FOS peptide was obtained from N. Zizlsperger.

3.2.3 Antibodies

All antibodies were purchased from Sigma Aldrich: \( \alpha \)-c-myc (M4439), \( \alpha \)-c-myc-Cy3 (C6594), \( \alpha \)-His (H1029), \( \alpha \)-His-FITC, \( \alpha \)-mouse-PE (P9287). Unless otherwise indicated, all antibody dilutions were 100-fold from the stock concentration, as provided by Sigma.

3.2.4 Membrane probing and blocking

Protocols for probing SPOT membranes were modified from Cold Spring Harbor Protocols [143]. Strips or sub-arrays were cut from the whole membrane using a clean razor blade. The membranes were then hydrated in 100% ethanol, transferred to TBS (137 mM NaCl, 2.7 mM KCl, 50 mM Tris, pH 7) and incubated at room temperature for 5 minutes. Membranes were then transferred into re-sealable sandwich bags. The volumes used for subsequent incubations were typically 1-5 mL. The volume was such that the membrane was always completely immersed and able to rock freely in the liquid.

The standard treatment for most membranes (after the hydration treatment described above) involved incubation in MBS (TBS with 0.2% Tween 20 and 2% dry milk) overnight at room temperature. After 16-18 hours, membranes were rinsed with T-TBS (TBS with 0.05% Tween-20) and then incubated with the probe protein in MBS for 3 hours at room temperature. For fluorescently labeled BCL-2 proteins, the membranes were rinsed with T-TBS and scanned wet on a Typhoon 9400 in Tania Baker’s laboratory. Membranes probed with unlabeled protein that require antibody detection were rinsed twice with T-TBS and then incubated with a primary antibody, 100-fold diluted in MBS, for one hour at room temperature. Membranes were rinsed with T-TBS and, if a labeled secondary antibody was required, incubated in 100-fold diluted antibody in MBS at room temperature for one hour. Membranes were scanned on the Typhoon 9400.

A few membranes were treated with a protocol modified to incorporate reducing agents. DTT-treated membranes were incubated at 37 °C in TBS containing 1 M dithiothreitol for 4-6
hours. These membranes were then incubated in MBS containing 0.2 M DTT at room temperature overnight for 16-18 hours. Membranes were rinsed with T-TBS containing 2 mM DTT, and the MBS for incubating with protein contained 2 mM DTT. All steps after BCL-2 protein incubation were the normal solutions without DTT.

3.2.5 Data processing

Images of the SPOT membranes from the Typhoon were analyzed using ImageQuant. In any quantitation shown, the data were normalized to a nearby reference spot (typically a wild-type BIM peptide in the strip or row being analyzed) or the average of multiple reference spots on the array (multiple wild-type BIM peptides).

3.3 Results

Data are presented in topics that address certain questions and issues pertaining to the application of the SPOT technique to the study of BH3 interactions, and are not in chronological order. While all membranes were printed on an Intavis peptide synthesizer, I often refer to Intavis membranes, made by the company in Germany, and Biopolymer membranes, made at the Koch Institute of Integrative Cancer Research. Once the technique was validated on strips cut from SPOT membranes, arrays of peptides were used to perform substitution analysis of the BIM BH3 peptide.

3.3.1 Peptide conditions

3.3.1.1 Peptide loading capacity

The initial SPOT membranes obtained from Intavis for this study came in two varieties: normal loading capacity and reduced loading capacity. Protein binding to these two membranes appeared comparable, with strips from each showing similar signal and background for the same probe (Figure 3.1). In some arrays, ‘rings’ – high signal around the edge of the spot – appeared on the membranes, making spot quantitation difficult. I tried several things to address this, one of which was to have peptides printed with different concentrations of the initial printed amino
Figure 3.1 **SPOT strips testing peptide lengths and linkers**

Results of various protein probes interacting with peptide printed on the membrane. Printed peptides are listed at the top. BIM neg is a negative control point mutant of BIM (L3aD). The numbers below the peptide names indicate the peptide construct. The first number is the length of the named region (i.e. 26-mer BIM BH3) and the second number is the length of the linker to the membrane. Peptide sequences are shown in Table 3.1. All probe concentrations were 5μM, except for mBCL-XL (murine), which was 40μM. The detection method is indicated by the fluorophore directly conjugated to the probe or the antibodies used to detect the probe. The α-mouse secondary is labeled with R-phycoerytherin for visualization. Expected interactions are shown above each set of SPOT tests, based on expected affinity: red, orange, and yellow are high, medium, and low affinity binders, respectively. White is used for SPOTs expected not to interact.

acid, similar to the method used by Intavis, since lower peptide loading has been implicated in reducing ring formation [144]. Peptides made using different loading capacities were synthesized with fluorescein at the N-terminus (Figure 3.2A). Additionally, I had short FLAG and c-myc epitopes printed, making it possible to examine signal from fluorescently-labeled antibody binding. Since these short peptide epitopes are less than half the length of the BH3 peptides, they provide a means of testing peptide synthesis conditions that is cheaper and faster to produce than BH3 peptide membranes. These results indicate that loading lower amounts of
peptide do not always lead to better signal or fewer rings (Figure 3.2B). I am unsure of the conditions that create these rings, though treatment with DTT is suspected to play a role, and is addressed later.

![Figure 3.2](image)

**Figure 3.2** Effect of decreased peptide loading.
(A) An array of peptides with fluorescein appended at the N-terminus, scanned prior to antibody probing. The numbers (600, 60, 2) correspond to the concentration (in mM) of the first residue printed on the membrane strip. (B) Fluorescence intensity of α-c-my-cy3 bound to peptides on a membrane, shown below the graph. c-myc and FLAG peptides were synthesized with different concentrations of the first residue, indicated after the peptide named.

### 3.3.1.2 BH3 peptide length

The membranes from Intavis included different lengths of BH3 sequences. BIM binding to the tested BCL-2 proteins, BCL-XL and MCL-1, did not seem to be affected by length (Figure 3.1). Similarly, BAD binding to BCL-XL was not decreased in the 20-mer SPOTs as compared to the 26-mers. However, for Noxa peptide, MCL-1 bound both of the 26-mer spots, but showed significantly decreased binding to the 20-mer spots (Figure 3.1). Solution binding studies using 18- and 26-mer BIM peptides showed tight binding to MCL-1 in each case [6]. However, in studies with BCL-XL, the 18-mer BIM had a lower affinity at 110 nM as compared to the 26-mer at 7 nM [6]. Thus, based on these SPOT experiments and others, all future BH3 peptides were synthesized as 26-mers.

### 3.3.1.3 Peptide linker to membrane

Different linker lengths between the BH3 C-terminus and the membrane were tested. The linkers were intended to provide the BCL-2 receptors sufficient room to bind. Comparisons indicated that both linker lengths were compatible with binding. Signals from the four-residue
and twelve-residue linkers were similar (Figure 3.1). Later membranes synthesized by Biopolymers included no linker and a PEG₃ linker in addition to the four-residue GSGS linker. Similar to the previous results indicating that both the four- and twelve-residue linkers were capable of binding the BCL-2 proteins, binding assays with these new membranes showed little difference between the linkers used (Figure 3.3). The no-linker and PEG₃ linker peptides show similar signal as for the four-residue linker. The no-linker peptides gave somewhat weaker signal, but the PEG₃ linker and GSGS linker were comparable. Based on these results, I designed all future membranes with the PEG₃ linker. Though the data from the three no-linker peptides looked good, the linker-containing spots were slightly stronger (especially BIM binding MCL-1). The PEG₃ provides the advantage over the four-residue linker of reducing the synthesis by three amino acids, likely increasing the purity of the peptide and decreasing time and cost of synthesis.

<table>
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<tr>
<th>Linker</th>
<th>BCL-X₁</th>
<th>BIM</th>
<th>BAD</th>
<th>Noxa</th>
<th>MCL-1</th>
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**Figure 3.3. Comparison of linkers**
Three BH3 peptides – BIM, BAD, and Noxa – were printed with different linkers. The binding of BCL-X₁ and MCL-1 to these peptides is shown. SPOT images were cut from the full arrays shown in Figure 3.7.

### 3.3.1.4 Oxidation of methionine

In every membrane synthesized at Biopolymers, a few peptides were synthesized with a cleavable Rink Linker at the C-terminus. Biopolymers personnel cut these spots from the membrane and treated them with a trifluoroacetic acid-containing mixture to cleave the synthesized peptide from the membrane, allowing for validation by MALDI. In these tests, we observed that all peptides containing methionines were about 16 Da larger than the calculated mass, corresponding to methionine oxidization to methionine sulfoxide. The treatment to cleave the peptides from the membrane is the same treatment used for deprotection of side chains post-
synthesis, thus it is possible that the methionine oxidation observed in the MALDI results is also present in the peptides on the membrane.

Oxidized methionines can potentially cause a problem in binding assays, since many of the BH3s have methionines in the middle of the sequences on the hydrophobic face. In order to minimize any problems that oxidized methionines may cause, I pretreated the membranes with reductant in an attempt to reduce the methionine sulfoxides. This treatment involved exposing the membranes to a high concentration of dithiothreitol (1 M DTT) at an elevated temperature (37 °C) before testing binding to MCL-1 and BCL-XL. In some cases, there was a decrease in binding from no DTT treatment to DTT treatment for peptides containing methionine, such as PUMA. Only in the case of MULE did a peptide with a methionine have increased binding on the DTT-treated membrane (Figure 3.4). If methionine oxidation was directly affecting the signals of methionine-containing BH3 peptides, I would have expected to see an increase in signal upon DTT treatment.

The difference between the DTT-treated membranes and the normally treated membranes was more than simply the presence of the reductant. A mock DTT control treatment that involved heating and increased incubation time in the absence of DTT showed some loss in signal (although less than with DTT), as seen in Figure 3.5 for BIM, PUMA, and BAK. This indicates that while DTT was likely contributing to the decreased signal with some of these peptides, other factors are also involved. In comparison, only MULE signal increased only when DTT was present, suggesting that the signal increase is actually due to DTT, although this is the only case in which significant signal increase was observed.

It should be noted that with the first DTT treatment, rings of increased signal intensity around spots appeared (Figures 3.4, 3.7, and 3.8). However, rings were also present on some membranes not treated with DTT, and DTT treatment does not always result in rings.
Protein [conc] treatment

Expected

BCL-XL

5μM normal
5μM DTT
200nM normal
200nM DTT

MCL-1

2μM normal
2μM DTT
200nM normal
200nM DTT
50nM normal
50nM mock
50nM DTT

Figure 3.4 SPOT strips binding BCL-XL and MCL-1 with different treatments
Binding to SPOT strips without DTT, with DTT, or mock DTT treatment (described in Methods). Expected binding by MCL-1 and BCL-XL, based on literature, is shown. Red, orange, and yellow are high, medium, and low affinity binders, respectively. Gray boxes are reported not to interact. White has not yet been reported. Sequences with a Met in the core of the sequence have an “M” above them. Sequences with Met outside of the core, that are expected not to be largely affected if oxidized, are indicated by a “*”. Noxa has a Cys in the core, as shown by the “C”. For BCL-XL, the 5 μM sample was His-tagged, while the 200 nM was c-myc-tagged. All MCL-1 probes used the c-myc-tagged version. Binding was detected by primary antibodies against the tag, followed by dye-labeled α-mouse secondary.

Figure 3.5 Quantitation of MCL-1 binding following different membrane treatments
Quantitation of the binding of 50 nM MCL-1 to the SPOTs shown in Figure 3.4.
3.3.2 Probe conditions

3.3.2.1 Direct labeling of probe

Initial SPOT binding tests used fluorescently labeled proteins. This method seemed ideal due to one-step detection. Binding of BCL-XL-Cy3 and MCL-1-fluorescein looked promising (Figure 3.1), but signal was observed for negative controls. This may have been due to free dye in the protein sample. In the case of fluorescein-labeled MCL-1, free dye may have bound to Noxa, the only peptide on the strip that contained a cysteine. This would have been indistinguishable from MCL-1 binding. This was further illustrated with a coiled-coil control peptide, cJUN, on the membrane. FOS labeled with fluorescein should have bound cJUN tightly [132], but there was little or no signal on the cJUN spot. However, there was significant binding to other spots, including Noxa (Figure 3.1). It should be noted that synthesis of the longer cJUN peptide (34 aa with 12 aa linker) was likely not complete, probably missing the N-terminal six amino acids due to an error during synthesis, according to Intavis.

3.3.2.2 Antibody detection of BH3 peptides

Half of the peptides synthesized by Intavis contained a FLAG tag, so the presence of peptide, or at least the tag, could be tested. Strips were probed with α-FLAG antibody, washed, and then incubated with R-phycoerytherin-labeled α-mouse antibody. Every peptide synthesized with a FLAG tag showed signal. All but one of the tagged peptides had the tag at the C-terminal end. This means that the large α-FLAG antibody was able to bind close to the membrane. Binding was also detected for a peptide with the FLAG tag at the N-terminus of a thirty amino acid linker, indicating that the sequence was also suitable for a longer peptide. Thus, antibody detection appeared to be promising.

3.3.2.3 Antibody detection of BCL-2 receptor probes

Most of the previous probe detection used fluorophore-labeled proteins, for which false positives were observed, such as BCL-XL (tcXH3) binding Noxa. The results from antibody detection of the FLAG tag peptides looked clean, so I proceeded to use epitope-tagged BCL-2 probes followed by antibody detection, in contrast to using directly labeled BCL-2 receptors.

In the case of MCL-1, the MBP fusion protein showed binding to BIM and Noxa, as expected. Unfortunately, there was also significant signal for BAD, which is reported to not bind
MCL-1. Testing binding of a Cy3-labeled MBP (no fusion to BCL-2 receptor) showed some binding to the peptide spots, especially the BAD spots. Thus, the MBP may have been responsible for some of the binding. A c-myc-tagged MCL-1 that was MBP-free and could be detected by antibodies was then used. With this protein, the signal for MCL-1 binding to BAD dropped to background levels (Figure 3.1). Unfortunately in later membranes, BAD binding to MCL-1 has been shown to be quite strong (Figure 3.4). The reasons behind this presumed false negative are still unknown.

Antibody detection of BCL-XL binding appeared promising. His-tagged BCL-XL (XH2) showed a cleaner signal when read out using an α-His-tag antibody followed by dye-labeled α-mouse antibody, as compared to the fluorophore-labeled BCL-XL proteins. The Noxa signal from antibody-detected BCL-XL was diminished as compared to fluorescently-labeled BCL-XL. To standardize the detection method, I began using a c-myc-tagged BCL-XL. This c-myc-tagged BCL-XL bound the BH3 peptides similarly to the previously used His-tagged version (Figure 3.4). Also, transition to Cy3-labeled α-c-myc antibody helped eliminate the need for a secondary incubation, yet maintained good binding signal.

3.3.2.4 Variation of solution protein concentrations

For the initial SPOT binding tests, 1-5 uM of BCL-2 receptor was used. Lower concentrations of protein would not only conserve purified protein, but could potentially allow for greater distinction between peptide binding affinities as observed on the SPOT membranes. Thus, I compared binding over a range of MCL-1 concentrations (Figure 3.4). While overall trends are clear in the picture of the SPOT membrane, quantitation of this signal indicated that when the concentration is very low, the overall signal is dampened (Figure 3.6A). Thus, the signal difference between interaction and non-interaction is decreased, and determining true interaction over the background becomes more difficult.

Antibody concentration testing showed a similar trend. All detection of BCL-2 receptor binding with BH3s used 100-fold diluted antibody, but to see if lower concentrations were possible, I used membranes printed with short peptide epitopes. These membranes have c-myc tag printed multiple times, varying the concentration of the first residue printed, which does not seem to affect signal (as previous discussed in section 3.3.1.1). Probing these strips with decreasing concentrations of α-c-myc antibody – 100-, 1000-, and 10000-fold dilutions – showed
that at the lower concentration, there was a decreased difference in signal (Figure 3.6B). In fact, at the 10,000-fold dilution, there was little signal difference between the c-myc-tag peptides, which should bind, and the FLAG-tagged peptides, which should not bind. Thus, the higher antibody concentrations were used for all subsequent experiments.

Figure 3.6 Varying concentration of probe.
(A) BH3 peptide strips were probed at varying concentrations of MCL-1. All of these strips underwent DTT treatment. The fluorescence signal is normalized to BAK, a peptide that has consistently strong signal and no core Met. (B) c-myc peptides probed with varying concentrations of α-c-myc antibody. The antibody dilution factors are shown. Peptides were synthesized with different concentrations of the first residue, the concentration (mM) indicated after the peptide name. Signal was normalized to the first c-myc peptide in the strip for each antibody dilution.
3.3.3 Larger test to compare binding of point mutations from literature

A membrane with mini-arrays of 130 unique peptides and a few repeats was printed by Biopolymers. The sequences printed included the BH3 regions from native proteins – BH3-only proteins, the pro-apoptotic BAX and BAK proteins, some anti-apoptotic proteins – BIM-based BCL-X\textsubscript{L} binding designs [8], alanine scan of BAK [145], point mutants of BAD and Noxa [4, 7], and alanine, glutamate, lysine scan of BIM [146, 147]. Most of the peptides printed have been previously tested for binding to BCL-X\textsubscript{L} and MCL-1, with affinities reported in Figure 3.7.

Data from these peptide arrays, especially the wild-type BH3 sequences, matched with the literature values (Figure 3.7). Most point mutations of BH3s were also largely consistent with the literature. Some notable discrepancies with the literature include the BIM F4aD and F4aK mutants; these mutants were expected to not bind BCL-X\textsubscript{L}, but as can be seen in Figures 3.7A and 3.7C, both of these F4a mutants bound BCL-X\textsubscript{L} quite well. Further, from the literature, I expected tight binding of L3aS to BCL-X\textsubscript{L} and no binding for L3aT. However, the SPOTs show the opposite, with L3aT binding fairly well and L3aS with relatively weak signal. The L3a mutants illustrate a situation where the signal read from the SPOTs is slightly higher or lower than that expected from the literature. This is generally the level of the discrepancy between the SPOT data and the literature. One case where the difference was larger was a BIM mutant that bound MCL-1 well, but not BCL-X\textsubscript{L} (BIM Mmut) [6]. On the SPOT arrays, this mutant bound BCL-X\textsubscript{L} as well as many of the stronger binders. This may be due to peptide length, since this study used 18-mer peptides. The affinity of the wild-type BIM peptide for BCL-X\textsubscript{L} is ten-fold tighter as a 26-mer [6]. Since the peptides on the membrane surface are 26-mers, this may be part of the reason for the discrepancy with the literature.

The greatest difference between the expected binding patterns and the observed signal were for the computationally designed BCL-X\textsubscript{L}-binding peptides [8]. These designs, as 26-mers, were tested in a pull-down assay, as well as a fluorescence polarization competition assay in solution, and found to bind BCL-X\textsubscript{L} [8]. Yet on the SPOT membrane, essentially no binding for any of these designs is observed (Figure 3.7). Even at a much higher concentration (40 μM) of murine BCL-X\textsubscript{L}, for which they were designed, still no binding is observed (data not shown). This had also been seen with the Intavis strips (Figure 3.1).
Figure 3.7 Reported affinities and actual signal of SPOT binding

Binding affinities of peptides for (A) BCL-XL and (C) MCL-1 taken from literature. Binding affinities are indicated by colors according to the heat map shown. White boxes indicate that the affinity for the interaction is unknown. All peptides have GSGS linkers to the membrane, unless the text is blue, as for PEG3 linker, or white, as for no linker. BAD mut1 and mut2 are mutations to BAK sequence [4]. Noxa m3 is Noxa variant capable of binding BCL-XL [7]. BIM 2A is mutant of BIM [5]. The BIM Mmut and Xmut are mutants from Boersma et al. [6]. The peptides in the last row X1 through N4 are sequences designed to interact with BCL-XL [8]. The probed SPOT arrays are shown for BCL-XL (B) and MCL-1 (D) below the expected with the same overall layout. His-tagged BCL-XL and c-myc-tagged MCL-1 were used with the appropriate antibodies. The bold black squares in (A) and (B) are peptide sequences discussed in the text.
3.3.4 Substitution Analysis of BIM

To systematically test the effect of all possible point mutations at all possible positions, substitution analysis was performed on a 26 amino-acid BIM BH3 peptide sequence. A membrane was synthesized in which 13 positions were changed – one at a time – to 19 amino acids (cysteine was excluded). Additionally, 40 spots were repeated for duplicate comparison. Two sets of 13 x 20 (19 + 1 wt column) arrays with 40 repeats were synthesized and probed with MCL-1 and BCL-X\textsubscript{L} at 1 \mu M. The substitution analysis results (Figure 3.8), even with some spot morphology problems (rings and lines through rows of spots), were promising. Some trends were as expected. For example, the conserved L3a position generally cannot accommodate charged or polar residues for either BCL-X\textsubscript{L} or MCL-1 binding. Also, the small G3e position cannot maintain binding when substituted with larger residues (this is especially true for MCL-1). More subtle trends were difficult to identify due to a variety of factors, including rings and what appears to be a horizontal white line through many of the spots (possibly due to misaligned pencil-drawn grids). Both of these make quantitation of the spot signals difficult due to the variable spot morphology. Further, the spot-to-spot variation in signal was high and seemed to vary semi-systematically with position on the array, as seen by comparing the column of wild-type peptides in each substitution analysis array (Figure 3.8).
A repeat substitution analysis was performed, avoiding methionine and cysteine, and therefore the potential need for any reducing agent. In this new array, a proline was included as a likely non-interaction control in each row. These arrays had 10 sites substituted with 18 amino acids. The first peptide in each row is the BIM wild-type sequence and the last peptide is a repeat of one of the single substitutions (not the same mutation at each position). These arrays, shown in Figure 3.9, were probed with 100 nM and 1 μM of receptor protein. These arrays were much cleaner than the initial BIM substitution analysis arrays, with reduced rings and no white lines to disrupt data processing. I am unsure of the reason for this cleaner data, but there are a few possibilities. The new arrays were not treated with DTT, unlike the first substitution analysis arrays. Also, these membranes were made later, giving more time for Biopolymers to gain experience with the relatively new peptide synthesizer. The signal from these spots appeared to be reproducible, as seen in Figure 3.10, where up to three identical peptide signals are compared. This can also be seen by looking at the wild-type column in the substitution analysis arrays.

From this analysis, some sites appear limited in the amino acid mutations allowed. For example, at the A2e position, it appears small amino acids are capable of binding MCL-1, but for BCL-X_L, the amino acid size is even more restricted with glycine preferred over wild-type alanine (Figure 3.9 and 3.11A). The opposite trend is observed for the G3e position, where MCL-1 binds essentially only the wild-type glycine, whereas BCL-X_L is slightly more permissive, allowing alanine and serine (Figure 3.9 and 3.10C). The I3d and F4a sites also appear interesting in specificity determination of BCL-X_L and MCL-1. In I3d, most substitutions reduce binding to MCL-1 significantly, but maintain, and in some cases even improve, binding to BCL-X_L (Figure 3.11B). The opposite is true for the F4a site, with most mutations binding to MCL-1 and not BCL-X_L (Figure 3.11D). These sites are interesting as potential specificity determinants, at least in the context of the BIM sequence.
Figure 3.9 Substitution analysis of BIM
Substitution analysis of BIM binding MCL-1 (A and C) and BCL-X\textsubscript{L} (B and D). The position being substituted is shown on the sides of each row. (A) and (B) were probed with 100 nM BCL-2 protein. (C) and (D) were probed with 1 \textmu M BCL-2 protein. \textalpha\text-myc labeled receptors were used for both MCL-1 and BCL-X\textsubscript{L} followed by Cy3-labeled \textalpha\text-c-myc antibody for detection.
Figure 3.10 SPOT duplicates from substitution analysis
Comparison of duplicate and triplicate spots for MCL-1 (A) and BCL-XL (B). Signal is normalized to the average wild-type BIM on that particular array (5 spots for membrane 1 and 20 for membrane 2). In the case of membrane 1, 18 BIM point mutations were tested in the same row, so the wild-type is the same for each grouping. For membrane 2A and 2B, data are from the same membrane, but when available, different spots of the same sequences are shown. For this, the BIM WT spots come from the row in which the listed site is substituted. Both membranes were probed with 100 nM protein. † indicates that there is no copy membrane 2 for this mutation. ○ indicates there is no membrane 1 data for this mutation.
Figure 3.11 Sites that influence BCL-XL versus MCL-1 binding specificity.
SPOT signal for substitutions at A2e (A), I3d (B), G3e (C), and F4a (D) binding to BCL-XL (blue) and MCL-1 (red) from figure 8C and 8D. Signal is normalized to the average wild-type BIM signal on the membrane.

3.4 Discussion

I have applied the SPOT technology to the study of the BH3/BCL-2 interaction. This was possible because new peptide synthesizers, such as the Intavis machine employed in this study, are capable of printing long (>25-mer) peptides. This is a good match for the length of BH3 peptide previously shown to be fundamental for BCL-2 binding. The protocol that has proven most successful was when membranes were probed with c-myc-tagged BCL-2 receptor. The probe was then visualized using an antibody to the c-myc tag. The SPOT technology looks promising for application to the BH3 peptides, and data from BIM BH3 substitution analysis reveals residues that provide discrimination between BCL-XL versus MCL-1 binding.

3.4.1 Specificity determinants identified in SPOT substitution analysis

The extension of the SPOT technology to perform substitution analysis of BIM demonstrates the power of this tool for analyzing the binding of BCL-2 receptors to many BIM
variants simultaneously. In the initial substitution analysis test (Figure 3.8), it appeared that the majority of amino acid substitutions did not hinder binding to either BCL-X_L or MCL-1. However, with the newer, cleaner substitution analysis data, it became clear that this is not the case. At 100 nM probe concentration, many of the peptides lose binding when compared to the wild-type BIM. When a higher concentration was used, many interactions not present at 100 nM could be detected at 1 μM. While most substitutions maintain or disrupt binding to both BCL-X_L and MCL-1, some sites appear as though they could play a role in establishing specificity; these are discussed below. This is especially evident from the 100 nM substitution analysis data.

3.4.1.1 Position I3d

Most substitutions at I3d, the third buried hydrophobic position, kill binding to MCL-1 while maintaining BCL-X_L binding when probed with 100 nM protein. Much of this pattern is dampened at 1μM probe concentration, but the trend is still clear that BCL-X_L is more flexible regarding the amino acids at this site. In the co-crystal structure, this site appears less tightly packed in the BCL-X_L, where it is located next to the less helical α2/α3 region of the BCL-X_L receptor, as compared to MCL-1. In native BH3 sequences, this site is generally hydrophobic, though it should be noted that Beclin, a BH3 that specifically binds BCL-X_L, has threonine at this site.

3.4.1.2 Position F4a

In contrast to the I3d position, almost any amino acid at the fourth buried position, F4a, can still bind MCL-1 but not BCL-X_L. This trend was observed by Lee et al. in a saturating mutagenesis study of the F4a position in BIM using phage ELISA assay [3]. Examination of the BIM co-crystal complexes indicate this site is more exposed in MCL-1 as compared to BCL-X_L. As discussed in Chapter 2, a charged glutamate residue is accommodated at this position by rotating out of the groove, as observed in the crystal structure of MCL-1 bound to the BIM F4aE mutant peptide. In native BH3 sequences, the F4a position is occupied by hydrophobic amino acids. However, the smallest residue present in the native BH3s is valine, only observed in MCL-1-specific peptides, such as MULE. With BCL-X_L observed to have preference for large
aromatic residues at this position in the SPOT arrays, it appears that this site may already be used by nature to impart specificity on the BH3 sequences.

3.4.1.3 Position A2e

The A2e position is generally small in native BH3 sequences (glycine, alanine, and serine). In the BIM co-crystal complexes with BCL-X<sub>L</sub> and MCL-1, alanine is tightly packed against the side of the hydrophobic groove at this site. Substitution analysis at 100nM probe concentration indicated that in BIM, BCL-X<sub>L</sub> has a striking preference for glycine and alanine, with serine, threonine, and proline additionally allowed at 1 μM probe concentration. MCL-1 can accommodate a larger range of amino acids, including valine, proline, and threonine at low probe concentration and all amino acids at higher concentration. This specificity is consistent with observations by Lee et al., where a BIM A2eE mutant retained tight binding for MCL-1, but lost the ability to bind BCL-X<sub>L</sub> and BCL-2 [5]. Thus, this site can confer specificity for MCL-1.

3.4.1.4 Position G3e

Position G3e is another site typically occupied by small amino acids in native BH3 sequences. On the arrays, this position can be mutated to alanine, and possibly serine, and still bind BCL-X<sub>L</sub>, while losing MCL-1 binding. In the BIM complexes with both MCL-1 and BCL-X<sub>L</sub>, this site is very constrained, with the glycine packing against the receptor backbone in the BH1 region. Mutation of this site would likely require shifting of the receptor groove to allow binding. However, in the native BH3 sequences, alanine is present in peptides capable of binding MCL-1, including the tight binding PUMA. The only larger residue in the native sequences is serine in BAD, a BCL-X<sub>L</sub> specific peptide. However, a previous study showed that a serine-to-glycine mutation in BAD is not sufficient to restore MCL-1 binding [2]. Thus, the context of the mutation (BIM versus BAD) may play a role in the mutation allowed at this site.

3.4.1.5 Position L3a

The L3a position is almost universally conserved in native BH3s. All BCL-2 proteins, except for anti-apoptotic BCL-W, have a leucine at this position. The substitution analysis, particularly the higher concentration data, shows that other amino acids are compatible with binding, consistent with earlier observation [5, 8]. While both BCL-X<sub>L</sub> and MCL-1 have
preference for hydrophobic amino acids at this position, there is a possibility for introducing specificity with the larger aromatics. Consistent with observations by Lee et al. [3], the SPOT binding shows that while BIM with a tyrosine at position L3a cannot bind MCL-1, other aromatics at this position are capable of binding MCL-1. However, it appears that tryptophan at this position may hinder binding to BCL-XL in comparison to the other hydrophobic residues, whereas for MCL-1, binding is still as strong as the other hydrophobics. Thus, the conserved L3a site may be capable of generating MCL-1- and BCL-XL- specific peptides with similar amino acids.

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Table 3.2 Available literature on BIM mutants binding to MCL-1 and BCL-XL
1. Saturating mutagenesis of L3a and F4a by Lee et al. [3].
2. Alanine scanning by Lee et al. [5].
3. Alanine, glutamate, and lysine scanning by Boersma et al. [6].
4. Computational design study by Fu et al. [8].
5. Chapter 2

3.4.2 Comparison to the literature

In recent years, researchers have begun more systematic analysis of amino acid requirements at particular sites in the BIM BH3 sequence. Table 3.2 provides a summary of mutant BIM BH3 peptides that have been previously characterized. However, it should be noted that peptide length and interaction detection methods vary among these studies. The two Lee studies use 26-mer peptides expressed on phage in a quantitative ELISA assay to determine IC50s [3, 5]. Boersma et al. determined the KdS of 18-mer peptides in a fluorescence polarization assay [6]. While many mutants have been tested previously, the significant majority of point mutations had not been tested until now (as seen in Table 3.2).
3.4.2.1 Alanine scanning mutagenesis

The effect on binding of alanine scanning in BIM BH3 to BCL-2 proteins MCL-1 and BCL-Xₐ have been previously reported two groups [5, 6]. The reported affinities of these mutants can be seen, represented as a heat map, compared to the SPOT results as can be seen in Figure 3.12. The SPOT data looks comparable to the previously reported data, especially when comparing the 100 nM MCL-1 SPOT data to the Boersma data. The mutants with the highest intensity SPOT signals corresponded to those with the lowest Kₐₜₜs. Interestingly, the Lee data matched the pattern of the 1 μM SPOT data better than either the Boersma or 100 nM SPOT data.

![Figure 3.12 Comparison of alanine scanning data](image)

SPOT data compared to literature binding studies.

3.4.2.2 Glutamate and lysine scanning

In addition to alanine scanning, Boersma et al. performed ‘hydrophile scanning’, mutating positions to glutamate and lysine [6]. These data did not correlate as well as the alanine scanning data (Figure 3.13). While most of the high intensity signal spots correspond to high affinity interactions from the solution FP study, some spots do not. In particular, the I3dE spot mutant shows strong binding to BCL-Xₐ, even at the lower probe concentration, while Boersma determined that the mutant did not bind. This may be due to differences in BH3 length. As previously mentioned, the wild-type BIM had ten-fold weaker affinity for BCL-Xₐ as an 18-mer, used in the Boersma scanning, as compared to the 26-mer, the length used in SPOT peptides [6].
Figure 3.13  Comparison of glutamate and lysine scanning data
SPOT data compared to literature binding studies. Affinities from literatures are represented in heat map in Figure 3.12. X indicates there is no data for this mutant from this study.

Figure 3.14  Comparison of saturating mutagenesis at L3a and F4a
SPOT data compared to literature binding studies. Affinities from literature are represented in heat map in Figure 3.12. X indicates there is no data for this mutant from this study.
3.4.2.3 L3a and F4a saturating mutagenesis

Saturating mutagenesis had previously been performed on two buried hydrophobic sites in the BIM BH3 sequence. Lee et al. determined IC50s of MCL-1 and BCL-XL binding of all L3a and F4a point mutations (no proline) in phage ELISA binding assay. The pattern of L3a mutants binding to both BCL-2 receptors reported is very similar to the SPOT data, with most hydrophobic mutations maintaining binding to both receptors, while polar and charged mutations lose binding. The most striking difference between the SPOT data and the previously reported data is L3aS. This mutant peptide was reported to bind BCL-XL with affinity similar to wild-type BIM, however this interaction was not observed on the SPOT membranes. This discrepancy was noticed previously in section 3.3.3. The F4a mutant binding pattern reported by Lee et al. also correlated with the SPOT data. All of tested F4a mutant peptides bound MCL-1, and a greater selectivity was observed for BCL-XL.

3.5 Future Work

3.5.1 Areas to improve

While the technique is quite promising, there are a few issues that should be resolved. Some of these improvements may lie in the array synthesis method. Others involve perfecting the membrane probing and data processing methods to improve the quality of the data available from SPOT arrays.

Conditions have been worked out to give respectable results using antibodies against epitope-tagged BCL-2 proteins. However, conditions can be further optimized. The reduced loading capacity membrane synthesized by Intavis appeared to have lower background signal, and decreased signal from expected non-interactions. Attempts to lower the loading capacity in the Biopolymers-printed membranes did not show the same effect, though there were no false positives, such as BAD, for comparison. Additionally, the means of lowering the loading was different. For the membrane made by Intavis, the first FMOC residue printed was diluted in an acetylated equivalent, preventing further extension. For the Biopolymers membrane, the first residue was simply diluted, thus there may not have been a decreased amount of peptide in the
SPOT. Future applications of the SPOT technique to the BH3 peptides should further explore the reduced loading capacity. This may help with some of the false positive signals and any rings.

Determining whether methionines are being oxidized is crucial if this work is to be applied to more BH3s, because many of the native sequences have methionines in the middle of the BH3. If oxidation is occurring on the membrane, then a method to prevent it or to reduce the oxidized methionines afterward is needed. Alternatively, norleucine can be examined as a possible methionine substitute.

Another recurring problem is that BAD BH3 appears to bind MCL-1 in the SPOT array. BAD is reported to not bind MCL-1, as confirmed in my own hands with a commercially available BAD peptide. Yet, BAD peptides on the membrane often bind, often as strongly as the high affinity BH3s (Figure 3.4). Mass spectrometry validated the sequence of BAD synthesized on the membranes, mostly with an oxidized methionine. For now, the origin of the BAD signal with MCL-1 probes remain unknown though it should be remembered that these experiments are probably not at equilibrium, and so cannot be expected to agree perfectly with solution binding data in all cases.

Another issue is that the designed BH3 peptides [8] never show binding, even for the low nanomolar affinity X1 design probed with 40 \( \mu \text{M} \) of murine BCL-X\(_L\). This may be due to oxidized methionine, as methionine is present in the majority of the designed peptides. Yet, because not all of the designs have methionine, including one of the low nanomolar affinity binding peptides (I3-I8A), some as-yet-unidentified factor is probably contributing. While the SPOT technique works well for most native BH3s, it would be beneficial to apply it to the design peptides, enabling the screening of hundreds of computationally designed BCL-2 receptor binding peptides to identify high affinity designs for further studies. Thus, getting all peptides, even non-native ones, to work in the SPOT would be greatly beneficial to the study of BH3 specificity.

An additional area to pursue is exploring the re-usability of the membranes. Preliminary studies indicate that it is difficult, indeed practically impossible, to remove bound antibody from the SPOT membranes using denaturants (data not shown). However, it has been suggested that the protein bound to peptides on the membrane can be transferred via blotting to another membrane, which can then be visualized with antibodies [143]. Since the protein has been
removed from the original SPOT membrane by electrophoresis, it is available to be probed again. This would enable re-using of arrays, which can be beneficial when one is interested in probing the same six hundred peptide array with four different proteins at two concentrations; only one array would be needed rather than eight, reducing the cost significantly.

3.5.2 Future applications

Now that the SPOT array appears to work satisfactorily for the BH3 peptides – especially for BIM and BIM point mutants – this technique can be applied to many other BH3 questions. Within the BIM context, the SPOT technique can be used to look at the coupling of different sites. By mutating multiple sites simultaneously, we can see if the effects are non-additive, as observed for alanine mutations of the L3a and F4a positions in BIM hindering BCL-XL binding [5]. This experiment has to be planned carefully, as the number of possible sequences can rapidly become unmanageable. This substitution analysis technique can also be applied to other BH3s to determine the effect of mutations in other contexts. For example, is the G3e position as restrictive in PUMA, Noxa, and Bad as it is in BIM? Or do we see the same specificity swap at the hydrophobic 3d and 4a positions for BCL-XL and MCL-1 in BID, or other BH3s? As mentioned previously, many computationally designed peptides can be tested with the SPOT arrays, once conditions have been worked out. This could be used as a screening method to ensure that peptides pursued for more rigorous study (large-scale synthesis, purification, solution binding and structural studies) are the candidates most likely to succeed.

All of these experiments have important considerations in the experimental design, including how to deal with possible methionine oxidation and peptide loading, as discussed previously. One important consideration that has become apparent is the probe concentration. The use of even two concentrations can report more subtle differences that may be masked at higher or lower concentrations. However, the SPOT technique provides a powerful tool to test binding of many BH3 peptides, as shown the substitution analysis of BIM.
Chapter 4

Conclusions and Possible Future Directions

In this thesis, I have described my efforts to further understand the specificity of the interactions among anti-apoptotic BCL-2 proteins and pro-apoptotic BH3 peptides, which are key apoptotic regulators. Much of this has focused on MCL-1 – an anti-apoptotic protein with an interaction profile distinct from BCL-XL, BCL-2, and BCL-W – and the BH3 peptide of BIM – a BH3-only that promiscuously interacts with all known anti-apoptotic BCL-2 proteins.

In Chapter 2, I showed the structures of MCL-1 bound to peptides that correspond to the BIM BH3 sequence, for which the latter was made as the wild-type and as three point mutants. In these structures, MCL-1 is observed to interact with the BIM peptides in the same manner as other BCL-2 proteins. A hydrophobic groove on the protein surface, formed in part by regions BH1-3, is occupied by the BIM BH3 peptide. Upon comparison of BIM-bound MCL-1 with free MCL-1 (murine NMR structure), it is clear that the groove widens, with both $\alpha_3$ and $\alpha_4$ shifting away from one another to accommodate binding of the BIM BH3 peptide. In contrast, when BIM binds to BCL-XL (murine structures), the groove straightens and $\alpha_3$ partially unravels, becoming less $\alpha$-helical.

Structures of the three mutant BIM BH3/MCL-1 complexes, as solved by X-ray crystallography, revealed further important information. Two of these mutations were at the I2d position (the first hydrophobic buried position in BIM), where this residue was mutated to alanine and tyrosine. In the alanine mutant (I2dA) complex, the structure is essentially identical to the wild-type complex. The most noticeable difference is a leucine on MCL-1 shifting to help fill the hole left by the mutation. The tyrosine mutant (I2dY) complex exhibits a much larger change from the wild-type complex. The N-terminal part of the BIM BH3 helix, where the mutation is located, and the $\alpha_3$ region of MCL-1 move away from each other to accommodate the large aromatic residue. The third mutation is at the fourth hydrophobic buried position; the wild-type phenylalanine was mutated to glutamate (F4aE). This residue rotates out of the hydrophobic groove without much disruption to the rest of the complex.
These MCL-1/BIM BH3 structures display flexibility in both the MCL-1 receptor and the BIM BH3 binding peptide. This flexibility makes prediction of allowable mutations difficult. For example, based on steric constraints in the wild-type BIM/MCL-1 structure, the I2dY mutant was expected to not bind MCL-1. Since prediction of the ability of point mutants to bind is limited, empirically measuring binding of the point mutant is required to provide information about the range of amino acids allowed.

In order to perform such a test, I applied SPOT technology to the BCL-2/BH3 peptide interaction. Interactions of BCL-2 proteins MCL-1 and BCL-XL with BH3 peptides synthesized on a membrane surface were tested. Assay development indicated that longer BH3 peptides were ideal for the SPOT application: 26-mer Noxa peptides bound MCL-1 (a positive control interaction), whereas 20-mer peptides did not. Comparing methods of detecting interactions on the membrane surface revealed that antibody detection of epitope-tagged BCL-2 proteins gives the best performance, with fewer false positives and a lower background signal than directly-labeled BCL-2 proteins. MCL-1 and BCL-XL were found to bind a variety of native BH3 sequences on the membrane.

The SPOT method was used to perform substitution analysis of the BIM BH3 interaction with MCL-1 and BCL-XL. Ten sites within the BH3 of BIM were mutated to 17 non-native amino acids and then probed for binding to MCL-1 and BCL-XL. While the results are qualitative, duplicate spots indicate that they are reproducible. From this, some sites within the BH3 BIM region appear as though they could play a role in specificity, and many observations are consistent with prior reports. MCL-1 appears more tolerant of different residues at the A2e position compared to BCL-XL. In contrast, BCL-XL accommodates more diversity at the BIM G3e position, compared to MCL-1. Similar trends are observed for two buried hydrophobic positions. I3d can be almost any natural amino acid, and still bind BCL-XL, whereas F4a can be almost any residue, and still bind MCL-1. These sites, individually and in combination, may be used to impart specificity on BIM BH3, as has been done in some previous studies [3, 5, 6]. These sites were identified using a probe concentration of 100 nM. A 10-fold higher probe concentration masked some of these specific sites (as expected since weaker interactions were then detected), and thus probe concentration is an important consideration for future studies.
Unfortunately, the high cost of SPOT arrays precludes using multiple concentrations to characterize large numbers of interactions.

Structural studies of BIM point mutants binding MCL-1 indicate that both small changes (rotation of side chains) and larger changes (shifting and bending of helices) can be used to accommodate mutations in BIM peptides. Examining the SPOT data in the context of the mutant structures may help predict the expected structure of the complex. For example, at the F4a position of BIM, the mutation of this site to a glutamate resulted in the rotation of this residue out of the hydrophobic groove. Thus, other residues at this position, especially those that are polar or charged, may be capable of rotating out of the hydrophobic groove in a similar fashion.

The MCL-1 or BCL-XL specificity arising from mutations at A2e and G3e, respectively, may indicate larger changes to the structure than that observed with F4aE. Because of the tight interface at these positions with both MCL-1 and BCL-XL, mutations at those sites still capable of binding the anti-apoptotic proteins may make larger changes to the structure to accommodate the new residue, as was observed with the BIM I2dY mutant with MCL-1. Examination of the wild-type BIM complex with MCL-1 suggested little room to allow residues larger than wild-type isoleucine at position 2d. However, the structure of the tyrosine mutation (I2dY) in BIM indicates that both the BIM BH3 peptide and MCL-1 \( \alpha3 \) move to accommodate the larger residue. Thus, when binding to A2e mutants, as observed in the SPOT arrays, either the BIM BH3 helix or the MCL-1 \( \alpha4 \) helix (or both) may shift away from one another to fit the larger residue. Helix \( \alpha4 \) of MCL-1 has already been observed to shift upon binding the BH3 peptides, and it may be able to shift even further, upon binding of valine or threonine mutants of the A2e position. Thus, combining information gathered from the structures with that gathered from the SPOT analysis may suggest regions of flexibility within the complex. X-ray crystal structures of A2e mutants (such as valine, threonine or proline) in complex with MCL-1 are required to confirm this.

Additionally, the highly conserved L3a site may provide further insight into the conformational flexibility of MCL-1. The SPOT data show that most hydrophobic residues are compatible with MCL-1 binding, including phenylalanine and tryptophan. However, tyrosine at L3a does not bind MCL-1 in the SPOT arrays, nor in the study by Lee et al. [3]. This is in
contrast to the case of BCL-XL, where all of the large aromatic mutations are capable of binding. Given the binding of phenylalanine and tryptophan mutants, it is possible that the phenolic group of the tyrosine mutant underlies the specificity against MCL-1. The tyrosine can possibly rotate to out of the groove, interacting with a backbone atom through the third helix of BCL-XL, which is not α-helical as discussed in Chapter 2. The equivalent region in MCL-1 is α-helical and has no capacity for hydrogen bonding. Structures of BIM peptides with leucine-to-tyrosine mutation at 3a in complex with BCL-XL would likely reveal the mechanism of such specificity.

SPOT technology provides a powerful tool to measure binding of hundreds of peptides in parallel. Here, I performed substitution analysis in the context of BIM, observing the effect on binding to MCL-1 and BCL-XL. However, there are many more applications of this technique that could further elaborate interaction specificity determinants for the BCL-2 family. The same BIM substitution analysis can be performed with other BCL-2 proteins in the future, including BCL-2, BCL-W, A1/BFL-1, and BCL-B. Additionally, the substitution analysis can be performed in other BH3-related contexts. The site-by-site mutation to all possible amino acids can be carried out on other native BH3 peptides, such as the MCL-1-specific Noxa or the BCL-XL-specific BAD. Furthermore, substitution analysis can be performed in the context of interesting point mutations. For example, the A2eV mutation in BIM may provide a different substitution analysis result as compared to native BIM, due to likely conformational differences (which could be described using crystallography). Similar studies can also be applied to the viral BCL-2 homologues: for example, SPOT synthesis of native BH3 proteins can be used to quickly profile the overall specificity of the viral BCL-2 proteins. Such studies can be followed by substitution analysis to uncover sites important for determining that specificity.

Additionally, the SPOT technology may be applied to the discovery of as-yet-unknown anti-apoptotic BCL-2 binding peptides. Currently, all identified BH3 sequences share a short signature motif, including the highly conserved leucine at 3a and aspartate at 3f. However, the SPOT studies, along with mutagenesis studies by others [3, 6, 8], have established that these sites need not remain so limited in order to bind the anti-apoptotic BCL-2 proteins. In an application similar to the SH3 study by Landgraf et al. [137], peptide yeast display [133] combined with substitution analysis data could be used to create a binding consensus sequence for each BCL-2 protein of interest. Peptides identified from the genome that match the consensus sequences can
then be synthesized on membranes, and tested to see if they actually bind the BCL-2 proteins. This could possibly link previously unidentified proteins to apoptosis, or associate the BCL-2 family members with other cellular processes.

My studies of the BIM BH3 interaction with MCL-1 indicate that structural flexibility within both the receptor protein and the binding peptide can allow binding for a variety of protein sequences. This has implications for designing new, specific BH3 peptides and/or small molecules, and for the discovery of new BCL-2 family interaction partners. However, currently available structural data are limited to a few point mutants of BIM. Two conformational states of MCL-1 bound to BIM have been identified (wild-type, I2dA, F4aE vs. I2dY). Structure determination of additional mutant complexes may elucidate more conformations of MCL-1. This expanded space of structural flexibility in MCL-1 could be exploited for design of specific inhibitors.

X-ray crystallography and SPOT arrays provide powerful tools to study interactions among the BCL-2 proteins. Crystal structures provide a detailed view of the interaction, while SPOTs provide a means to test the binding of many peptides simultaneously. Together, these techniques can be used to predict and test the interplay between specificity and flexibility in the interactions between BH3 peptides and BCL-2 receptor proteins.
Appendix

Modeling BCL-2 proteins in TRAIL-induced apoptosis

Collaborators:
I determined the BCL-2 interaction network, and associated rates, to be modeled from the literature. Carlos Lopez wrote and ran the code in Little b.
5.1 Introduction

As described elsewhere in this thesis, a number of pro- and anti-apoptotic BCL-2 proteins regulate cell death decisions. Most BCL-2 proteins are involved in the intrinsic apoptosis pathway, in which various BH3-only proteins are activated by different damage cues, such as anoikis, DNA damage, or UV irradiation. However, the BCL-2 family also links to the extrinsic pathway, in which death ligands, such as TRAIL and TNF, bind receptors on the cell surface, oligomerizing DISC and activating caspase 8. BID, a BH3-only protein, is cleaved and thereby activated by caspase 8. The active form of BID, known as tBID, then activates pro-apoptotic BCL-2 proteins and inhibits anti-apoptotic proteins.

A model of receptor-mediated apoptosis was built using ordinary differential equations (ODEs) by Albeck et al. [1]. This model starts with an input of TRAIL ligand and leads to the eventual cleavage of PARP as the system output (Figure 5.1). From this “extrinsic apoptosis reaction model” (EARM), it was determined that mechanistic details included in the model, such as oligomerization of proteins and compartmentalization of the molecules, were essential to reproducing features of TRAIL-induced MOMP (mitochondrial outer membrane permeabilization). MOMP, regulated by BCL-2 proteins, appeared as the point of transition from a graded response to TRAIL (resulting from accumulation of active caspase 8) to a snap-action response (cell-death or life decision) in the model.

While TRAIL-induced apoptosis is considered an extrinsic apoptosis pathway, it intersects with regulation centered at the mitochondria, which is part of the intrinsic apoptotic pathway. In the Albeck et al. model, MOMP is important for the rapidity of cell death in the intrinsic pathway. But the EARM as implemented includes only three BCL-2 family members: pro-apoptotic BH3-only BID, pro-apoptotic BAX, and anti-apoptotic BCL-2. Expanding this to include the roles of other BCL-2 family members may improve understanding of the extrinsic apoptotic pathway. Thus, in this appendix, I describe the expansion of the EARM to include more BCL-2 proteins. The model was constructed in Little b, a rules-based language for modeling complex biological systems [148].
5.2 Materials & Methods

The model was written as rules in the LISP-based scripting language, Little b [148]. An ODE-based model is written by Little b, based on these rules. The ODE model is then run in Jacobian [149].

5.3 Results

5.3.1 Building an expanded BCL-2 model

I set out to build a model with more representative BCL-2 family members. I incorporated three different anti-apoptotic proteins (BCL-XL, BCL-2, and MCL-1), two multi-
domain pro-apoptotic proteins (BAX and BAK), and five different BH3-only proteins (BID, BIM, BAD, Noxa, and MULE), as shown in Figure 5.2. This subset of BCL-2 proteins was chosen because each has a unique interaction profile, function, or cellular localization.

**Figure 5.2 Expanded BCL-2 family**

Comparison of the EARM pathway (A) and the expanded BCL-2 pathway (B).

I initially wrote the ODEs for the BCL-2 interactions in Matlab under the guidance of John Burke. This model started with activated caspase 8 (cleaved) and ended with cytochrome c (Cyt c) and Smac released from the mitochondria. The idea was that this model can be easily incorporated into the original model, adding the interactions up- and down-stream of the mitochondria. The model had 96 ODEs (for 96 species), 55 on and 55 off rates, 24 catalytic rates, 25 translocation constants, and 15 non-zero initial conditions. While many of the BH3-only proteins are regulated via intrinsic death cues, these cues were not included. However, levels of BH3-only proteins, which potentially play an important role in extrinsic apoptosis, could be set using initial conditions. Values for rate constants and initial concentrations were taken from the literature (described in section 5.3.5), or copied from analogous values used by Albeck et al. [1].

After writing the differential equations, and experiencing first-hand the difficulty of making changes to the interaction model when it was described by 96 equations, a rule-based model was written in Little b by Carlos Lopez. C. Lopez took the reactions I modeled with ODEs and implemented them in Little b, including the rest of the EARM modeled by Albeck et al., such that we had the original model with the expanded BCL-2 proteins in a rule-based
language. The initial cellular concentrations of proteins are taken from EARM for the overlapping proteins, while new BCL-2 protein concentrations were estimated. The rules are listed below (section 5.3.3).

The interactions among the BCL-2 proteins are based on the literature. Associated with the Little b model is a wiki document (wikidoc shown in section 5.3.5) that describes these literature sources.

5.3.2 Reading the rules

The Little b rules file sets up the cell, the molecular species, and rules governing interactions and catalytic reactions involving the molecular species. The cell includes the extracellular region (dish), the cell membrane, the cytoplasm, the mitochondrial membrane, and the mitochondria. The areas and volumes for each of these set to one, with the rates accounting for volume or area of the each compartment. The species are defined by “defmonomer”. For example,

```plaintext
(defmonomer BIDC "BID cytosolic"
  CCSP8 CPRO CANTI (asite :states (member :active :inactive) :default :inactive))
```

defines the species BIDC, which is described as “BID cytosolic”. Variables starting with a “C” are complexation sites: CCSP8 is the caspase 8 binding site on BID, CPRO is the pro-apoptotic BCL-2 protein (BAX and BAK) binding site on BID, and CANTI is the anti-apoptotic BCL-2 binding site on BID. BID also has an “asite” variable associated with it that defines the state of BID. Possible states for this parameter include active and inactive, with the default state as inactive. The rules define interactions, which follow standard kinetics. Two monomers with defined states (complexation and asite) bind to form a complex, which then may undergo a catalytic conversion.

```plaintext
;; CSP3 activates CSP6
[[[caspase3 CCSP6._ asite.active] + [caspase6 CCSP3._ asite.inactive]
  <=>> [[caspase3 CCSP6.1 asite.active][caspase6 CCSP3.1 asite.inactive]]}
  (.set-rate-function 'mass-action :fwd KCSP3CSP6F :rev KCSP3CSP6R)]
[[[[caspase3 CCSP6.1 asite.active][caspase6 CCSP3.1 asite.inactive]] ->>
  [caspase3 CCSP6._ asite.active] + [caspase6 CCSP3._ asite.active]]
  (.set-rate-function 'mass-action KCSP3CSP6C)]
```

In this reaction, a caspase3 monomer that is active (asite.active) with nothing in its caspase 6 binding site (CCSP6._), binds to a caspase6 monomer that is inactive (asite.inactive) and nothing
In its caspase 3 binding site (CCSP3._) to form a complex in which the complexation sites are now occupied (CCSP6.1 and CCSP3.1) with a forward rate of KCSP3CSP6F and a reverse rate of KCSP3CSP6R, as defined in the rates file (5.3.4). This complex can then proceed with the catalytic rate KCSP3CSP6C to convert caspase 6 to its active state, and the monomers dissociate leaving their complexation sites empty. Additionally, Little b allows the use of tables, so that monomers involved in similar reactions can be put into a table. Each monomer in a row interacts with each monomer in a column, with defined rates. The following is a table that defines inhibitory interactions of anti-apoptotic BCL-2 proteins with BAX and BAK.

```
;; ANTIAPOPTOTIC inhibiting BAX, BAK
;; \wikidoc{BCLXL_binds_BAX}
;; \wikidoc{BCL2_binds_BAX}
;; \wikidoc{BCLXL_binds_BAK}
;; \wikidoc{MCL1_binds_BAK}
;; \wikidoc{MCL1_doesnot_inhibit_BAX}
;; \wikidoc{BCL2_doesnot_inhibit_BAK}
(with-data-table (:rows ($R1 @A) :cols ($R2 @B) :cells ($Kf $Kr @C @D) :ignore _)
  ((                       (BAXC NIL)
    (BAXM (CDIM.* CTETRA.*))
    (BAXM (CDIM.* CTETRA.*))
    ((BCLXLC NIL)           (KBCLXLCBAXCF KBCLXLCBAXCR NIL NIL)
      (KBCLXLCBAXMF KBCLXLCBAXMR (@ :outer) NIL)
      (KBCLXLCBAKF KBCLXLCBAKR (@ :outer) NIL))
    ((BCLXLM NIL)           (KBCLXLMBAXCF KBCLXLMBAXCR NIL (@ :outer))
      (KBCLXLMBAXMF KBCLXLMBAXMF NIL NIL)
      (KBCLXLMBAKF KBCLXLMBAKR NIL NIL))
    ((BCL2 NIL)             (KBCL2BAXCF KBCL2BAXCR NIL (@ :outer))
      (KBCL2BAXMF KBCL2BAXMF NIL NIL)
      (KBCL2BAKF KBCL2BAKR NIL NIL))
    ((MCL1C (asite.active)) (KMCL1CBAXCF KMCL1CBAXCR NIL NIL)
      (KMCL1CBAXMF KMCL1CBAXMF (@ :outer) NIL)
      (KMCL1CBAKF KMCL1CBAKR (@ :outer) NIL))
    ((MCL1M (asite.active)) (KMCL1MBAXCF KMCL1MBAXCR NIL (@ :outer))
      (KMCL1MBAXMF KMCL1MBAXMF NIL NIL)
      (KMCL1MBAKF KMCL1MBAKR NIL NIL))
  )
[[[$R1 @A CPRO._ __] @C + [$R2 @B CANTI._ asite.* __] @D <->>
  [[[$R1 @A CPRO.1 __]][$R2 @B CANTI.1 asite.* __]]])
(.set-rate-function 'mass-action :fwd $Kf :rev $Kr)]]
```

In this table, the five anti-apoptotic monomers are the rows ($R1$). There are three different proteins (BCL-XL, BCL-2, and MCL-1), but two have more than one localization (cytosolic and mitochondrial), giving five monomer types. The pro-apoptotic proteins are the columns ($R2$), with two versions of BAX and one of BAK. (To prevent text wrapping, the three column monomers are staggered, each on a different line.) Little b is able account for wild-cards (*). For example, with BAXM and BAK, the dimer and tetramer binding sites can be in any state,
bound or unbound, as represented by CDIM.* and CTETRA.*. Information about the interaction and rates is described in the wikidoc{filename} (section 5.3.5).

5.3.3 Rules

The text of the rules files is listed.
(in-package :b-user)
(format t "READING RULES -\%")
(include b-user/ode-biochem)

;;;; ---BIOLOGY HERE:
;;;;

;;;; RATES FILE
(include @FOLDER/bcl2-5_rates)
(format t "DONE READING RATES -\%")

;;;; cell definition
;;;; all lengths in um, all areas in um^2 and all volumes in um^3
(defcon cell-location (location)
  (&optional (id := *name*)
     &property
     (cytoplasm compartment :#= [[compartment] {.size.value := 1.0}}); 1000.0, um^3 cell volume of
     1pL (in um^3)
     (dish compartment :#= [[compartment] {.size.value := 1.0}}); 1.0E6, um^3 1E-9L = dish
     volume
     (mitochondria compartment :#= [compartment])
     (cell-membrane membrane :#= (let ((d .dish)
       (c .cytoplasm))
       [[membrane] :outer d :inner c {.size.value := 1.0}})); 706 um^2, 7.5um
     cell radius
     (mito-membrane membrane :#= (let ((e .mitochondria)
       (c .cytoplasm))
       [[membrane] :inner e :outer c {.size.value := 1.0}})); 900 um^2, 300 mito,
     3um^2/mito
     (inverse-mito-membrane membrane :#= .mito-membrane.inverse))) ; invert inner and outer
;;
(format t "DONE SETTING CELL -\%")

;;;; SPECIES DEFINITIONS
;;;; RECEPTOR LAYER
(defmonomer TRAIL "trail ligand"
  CDREC )
(defmonomer (DRECEPTOR membrane) "Death receptor for TRAIL"
  CTRAIL CCSP8 (asite :states (member :active :inactive) :default :inactive))
(defmonomer caspase8 "caspase 8 with on/off site"
  CSUB CMCL1 CCSP6 CDREC (asite :states (member :active :inactive) :default :inactive))
(defmonomer BIDC "BID cytosolic"
  CCSP8 CPRO CANTI (asite :states (member :active :inactive) :default :inactive)) ; inactive = untruncated
(defmonomer (BIDM membrane) "BID membrane-associated"
  CCSP8 CPRO CANTI (asite :states (member :active :inactive) :default :inactive)) ; inactive = untruncated
(defmonomer BAXC "BAX documentation"
  CACTIVATOR CANTI (asite :states (member :active :inactive) :default :inactive))
(defmonomer BAXM "BAXM documentation"
  CDIM CTETRA CACTIVATOR CANTI (asite :states (member :active :inactive) :default :inactive))
(defmonomer BIM "BIM documentation"
  CPRO CANTI)
(defmonomer (BAK membrane) "BAK documentation"
  CDIM CTETRA CACTIVATOR CANTI (asite :states (member :active :inactive) :default :inactive))
(defmonomer (BCL2 membrane) "BCL2 documentation"
  CBH3 CPRO)
(defmonomer (BCLXLC "BCL-XL documentation"
  CBH3 CPRO)
(defmonomer (BCLXLM membrane) "BCL-XL documentation"
  CBH3 CPRO)
(defmonomer MCL1C "MCL1 documentation"
  CCSP8 CBH3 CPRO (asite :states (member :active :degraded) :default :active))
(defmonomer (MCL1M membrane) "MCL1 documentation"
  CCSP8 CBH3 CPRO (asite :states (member :active :degraded) :default :active))
(defmonomer BAD "BAD documentation"
  CPRO CANTI)
(defmonomer NOXA "NOXA documentation"
  CANTI)
(defmonomer MULE "MULE documentation"
  CANTI)
(defmonomer caspase3 "caspase 3"
  CCSP8 CCSP6 CMCL1 CXIAP CPARP CAPOP
  (asite :states (member :active :inactive) :default :inactive) ;activation site
  (dsite :states (member :ubiq :nonubiq) :default :nonubiq)) ;ubiquitination site
(defmonomer caspase6 "caspase 6"
  CCSP3 CCSP8 (asite :states (member :active :inactive) :default :inactive))
(defmonomer CYTC "cytochrome C documentation"
  CPORE CAPAF)
(defmonomer SMAC "SMAC/DIABLO documentation"
  CPORE CXIAP)
(defmonomer (BAXMPORE membrane) "pore"
  CMITOP)
(defmonomer (BAKPORE membrane) "pore"
  CMITOP)
(defmonomer XIAP "x-linked inhibitor of apoptosis proteins"
  CCSP3 CAPOP CSMAC)
(defmonomer PARP "Poly(ADP-ribose) polymerase"
  CCSP3 (asite :states (member :active :inactive) :default :active))
(defmonomer APAF "Apoptosis activating factor 1"
  CYYTC CCSP9 (asite :states (member :active :inactive) :default :inactive))
(defmonomer caspase9 "caspase 9"
  CAPAF)
(defmonomer apoptosome "Apoptosome"
  CCSP3 CXIAP)

(format t "DONE SETTING MONOMERS ~%")

;;;;  ************************************************************************
;;;;  RULEZ
;;;;  **************************************************

;;;; TRAIL BINDING TO RECEPTOR
[[[TRAIL CDREC._ __] @ :outer + [DRECEPTOR CTRAIL._ __] <<-->> [[TRAIL CDREC.1][DRECEPTOR CTRAIL.1]]]
(.set-rate-function 'mass-action :fwd KTRAILRECF :rev KTRAILRECR)
[[[TRAIL CDREC.1][DRECEPTOR CTRAIL.1]] --> [DRECEPTOR CTRAIL._ asite.active]] ;NOTICE TRAIL DISAPPEARS
(.set-rate-function 'mass-action 'mass-action KTRAILRECC)

;;;; EXCLUDING FLIP INHIBITOR OF DEATH RECEPTOR
;;;; CSP 8 activation
[[[DRECEPTOR CCSP8._ asite.active] + [caspase8 CDREC._ asite.inactive] @ :inner <<-->>
  [[DRECEPTOR CCSP8.1 asite.active][caspase8 CDREC.1 asite.inactive]]]
(.set-rate-function 'mass-action :fwd KDRECCSP8F :rev KDRECCSP8R)
[[[DRECEPTOR CCSP8.1 asite.active][caspase8 CDREC.1 asite.inactive]] -->
  [DRECEPTOR CCSP8._ asite.active] + [caspase8 CDREC._ asite.active] @ :inner }
(.set-rate-function 'mass-action 'mass-action KDRECCSP8C)

;;;; MCL1 degradation
;;;; \wikidoc{MCL1 degradation}
;;;; MCL1 gets degraded in the presence of cycloheximide
[[[MCL1C asite.active] --> [MCL1C asite.degraded]]
; (.set-rate-function 'mass-action KMCL1CAMCL1CD)]
[[[MCL1M asite.active] --> [MCL1M asite.degraded]]
;; BH3 ACTIVATORS BINDING BAX BAK
;; \wikidoc{bid_activates_bax}
;; \wikidoc{bim_activates_bax}
;; CYTOSOL
;; can happen both in the cytoplasm and in the membrane
(with-data-table
  (:rows ($R1 @A) :cols $R2 :cells ($Kf $Kr $Kc @B @C) :ignore _)
  ((BAXC BAK)
    ((BIDC (asite.active)) (KBIDCBAXCF KBIDCBAXCR KBIDCBAXCC NIL NIL) (KBIDCBAKF KBIDCBAKR KBIDCBAKC)
      (@ :outer) NIL))
  ((BIM NIL)
    (KBIMBAXCF KBIMBAXCR KBIMBAXCC NIL NIL)
    (KBIMBAKF KBIMBAKR KBIMBAKC)
    (@ :outer) NIL))
;; FORWARD (BINDING)
[[$R1 CPRO._ @A] @B + [$R2 CACTIVATOR._ asite.inactive] @C <<->> [[$R1 CPRO.1 @A]$R2 CACTIVATOR.1 asite.inactive]]
(.set-rate-function 'mass-action :fwd $Kf :rev $Kr)
[[$R1 CPRO.1 @A]$R2 CACTIVATOR.1 asite.inactive] --> [[$R1 CPRO._ @A] @B + [$R2 CACTIVATOR._ asite.active] @C]
(.set-rate-function 'mass-action $Kc))

;; Translocations
;; \wikidoc{BAX_translocation}
(with-substitution-table
  ($R1 @A $R2 $Kf $Kr)
  (BIDC (asite.active) BIDM KBIDCBIDMF KBIDCBIDMR)
  (BAXC (asite.active) BAXM KBAxCBAXMF KBAxCBAXMR)
  (BCLXLC NIL BCLXLM KBCLXLCBCLXLMF KBCLXLCBCLXLMR)
  (MCL1C NIL MCL1M KMCL1CMCL1MF KMCL1CMCL1MR)
)[[$R1 @A] @ cell-location.cytoplasm <<->> [[$R2 @A] @ :mito-membrane]
(.set-rate-function 'mass-action :fwd $Kf :rev $Kr))

;; AUTO-ACTIVATION
;; \wikidoc{autoactivation}
;; BAK/BAX "spontaneously" activates itself (Australian model)
(with-substitution-table
  ($R1 $Kf $Kr)
  (BAXC KBAxAutoACTF KBAxAutoACTR))
(BAK KBAKAUTOACTF KBAKAUTOACTR))

{[$R1 asite.inactive __] <-> [$R1 asite.active __]}
(.set-rate-function 'mass-action :fwd $Kf :rev $Kr))

;; BAK/BAX Oligomerization (dimer formation)
(with-substitution-table
  (($R1  $Kf  $Kr ))
  (BAXM KBAXMDIMF KBAXMDIMR)
  (BAK KBAKDIMF KBAKDIMR))

{[$R1 CDIM._ asite.active __]+[$R1 CDIM._ asite.active __] <->
  [$R1 CDIM.1 asite.active __][[$R1 CDIM.1 asite.active __]]}
(.set-rate-function 'mass-action :fwd $Kf :rev $Kr))

;; BAK/BAXM Oligomerization (tetramer formation)
;; littleb gets freaked out if you allow multibonds in this situation
;; through a tetramer. I replaced the tetramer with
(with-substitution-table
  (($R1  $Kf  $Kr ))
  (BAXM KBAXMTETF KBAXMTETR)
  (BAK KBAKTETF KBAKTETR))

{[$R1 CDIM.1 asite.active __][[$R1 CDIM.1 asite.active __]]+
  [$R1 CDIM.2 asite.active __][[$R1 CDIM.2 asite.active __]] <->
  [$R1 CDIM.1 CTETRA.3 asite.active __][[$R1 CDIM.1 CTETRA.4 asite.active __]]
  [$R1 CDIM.2 CTETRA.3 asite.active __][[$R1 CDIM.2 CTETRA.4 asite.active __]]}
(.set-rate-function 'mass-action :fwd $Kf :rev $Kr))

;; BAK/BAXM tetramer becomes a "pore"
(with-substitution-table
  (($R1  $R2  $Kf ))
  (BAXM BAXMPORE KBAXMPORE)
  (BAK BAKPORE KBAKPORE))

{[$R1 CDIM.1 CTETRA.3 asite.active __][[$R1 CDIM.1 CTETRA.4 asite.active __]]
  [$R1 CDIM.2 CTETRA.3 asite.active __][[$R1 CDIM.2 CTETRA.4 asite.active __]]
  -> [$R2 __]}
(.set-rate-function 'mass-action $Kf )))

;; ANTIAPOPTOTIC inhibiting BAX, BAK
;; \wikidoc{BCLXL_binds_BAX}
;; \wikidoc{BCL2_binds_BAX}
;; \wikidoc{BCLXL_binds_BAK}
;; \wikidoc{MCL1_binds_BAK}
;; \wikidoc(MCL1_doesnot_inhibit_BAX)
;; \wikidoc(BCL2_doesnot_inhibit_BAK)
(with-data-table (:rows ($R1 @A) :cols ($R2 @B) :cells ($Kf $Kr @C @D) :ignore _)
((
    (BAXC NIL)      (BAXM (CDIM.* CTETRA.*))     (BAK (CDIM.* CTETRA.*))

    ((BCLXL NIL)    (BCLXLBCAXCF KBCXLBCAXCR NIL NIL)
     (KBCXLBCAXMF KBCXLBCAXMR (@ :outer) NIL)
     (KBCXLBCAXMR (@ :outer) NIL))

    ((BCLXM NIL)    (BCLXLMBAXCF KBCXLMBAXCR NIL (@ :outer))
     (KBCXLMBAXMF KBCXLMBAXMR NIL NIL)
     (KBCXLMBAXMF KBCXLMBAXMR NIL NIL))

    ((BCL2 NIL)     (BCL2BAXCF KBCL2BAXCR NIL (@ :outer))
     (KBCL2BAXMF KBCL2BAXMF NIL NIL)
     (KBCL2BAKR NIL NIL))

    ((MCL1C (asite.active)) (KMCL1CBAXCF KMCL1CBAXCR NIL NIL)
     (KMCL1CBAXMF KMCL1CBAXMR (asite.*))
     (KMCL1CBAXMF KMCL1CBAXMR (asite.*))
     (KMCL1CBAXMF KMCL1CBAXMR (asite.*)))

    ((MCL1M (asite.active)) (KMCL1MBAXCF KMCL1MBAXCR NIL (@ :outer))
     (KMCL1MBAXMF KMCL1MBAXMR NIL NIL)
     (KMCL1MBAXMF KMCL1MBAXMR NIL NIL)))
[[$R1 @A CPRO._ __] @C + [$R2 @B CANTI._ asite.*  __] @D <<->> [[[$R1 @A CPRO.1 __] [$R2 @B CANTI.1 asite.* __] @D]])
(.set-rate-function 'mass-action :fwd $Kf :rev $Kr)))

;; ANTIAPOPTOTIC binding BH3s
;; \wikidoc{BID_inhibits_BCLXL}
;; \wikidoc{BID_inhibits_MCL1}
;; \wikidoc{BID_inhibits_BCL2}
;; \wikidoc{BIDM_inhibits_BCLXL}
;; \wikidoc{BIDM_inhibits_MCL1}
;; \wikidoc{BAD_inhibits_BCLXL}
;; \wikidoc{BAD_inhibits_BCL2}
(with-data-table (:rows ($R1 @A) :cols ($R2 @B) :cells ($Kf $Kr @C @D) :ignore _)
((
    (BIDM (asite.active)) (BIDM (asite.active)) (BIM NIL) (BAD NIL) (NOXA NIL) (MULE NIL))

    ((BCLXL NIL)    (KBCXLBCIDCF KBCXLBCIDCR NIL NIL) ; with BIDC
     (KBCXLBCIDMF KBCXLBCIDMR (@ :outer) NIL) ; with BIDM
     (KBCXLBCIDMR (@ :outer) NIL) ; with BIDM
     (KBCXLBCADF KBCXLBCADR NIL NIL) ; with BAD
     (KBCXLBCADR NIL NIL) ; with NOXA
     _ _ ) ; with MULE

    ((BCLXM NIL)    (KBCXLMBIDCF KBCXLMBIDCR NIL (@ :outer)) ; with BIDC
(KBCLXLMBIDMF KBCLXLMBIDMR NIL NIL) ; with BIDM
(KBCLXLMBIMF KBCLXLMBIMR NIL (@ :outer)) ; with BIM
(KBCLXLMBADF KBCLXLMBADR NIL (@ :outer)) ; with BAD

((BCL2 NIL)
 (KBCL2BIDCF KBCL2BIDCR NIL (@ :outer)) ; with BIDC
(KBCL2BIDMF KBCL2BIDMR (@ :outer) NIL) ; with BIDM
(KBCL2BIMF KBCL2BIMR NIL (@ :outer)) ; with BIM
(KBCL2BADF KBCL2BADR NIL (@ :outer)) ; with BAD

(MCL1C (asite.active))
(KMCL1CBIDCF KMCL1CBIDCR NIL NIL) ; with BIDC
(KMCL1CBIDMF KMCL1CBIDMR (@ :outer) NIL) ; with BIDM
(KMCL1CBIMF KMCL1CBIMR NIL NIL) ; with BIM

(MCL1M (asite.active))
(KMCL1MBIDCF KMCL1MBIDCR NIL (@ :outer)) ; with BIDC
(KMCL1MBIDMF KMCL1MBIDMR NIL NIL) ; with BIDM
(KMCL1MBIMF KMCL1MBIMR NIL (@ :outer)) ; with BIM

[[[$R1 @A CBH3.1 __] @C + [$R2 @B CANTI.1 __] @D <-> [[[[$R1 @A CBH3.1 __][$R2 CANTI.1 @B __]]
(.set-rate-function 'mass-action :fwd $Kf :rev $Kr))]]

;; MCL1 degradation
;; \wikidoc{NOXA_inhibits_MCL1}
;; \wikidoc{MULE_inhibits_MCL1}
(\with-data-table (:rows $R1 :cols $R2 :cells ($Kc @A @B) :ignore _)
 ((
   NOXA
   MULE)
   (MCL1C KMCL1CNOXAC NIL NIL) (KMCL1CMULEC NIL NIL))
 (MCL1M KMCL1MNNOXAC NIL (@ :outer)) (KMCL1MMULEC NIL (@ :outer))))

[[[$R1 asite.active CBH3.1 __] [SR2 CANTI.1 __]] ->> [$R1 asite.degraded CBH3._ __] @A + [$R2 CANTI._ __]
 @B)
(.set-rate-function 'mass-action $Kc))

;; CSP8 activates BIDC and CSP3
(\with-data-table (:rows $R1 :cols $R2 :cells ($Kf $Kr $Kc) :ignore _)
 ((
   caspase8
   ))
(BIDC         (KBIDCCSP8F KBIDCCSP8R KBIDCCSP8C ))
(caspase3     (KSCP3CSP8F KSCP3CSP8R KSCP3CSP8C))

[({$R1 asite.inactive CCSP8._ __} + {$R2 asite.active CSUB._ __} <=> {$R1 asite.inactive
CCSP8.1}{$R2 asite.active CSUB.1})
 (.set-rate-function 'mass-action :fwd $Kf  :rev $Kr)

[({{$R1 asite.inactive CCSP8.1}{$R2 asite.active CSUB.1}} -->> {$R1 asite.active CCSP8._ ] + {$R2
asite.active CSUB.}_])
 (.set-rate-function 'mass-action $Kc))]

;; CSP3 activates CSP6
[({caspase3 CCSP6._ asite.active] + [caspase6 CCSP3._ asite.inactive] <=>>
 [caspase3 CCSP6.1 asite.active][caspase6 CCSP3.1 asite.inactive]])
(.set-rate-function 'mass-action :fwd KCSP3CSP6F :rev KCSP3CSP6R)

[({caspase3 CCSP6.1 asite.active][caspase6 CCSP3.1 asite.inactive]] -->>
 [caspase3 CCSP6._ asite.active] + [caspase6 CCSP3._ asite.active])
(.set-rate-function 'mass-action KCSP3CSP6C)

;; CSP3 gets tagged for ubiquitination by XIAP
[({caspase3 CXIAP._ asite.active dsite.nonubiq] + [XIAP CCSP3._ ] <=>>
 [caspase3 CXIAP.1 asite.active dsite.nonubiq][XIAP CCSP3.1]])
(.set-rate-function 'mass-action :fwd KCSP3XIAPF :rev KCSP3XIAPR)

[({caspase3 CXIAP.1 asite.inactive dsite.nonubiq][XIAP CCSP3.1]] -->>
 [XIAP CCSP3._] + [caspase3 CXIAP. asite.inactive dsite.ubiq]}
(.set-rate-function 'mass-action KCSP3XIAPC)

;; CSP3 cleaves PARP and inactivates it
[({caspase3 CPARP._ asite.active] + [PARP CCSP3._ ] <=>>
 [caspase3 CPARP.1 asite.active][PARP CCSP3.1]])
(.set-rate-function 'mass-action :fwd KCSP3PARPF :rev KCSP3PARPR)

[({caspase3 CPARP.1 asite.active][PARP CCSP3.1]] -->>
 [caspase3 CPARP._ asite.active] + [PARP CCSP3._ asite.inactive})
(.set-rate-function 'mass-action KCSP3PARPC)

;; CSP6 activates CSP8
[({caspase6 CCSP8._ asite.active] + [caspase8 CCSP6._ asite.inactive] <=>>
 [caspase6 CCSP8.1 asite.active][caspase8 CCSP6.1 asite.inactive]])
(.set-rate-function 'mass-action :fwd KSCP6CSP8F :rev KSCP6CSP8R)

[({caspase6 CCSP8.1 asite.active][caspase8 CCSP6.1 asite.inactive]] -->>
 [caspase6 CCSP8._ asite.active] + [caspase8 CCSP6._ asite.active])
(.set-rate-function 'mass-action KSCP6CSP8C)
caspase8/caspase3 induces MCL1 degradation

(with-data-table (:rows $R1 :cols $R2 :cells ($Kf $Kr $Kc @A) :ignore _)

<table>
<thead>
<tr>
<th>caspase8</th>
<th>caspase3</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCL1M</td>
<td>(KMCL1MCSP8F KMCL1MCSP8R KMCL1MCSP8C (@ :outer)) (KMCL1MCSP3F KMCL1MCSP3R KMCL1MCSP3C (@ :outer))</td>
</tr>
<tr>
<td>MCL1C</td>
<td>(KMCL1CCSP8F KMCL1CCSP8R KMCL1CCSP8C NUL @ :outer) (KMCL1CCSP3F KMCL1CCSP3R KMCL1CCSP3C NUL)</td>
</tr>
</tbody>
</table>

[[$R1 CCSP8._ asite.active] + [$R2 asite.active CMCL1._] @A  <<->> [[[$R1 CCSP8.1 asite.active][SR2 asite.active CMCL1.1]]]_{A}

(set-rate-function 'mass-action :fwd $Kf :rev $Kr)

[[[$R1 CCSP8.1 asite.active][SR2 asite.active CMCL1.1]]]_{A}  >> [$R1 CCSP8._ asite.degraded] + [$R2 asite.active CMCL1._]_{A}

(set-rate-function 'mass-action :fwd $Kc)

BAXP releases CYTC

(with-data-table (:rows $R1 :cols $R2 :cells ($Kf $Kr $Kc) :ignore _)

<table>
<thead>
<tr>
<th>BAXMPORE</th>
<th>BAKPORE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYTC</td>
<td>(KCYTCBAXPF KCYTCBAXPR KCYTCBAXPC) (KCYTCBAKPF KCYTCBAKPR KCYTCBAKPC)</td>
</tr>
<tr>
<td>SMAC</td>
<td>(KSMACBAXPF KSMACBAXPR KSMACBAXPC) (KSMACBAKPF KSMACBAKPR KSMACBAKPC)</td>
</tr>
</tbody>
</table>

[[$R1 CPORE._ @ :inner + [$R2 CMITOP._] @ :inner] <<<->> [[[$R1 CPORE.1 @ :inner + [$R2 CMITOP.1 @ :inner]]]_{A}]

(set-rate-function 'mass-action :fwd $Kf :rev $Kr)

[[[$R1 CPORE.1 @ :inner] + [$R2 CMITOP.1 @ :inner]]]_{A}  >> [$R1 CPORE._ @ :outer + [$R2 CMITOP._] @ :outer]

(set-rate-function 'mass-action :fwd $Kc)

CYTOCHROME C/SMAC released via BAXMPORE/BAKPORE

CYTOCHROME C complexes with APAF

[[[CYTC CAPAF._] @ cell-location.cytoplasm + [APAF CCYC._ asite.inactive] @ cell-location.cytoplasm <<<->> [[CYTC CAPAF.1] + [APAF CCYC.1 asite.inactive] @ cell-location.cytoplasm]

(set-rate-function 'mass-action :fwd KYTCAPAFF :rev KYTCAPAFF)

APAF and CSP9 form the apoptosome

[[[CYTC CAPAF.1] + [APAF CCYC.1 asite.inactive] @ cell-location.cytoplasm + [caspase9 CAPAF._] @ cell-location.cytoplasm <<<->> [apoptosome] @ cell-location.cytoplasm]

(set-rate-function 'mass-action :fwd KAPAFCS9F :rev KAPAFCS9R)

apoptosome activates caspase3

[[[apoptosome CCSP3._] + [caspase3 CAPOP._ asite.inactive] <<<->> [[apoptosome CCSP3.1][caspase3 CAPOP.1 asite.inactive]]]

(set-rate-function 'mass-action :fwd KAPOPCCSP3F :rev KAPOPCCSP3R)

[[[apoptosome CCSP3.1][caspase3 CAPOP.1 asite.inactive]]]_A  >>
[apoptosome CCSP3._] + [caspase3 CAPOP._ asite.active]
(.set-rate-function 'mass-action KAPOPCSP3C))

;; apoptosome binds to XIAP
[[[apoptosome CXIAP._] + [XIAP CAPOP._] <<-->> [[apoptosome CXIAP.1][XIAP CAPOP.1]]]
(.set-rate-function 'mass-action :fwd KAPOPXIAPF :rev KAPOPXIAPR)

;; smac binds to XIAP
[[[SMAC CXIAP._] + [XIAP CSMAC._] <<-->> [[SMAC CXIAP.1][XIAP CSMAC.1]]]
(.set-rate-function 'mass-action :fwd KSMACXIAPF :rev KSMACXIAPR)

;; INITIAL CONDITIONS

;; DEFINE CELL AND SPECIES IN COMPARTMENTS
(define cell [cell-location])
cell.dish.(contains [TRAIL])
cell.cell-membrane.(contains [DRECEPTOR])
cell.cytoplasm.(contains [caspase8] [caspase3] [caspase6] [caspase9] [BIDC] [BAXC] [BIM]
[BCLXLC] [MCLIC] [NOXA] [BAD] [MULE] [XIAP] [PARP]
[APAF] [apoptosome])
cell.mito-membrane.(contains [BIDM] [BAXM] [BAK] [BCLXLM] [BCL2] [MCL1M] [BAXMPORE] [BAKPORE])
cell.mitochondria.(contains [CYTC] [SMAC])

;; INITIAL CONCENTRATIONS/AMOUNTS
[[TRAIL].(in cell.dish).conc.t0 := 3000] ;; molecules/cell original: 3000
[[DRECEPTOR].(in cell.cell-membrane).conc.t0 := 200)] ;; molecules/cell
[[caspase8].(in cell.cytoplasm).conc.t0 := 2E4 } ;; molecules/cell
[[caspase3].(in cell.cytoplasm).conc.t0 := 1E4} ;; molecules/cell
[[caspase6].(in cell.cytoplasm).conc.t0 := 1E4 } ;; molecules/cell
[[caspase9].(in cell.cytoplasm).conc.t0 := 1E5 } ;; molecules/cell
[[BIDC].(in cell.cytoplasm).conc.t0 := 4E4 } ;; molecules/cell
[[BIDM].(in cell.mito-membrane).conc.t0 := 0 } ;; molecules/cell
[[BAXC].(in cell.cytoplasm).conc.t0 := 1.0E5 } ;; molecules/cell
[[BAXM].(in cell.mito-membrane).conc.t0 := 0)} ;; molecules/cell
[[BIM].(in cell.cytoplasm).conc.t0 := 0)} ;; molecules/cell
[[BAK].(in cell.mito-membrane).conc.t0 := 5E4)} ;; molecules/cell
[[BCLXLC].(in cell.cytoplasm).conc.t0 := 1.98E4)} ;; molecules/cell
[[BCLXLM].(in cell.mito-membrane).conc.t0 := 200)} ;; molecules/cell
\{[BCL2]\}.(in cell.mito-membrane).conc.t0 := 2E4} ;; molecules/cell
\{[MCL1C]\}.(in cell.cytoplasm).conc.t0 := 1.98E4} ;; molecules/cell
\{[MCL1M]\}.(in cell.mito-membrane).conc.t0 := 200} ;; molecules/cell
\{[BAD]\}.(in cell.cytoplasm).conc.t0 := 5E4} ;; molecules/cell
\{[NOXA]\}.(in cell.cytoplasm).conc.t0 := 5E4} ;; molecules/cell
\{[MULE]\}.(in cell.cytoplasm).conc.t0 := 1E4} ;; molecules/cell
\{[XIAP]\}.(in cell.cytoplasm).conc.t0 := 1E5} ;; molecules/cell
\{[PARP]\}.(in cell.cytoplasm).conc.t0 := 1E6} ;; molecules/cell
\{[APAF]\}.(in cell.cytoplasm).conc.t0 := 1E5} ;; molecules/cell
\{[apoptosome]\}.(in cell.cytoplasm).conc.t0 := 0} ;; molecules/cell
\{[CYTC]\}.(in cell.mitochondria).conc.t0 := 5E5} ;; molecules/cell
\{[SMAC]\}.(in cell.mitochondria).conc.t0 := 1E5} ;; molecules/cell
\{[BAXMPORE]\}.(in cell.mito-membrane).conc.t0 := 0} ;; molecules/cell
\{[BAKPORE]\}.(in cell.mito-membrane).conc.t0 := 0} ;; molecules/cell

;; \wikidoc{create_model}
;(include b/matlab/ode-translation)
;(create-ode-model "input/erb6_rcpt" :ode-comments nil :overwrite t)
(include b/numerica/ode-translation)
(setf *NUMERICA-RATE-STRING-MAX-LENGTH* NIL)
(create-numerica-model "input/bcl2_5" :ode-comments nil :overwrite t :vars (query species.moles).alpha-order
   :sim-options "(CSVOUTPUT := TRUE)" :sim-steps 56600)
;(create-numerica-model "input/bcl2_4" :ode-comments nil :overwrite t :vars (query species.conc)
   ; :sim-options "(CSVOUTPUT := TRUE" "DYNAMIC_REPORTING_INTERVAL := 600.0") :sim-steps 1800)
5.3.4 Rates

The text of the rates file is listed.

;;; BCL2 MODEL RATES
(in-package :b-user)
(define-macro define-rates (&rest var-defs)
  `(progn ,@(loop for (name value) in var-defs
      collect `(define ,name [[reference-var] :value value])))

(define-rates
  (KBIDCCSP8F 3.0E-8); (#/cell)^-1 sec^-1
  (KBIDCCSP8R 1.0E-3); sec^-1
  (KBIDCCSP8C 1.0); sec^-1
  (KBIDCBAXCF 4.5E-8); (#/cell)^-1 sec^-1
  (KBIDCBAXCR 1.0E-3); sec^-1
  (KBIDCBAXCC 1.0); sec^-1
  (KBIDMBAXCF 2.2E-8); (#/cell)^-1 sec^-1
  (KBIDMBAXCR 1.0E-3); sec^-1
  (KBIDMBAXCC 1.0); sec^-1
  (KBIDCBAKF 2.0E-6); (#/cell)^-1 sec^-1
  (KBIDCBARKR 1.0E-3); sec^-1
  (KBIDCBARI 1.0); sec^-1
  (KBIDMBAKF 2.0E-6); (#/cell)^-1 sec^-1
  (KBIDMBAKR 1.0E-3); sec^-1
  (KBIDMBARI 1.0); sec^-1
  (KBIMBAXCF 0.0); (#/cell)^-1 sec^-1
  (KBIMBAXCR 0.0); sec^-1
  (KBIMBAXCC 0.0); sec^-1
  (KBIMBAKF 0.0); (#/cell)^-1 sec^-1
  (KBIMBARKR 0.0); sec^-1
  (KBIMBARI 0.0); sec^-1
  (KBAXAUTOACTF 0.0); sec^-1
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5.3.5 Wikidoc

The text of the wikidoc file is listed, providing explanations for the interactions and rates not used in the original EARM. These are referenced in the rules file (5.3.3).

% \wikidoc{MCL1 degradation}
MCL1 had a short half-life in cells. A degradation rate of $1664.0 \text{ s}^{-1}$ was calculated assuming exponential decay with half life of 40 minutes.

% \wikidoc{bid_activates_bax}
The forward rate that was included in this reaction was obtained from Walensky (\it et al) in Mol. Cell. (2006) based on a 77nM $K_{(d)}$. A default reverse rate of $1E-3$ was used to calculate the forward rate. An alternative forward rate of $8.3E-10$ can be used based on Willis (\it et al) (Science 2007). With an IC50 of 2\micro M. This IC50 was used as a pseudo $K_{(d)}$ to calculate the forward rate using that $1E-3$ as a reverse rate. \cite{101, 150}

% \wikidoc{bim_activates_bax}
The rates for BIM interaction with BAX is set at zero. However, the forward rate has been calculated for this reaction obtained from Walensky (\it et al) in Mol. Cell. based on a 7nM $K_{(d)}$. A default reverse rate of $1E-3$ was used to calculate the forward rate of $2.4E-7$. An alternative forward rate of $8.3E-10$ can be used based on Willis (\it et al) (Science 2007). With and IC50 of 2\micro M. This IC50 was used as a pseudo $K_{(d)}$ to calculate the forward rate using that $1E-3$ as a reverse rate. \cite{101, 150}
This reaction was made as a bimolecular reaction with the membrane (M) as a component of the reaction and BAX forming a complex with this molecule. In our case we have a simple unimolecular component transfer reaction. This parameter will have to be fit at estimation.

This reaction rate is very loosely based on \(2 \mu M \, K_{d}\) extrapolated from \(13 \mu M \, K_{d}\) in Sattler \(1^{st}\) et al. Science (1997). A default reverse rate of \(1E-3\) was used to calculate the forward rate. [53]

This reaction rate is loosely based on \(1 \mu M \, K_{d}\) extrapolated from a 5.2 \(2 \mu M \, K_{d}\) in Huang \(1^{st}\) et al. PNAS (2002) using a 16mer peptide. A default reverse rate of \(1E-3\) was used to calculate the forward rate. [71]

This reaction rate is loosely based on a \(50 \, \text{nM} \, K_{d}\) from Willis \(1^{st}\) et al. Genes and Development (2005). A default reverse rate of \(1E-3\) was used to calculate the forward rate. [9]

This reaction rate is loosely based on a \(10 \, \text{nM} \, K_{d}\) from Willis \(1^{st}\) et al. Genes and Development (2005). A default reverse rate of \(1E-3\) was used to calculate the forward rate. [9]

This reaction rate is based on molar rates from Kuwana \(1^{st}\) et al. Mol. Cell v17 p 525 (2005). The rates were converted to the numbers per cell rates using a cell volume of \(10^{-12} L\). [11]

The forward rate of \(1.7E-6 \, (\#/Cell)^{-1} \text{sec}^{-1}\) used is based on a \(10 \, \text{nM} \, K_{d}\) from Certo \(1^{st}\) et al. Cancer Cell (2006). A default reverse rate of \(1E-3 \, s^{-1}\) was used to calculate the forward rate. An additional forward rate of \(8.3E-10 \, (\#/Cell)^{-1} \text{sec}^{-1}\) can be used based on a 2 \(\mu M \, K_{d}\) from Chen \(1^{st}\) et al. Mol. Cell (2005). NOTE: No interaction was detected by Kuwana \(1^{st}\) et al. Mol. Cell (2005). [10, 11]

The forward rate of \(1.9E-7 \, (\#/Cell)^{-1} \text{sec}^{-1}\) used is based on a \(66 \, \text{nM} \, K_{d}\) from Certo \(1^{st}\) et al. Cancer Cell (2006). A default reverse rate of \(1E-3 \, s^{-1}\) was used to calculate the forward rate. An additional forward rate of \(7.5E-9 \, (\#/Cell)^{-1} \text{sec}^{-1}\) can be used based on a \(220 \, \text{nM} \, K_{d}\) from Letai \(1^{st}\) et al. Cancer Cell (2002). Another forward rate of \(2.4E-10 \, (\#/Cell)^{-1} \text{sec}^{-1}\) based on \(6.8 \mu M \, IC_{50}\) from Chen \(1^{st}\) et al. Mol. Cell (2005) can be used. We have chosen to use the first rate based on the experimental technique being fluorescence polarization assay as opposed to surface plasmon resonance. [7, 10, 78]

The forward rate of \(5.3E-7 \, (\#/Cell)^{-1} \text{sec}^{-1}\) and reverse rate of \(2.1E-3 \, \text{sec}^{-1}\) were based on the molar rates by Kuwana \(1^{st}\) et al.
al.} Mol. Cell (2005). Additional rates of $9.5E-7\mbox{ (#/Cell)^{-1} sec^{-1}}$ and reverse rate of $4.4E-4\mbox{ sec^{-1}}$ can also be used. The latter rates are based on the molar rates determined by Chen {\it et al.} Mol. Cell (2005). [7, 11]

These rates were based on Chen {\it et al.} Mol. Cell (2005). [7]

The forward rate of $1.3E-6\mbox{ (#/Cell)^{-1} sec^{-1}}$ and reverse rate of $2.2E-3\mbox{ sec^{-1}}$ were based on molar rates by Kuwana {\it et al.} Mol. Cell (2005). Additional rates of $2.2E-6\mbox{ (#/Cell)^{-1} sec^{-1}}$ and reverse rate of $2.6E-4\mbox{ sec^{-1}}$ can also be used. The latter rates are based on the molar rates determined by Chen {\it et al.} Mol. Cell (2005). [7, 11]

The forward rate of $6.8E-7\mbox{ (#/Cell)^{-1} sec^{-1}}$ and reverse rate of $6.5E-4\mbox{ sec^{-1}}$ were based on molar rates by Kuwana {\it et al.} Mol. Cell (2005). There are also $IC_{50}$ values available in Chen {\it et al.} Mol. Cell (2005). [7, 11]

The forward rate of $1.3E-7\mbox{ (#/Cell)^{-1} sec^{-1}}$ used is based on a 13 $\nano M$ $K_{d}$ averaged from the 11$\nano M$ $K_{d}$ in Certo {\it et al.} Cancer Cell (2006) and the 16$\nano M$ $IC_{50}$ from Chen {\it et al.} Mol. Cell (2005). A default reverse rate of $1E-3\mbox{ s^{-1}}$ was used to calculate the forward rate. [7, 10]

The forward rate of $1.3e-8\mbox{ (#/Cell)^{-1} sec^{-1}}$ and reverse rate of $3.7\mbox{ sec^{-1}}$ were based on molar rates by Kuwana {\it et al.} Mol. Cell (2005). This interaction is known to induce degradation of MCL-1 (Czabotar {\it et al.} (2007)), however the catalytic rate is unknown and will be estimated at calibration, though it is currently set at $1E-4\mbox{ s^{-1}}$. There are also $IC_{50}$ values available in Chen {\it et al.} Mol. Cell (2005). [7, 11, 85]

MULE is known to interact with and induce degradation of MCL-1 (Warr {\it et al.} FEBS Lett. (2005) and Zhong {\it et al.} Cell (2005)), however the rates and affinities are unknown. Thus, the current values were estimated by E. Fire. [151, 152]

MCL-1 can be degraded by caspase 8 (Han {\it et al.} JBC (2006)), though reaction rates are not reported. These current values were estimated by E. Fire. [153]

MCL-1 can be degraded by caspase 3 (Han {\it et al.} JBC (2006)), though reaction rates are not reported. These current values were estimated by E. Fire. [153]

The autoactivation is proposed by Willis {\it et al.} Genes&Dev. (2005). No rates are available, and are currently at zero for this model. [9]
There is no reported interaction between MCL1 and BAX.

There is no reported interaction between BCL2 and BAK. Willis et al. Genes&Dev. (2005) detected no interaction. [9]

### 5.3.6 Testing the Model

C. Lopez ran the Little b model to generate the ODEs for the modified EARM. This model shows the release of cytochrome c and Smac from the mitochondria following the formation of just a few BAX and BAK pores (Figure 5.3). We were able to recapitulate the original EARM model (Figure 5.4). MOMP occurs quickly, after little more than three hours. tBID and cPARP kinetics are not exactly the same as the original model, but still on a similar scale.

**Figure 5.3 Running the expanded BCL-2 model**
Simulation of the model shows that release of mitochondrial proteins coincides with pore formation. The number of BAX and BAK pores is plotted on the left Y-axis. The number of Cyt C or Smac in the mitochondria or released into the cytosol is plotted on the right Y-axis.
Figure 5.4 Comparison of Little b model to EARM
Simulation of expanded BCL-2 model (A) compared to EARM (B) from Figure 4B of Albeck et al. [1]. Plots show fraction of the indicated form of the protein as compared to the total amount of that protein versus time. The red line in each graph indicates the fraction of total Smac in the cytosolic compartment. The blue line indicates the fraction of total BID that has been cleaved to become active tBID. The green (A) and yellow (B) lines indicate the fraction of total PARP that is cleaved.

5.4 Discussion

Using Little b has many benefits for constructing complex biological processes. The language allows for many compartments. For example, this model defines the outside of the cell, the cytoplasm, the mitochondria, and the cell and mitochondrial membranes as independent compartments. Interaction of molecules at the membrane (a 2D space) with molecules in the cytosol (a 3D space) is modeled appropriately. This is quite different from the ODE model previously written, which could not handle this type of interaction. In contrast, the EARM defined a “mitochondrial compartment”, i.e. a space surrounding the mitochondria in which mitochondrial membrane proteins resided and into which other proteins could diffuse to allow for interaction. Additionally, Little b enables easy modification of the model by adding or removing components or reactions. If a reaction or interaction is to be added, a rule describing the interaction is written, and then Little b derives the correct ODEs, rather than having to modify all ODEs involving that change by hand.

Though still in the early stages of development, this model is set up to test the role that different BCL-2 proteins play in receptor-mediated apoptosis. Additionally, this model may
allow testing of models of BAX and BAK activation by direct interaction with tBID and BIM (direct model) or auto-activation of BAX and BAK with no interaction with the BH3s (indirect model). Either model can be tested by zeroing the rate constants for interactions or conversions that do not exist in that model.
References


133. Dutta, S., Keating, A. E., manuscript in preparation.


