Theoretical study of the binding of BH3 peptides to BCL-2 proteins towards a novel anticancer strategy

Cui, Jinglan

2017

Cui, J. (2017). Theoretical study of the binding of BH3 peptides to BCL-2 proteins towards a novel anticancer strategy. Doctoral thesis, Nanyang Technological University, Singapore.

<http://hdl.handle.net/10356/70895>

<https://doi.org/10.32657/10356/70895>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**THEORETICAL STUDY OF THE BINDING OF
BH3 PEPTIDES TO BCL-2 PROTEINS
TOWARDS A NOVEL ANTICANCER
STRATEGY**

CUI JINGLAN
SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES
2016

**THEORETICAL STUDY OF THE BINDING of
BH3 PEPTIDES TO BCL-2 PROTEINS
TOWARDS A NOVEL ANTICANCER
STRATEGY**

CUI JINGLAN

School of Physical and Mathematical Sciences

A thesis submitted to the Nanyang Technological University
in fulfilment of the requirement for the degree of
Doctor of Philosophy

2016

Abstract

BCL-2 proteins play a significant role in regulating apoptosis through complex interactions between antiapoptotic and apoptotic members. In cancer cells, the overexpression of antiapoptotic proteins inhibit the heterodimerization of apoptotic proteins, preventing cell death. Hence, through studies focusing on key interactions between BH3 domains of apoptotic proteins and antiapoptotic proteins, insights which are constructive towards the development of novel anticancer drugs that mimic the functionality of BH3 domains can be attained. Molecular dynamics (MD) simulations have been well developed along with robust computational analytical tools, and become a standard approach used to study peptide-protein binding. In this thesis, through MD simulations, the impact caused by the length of the BH3 peptide in BAX when bound to antiapoptotic protein BCL-2 was studied. By comparing the 26-mer and 21-mer BAX peptides, the C-terminus residues of BH3 peptide were revealed having the ability to maintain the α helix and, at the same time, stabilise the salt-bridge between peptide and protein. Further, more BCL-2/BH3 complexes are studied to identify the influence of peptide sequence on binding affinity. Detailed information on both hydrophobic and hydrophilic residues was obtained by analysing multiple simulations for each complex. Furthermore, hotspots of BCL-2 were identified. Although having similar binding scenario, BH3 peptides from different BH3-only proteins selectively bind to antiapoptotic proteins and through MD simulations and energy decomposition, this recognition between BH3-only proteins and antiapoptotic proteins were identified.

Crucial sequence and dynamic difference of BCL-xL and MCL-1 were also discussed.

All the detailed information on the BCL-2 family at molecular level was intended to offer insights towards future drug design based on the binding mechanism between BCL-2 family members.

Acknowledgement

First and foremost, I would like to thank my supervisor, Associate Professor Li Tianhu for giving me a chance to work in his group and supporting my PhD study.

Second I would like to express my greatest appreciation to Assistant Professor Zhang Dawei. His sincere guidance and continuous support during my PhD study allow me to grow as a computational chemist. The completion of this thesis can never be achieved without his illuminating instructions and encouragement. Besides being an excellent research mentor, he also shares invaluable opinions on career and attitude of life. All of these help has walked me through all the stages of my PhD life.

I would be grateful to all my colleagues Dr Wei Caiyi, Dr Sun Tiedong, Dr Raudah Lazim, Dr Duan Rui and Yip Yew Mun, Yang Hong. Thanks for their invaluable contributions toward my research and also for their precious friendship.

Last but never least, I wish to express my special gratitude to my family. Thanks to my parents for their great sacrifice and encouragement. Thank you very much for giving me full support and priceless love. Also, many thanks to my dear friends Wang Lei, Qi Xiaofei and Wu Jingyuan.

Contents

Abstract	i
Acknowledgement	iii
Contents	iv
List of Figures	viii
List of Tables	xiv
List of Abbreviations	xv
Chapter 1	1
Introduction.....	1
1.1 BCL-2 family and cell death	1
1.2 Subgroups of BCL-2 family	3
1.3 Structural biology of BCL-2 family	6
1.4 BCL-2 family deregulation in cancer	9
1.5 Molecular dynamics simulation.....	12
1.6 Principle of molecular dynamics simulations	14
1.7 Binding free energy	17
1.8 Outline of thesis	19
Chapter 2	21
Influence of BH3 peptide length in binding with antiapoptotic protein BCL-2	21

2.1 Introduction	21
2.2 Materials and Methods	24
2.2.1 Preparation of initial structures.....	25
2.2.2 Molecular dynamics simulations	25
2.2.3 Binding free energy using Sietraj	26
2.2.4 Alanine scanning calculation	27
2.2.5 Hydrogen bond analysis and salt-bridge analysis.....	28
2.2.6 Helical content analysis	28
2.3 Results and Discussion	28
2.3.1 MD simulation and binding free energy	29
2.3.2 Residues in charge of binding differences	31
2.3.3 Binding affinity influenced by helical content	36
2.4 Conclusion	37
Chapter 3	40
Antiapoptotic protein BCL-2 in binding with BH3 peptides of proapoptotic	
 proteins	40
3.1 Introduction	40
3.2 Methodology	44
3.2.1 Preparation of initial structures.....	44

3.2.2 Molecular dynamics simulations	44
3.2.3 Binding free energy using Sietraj	45
3.2.4 Alanine scanning calculation	46
3.2.5 Free energy decomposition	47
3.2.6 Analysis of molecular dynamics simulations.....	47
3.3 Results and Discussion	48
3.3.1 Binding free energy.....	48
3.3.2 Conserved hydrophobic residues	51
3.3.3 Influence of hydrophilic residues.....	57
3.3.4 Helical content	62
3.3.5 In-silico design of BH3-like peptide.....	64
3.3.6 Hotspots of BCL-2.....	66
3.4 Conclusion	69
Chapter 4	71
The selectivity of BH3 peptides to antiapoptotic proteins BCL-xL and MCL-1	71
4.1 Introduction	71
4.2 Methodology	76
4.2.1 Preparation of initial structures.....	76
4.2.2 Multiple molecular simulations	77

4.2.3 Binding free energy calculation using MM_PBSA	77
4.2.4 Free energy decomposition	78
4.3 Results and Discussion	79
4.3.1 BCL-xL/BAD·BH3 versus MCL-1/BAD·BH3	81
4.3.2 BCL-xL/NOXA·BH3 versus MCL-1/NOXA·BH3	95
4.4 Conclusion	106
Chapter 5	109
Summary	109
Publications	113
References	114

List of Figures

Figure 1.1. Mitochondrial Pathway of Apoptosis: Diverse cell death stimulus activate BCL-2 family-regulated mitochondrial pathway. Once receive stimulus from upstream, BH3-only proteins will inhibit antiapoptotic proteins and activate effector proteins. Effector proteins, BAX and BAK will then permeabilize mitochondrial membrane and release cytochrome c which activate APAF1. The downstream caspase 9 will then be activated and deliver signal to effector caspases, thus apoptosis is promoted. (Adapted) [3].....	2
Figure 1.2. Three subclasses of BCL-2 family. BCL-2 family proteins are functionally distributed into antiapoptotic proteins and proapoptotic proteins. Proapoptotic proteins can further be classified into effector proteins and BH3-only proteins according to the BCL-2 homology domains defined by sequence similarities. The schematic also show three dimensional structures corresponding to the homology domain. Antiapoptotic proteins and effector proteins share similar structures comprising nine α helices while BH3-only proteins, only contain one BH domain are disordered when it is free....	3
Figure 1.3. Activation models addressing how interactions between BCL-2 family proteins regulate oligmerization of effector proteins. BCL-2, BAX are used to represent antiapoptotic proteins and effector proteins respectively. BAD represents ‘sensitizers’ of BH3-only proteins while BIM represents ‘activators’.	5
Figure 1.4. Three dimensional structure of BCL-xL (PDB code: 1MAZ). The protein compromise eight α helices with $\alpha 5$ in the central (highlight in purple).	7

Figure 1.5. The conformational change of BAX along with activation and dimerisation. Core domains comprise $\alpha 1$ - $\alpha 5$ and the latch domain contains $\alpha 6$ - $\alpha 8$. (Adapted)[23]	9
Figure 1.6. Illustration of terms related to potential energy calculated by equation 1.5 (Adapted)[94].	16
Figure 2.1. The structure of BCL-2 in binding with BH3 peptides of BAX. Protein BCL-2 is shown in purple ribbon. Longer BAX·BH3 (with residue 78-82) is shown in yellow and shorter BAX·BH3 (without residue 78-82) is shown in pink. Below is the sequence of BAX·BH3.	24
Figure 2.2. RMSD plotted against time for (A) BCL-2/BAX·BH3 (26-mer) and (B) BCL-2/BAX·BH3 (21-mer) Results for BCL-2 are shown in black and for BH3 peptides are shown in red.	29
Figure 2.3. Binding free energy of 26-mer BCL-2/BAX·BH3 (Black) and 21-mer BCL-2/BAX·BH3 (Red). (A) Total binding free energy represented as time function (B) Distribution of binding free energy.	30
Figure 2.4. Alignment of alanine scanning results of 26-mer and 21-mer BAX·BH3. .	34
Figure 2.5. (A) Three residues of BAX·BH3 in charge of binding difference (B) Hydrogen bond stability of R78 and Glu200 (C) Hydrogen bond stability of R64 and Glu136 (D) Salt-bridge stability of D68 and Arg146	36
Figure 2.5. Helical content of the common part of 26-mer BAX·BH3 (Black) and 21- mer BAX·BH3 (Red)	37

Figure 3.1. Binding free energy from experimental data (shown in blue line) and Sietraj calculation (shown in green line) for four complexes	50
Figure 3.2. Alanine scanning results for four BH3 peptides. Critical hydrophobic residues are highlighted with lines and critical hydrophilic residues are highlighted with dot lines.....	52
Figure 3.3. Five conserved hydrophobic residues of BH3 peptides in complex BCL-2/BAX·BH3. Basic residues of BCL-2 are shown in iceblue, acidic residues are shown in pink, polar residues are in grey, and nonpolar residues of BCL-2 are in white surface. .	54
Figure 3.4. (A) Residue located at F14 of BIM·BH3 interacts with Phe104 of BCL-2. (B) π - π stacking between Y18 from BIM·BH3 and Tyr202 from BCL-2. (C) π - π stacking between W18 from NOXA·BH3 and Tyr202 from BCL-2. Residues on BH3 peptides are shown in Licorice and on BCL-2 protein are shown in CPK	55
Figure 3.5. (A) The H-bond between P6 and BCL-2 in complex BCL-2/BIK·BH3 (B) Salt-bridge between R8 and Asp140 in complex BCL-2/BIM·BH3(C) The H-bond between R8 and Glu136 in complex BCL-2/BAX·BH3	59
Figure 3.6. (A) The distance between NH2 of P12 and OD2 of Arg146 in four complexes. (B) Distance analysis of OE2 of P13 and NH2 of Arg110 (C) NH1 of P13 and OE1 of Arg107. (D) Distance of NH2 of P22 and OE2 of Glu200 in complex BCL-2/BAX·BH3 and BCL-2/BIK·BH3.....	60
Figure 3.7. Helical Content analysis for four BH3 peptides	63
Figure 3.8. Alanine scanning of the in-silico designed new peptide	65

Figure 3.9. Free energy decomposition of BCL-2 in each complex.	67
Figure 4.1. Some BH3-only proteins can bind and inhibit all prosurvival proteins (tBID, BIM and PUMA) while BAD only has the affinity to BCL-2, BCL-xL and BCL-w. NOXA can only bind with MCL-1 and A1.	73
Figure 4.2. Sequence Comparison of BCL-xL and MCL-1. Identical residues are shown in green, similar residues are in pink, mismatch residues are in blue and inserted or deleted residues are in brown.	80
Figure 4.3. Sequence alignment of BCL-xL and MCL-1. Sequence and original residue number of BCL-xL are highlighted in green and of MCL-2 are in yellow.	80
Figure 4.4. Binding free energy decomposition for (A) MD1 of BCL-xL (B) MD2 of BCL-xL(C) MD3 of BCL-xL (D) Average of BCL-xL (a) MD1 of MCL-1 (b) MD1 of MCL-1 (c) MD1 of MCL-1 (d) Average of MCL-1. Residue number follows sequence alignment number.	84
Figure 4.5. Difference between contribution of each residue on BCL-xL and MCL-1 in associating with BAD·BH3.	85
Figure 4.6. Pair-wise binding free energy decomposition. More negative means this pair of residues contribute more to binding	85
Figure 4.7. Salt-bridge between Arg53 of protein and D16 of peptide. The loop between $\alpha 4$ and $\alpha 5$ is highlighted in cyan. Residues on the protein are shown in CPK and residues on peptide are shown in Licorice.	87

Figure 4.8. (A) Salt-bridge between Arg53 and D16 (B) Distance between NH ₂ of Arg53 and OE2 of E19 (C) Hydrogen bond between ND2 of Asn 50 and CD of E19 (D) Hydrogen bond between N of Gly52 and CD of E19.....	88
Figure 4.9. Backbone analysis of residue Asn50 on (A) BCL-xL (B) MCL-1. Colours are used to illustrate the popularity of dihedral angles. Higher popularity is shown in smaller number. (C) RMSF analysis of proteins (BCL-xL in black and MCL-1 in red).....	90
Figure 4.10. (A) SASA of F18 from BH3 peptide in both complex (B) SASA of residue10 on BCL-xL and MCL-1. Interactions between residue10 and F18 in complex (C) BCL-xL/BAD·BH3 (D) MCL-1/BAD·BH3	90
Figure 4.11. DSSP analysis for BAD·BH3 in complex (A) BCL-xL/BAD·BH3 and (B) MCL-1/BAD·BH3	91
Figure 4.12. Ionic interactions between BAD·BH3 and residue 42 from (A) BCL-xL (B) MCL-1.	93
Figure 4.13. The interactions between Y7 and residue 17, 21 of (A) BCL-xL (B) MCL-1. α 3 in each protein is highlighted in iceblue. DSSP result for α 3 of (C) BCL-xL and (D) MCL-1	93
Figure 4.14. Binding free energy decomposition for (A) MD1 of BCL-xL (B) MD2 of BCL-xL(C) MD3 of BCL-xL (D) Average of BCL-xL (a) MD1 of MCL-1 (b) MD1 of MCL-1 (c) MD1 of MCL-1 (d) Average of MCL-1. Residue number follows sequence alignment number.	97

Figure 4.15. Difference between the contribution of each residue on BCL-xL and MCL-1 in associating with NOXA·BH3.	98
Figure 4.16. Pair-wise binding free energy decomposition for complex (A) BCL-xL / NOXA·BH3 and (B) MCL-1/NOXA·BH3	98
Figure 4.17. (A) Distance analysis of salt-bridge between Arg53 and D16. Illustration of interactions between Arg53, Asn50 of protein and D16, Y19 of peptide in complex (B) BCL-xL/NOXA·BH3 and (C) MCL-1/NOXA·BH3.	101
Figure 4.18. Backbone analysis of residue Asn50 on (A) BCL-xL (B) MCL-1. (C) RMSF analysis of proteins (BCL-xL in black and MCL-1 in red)	102
Figure 4.19. With the break of salt-bridge between Arg53 and D16 in complex BCL-xL/NOXA·BH3, water molecules penetrate into the binding site.	102
Figure 4.20. (A) The salt-bridge and H-bond analysis of E2 in contact with protein MCL-1. (B) DSSP for the BH3 peptide NOXA·BH3 when in complex with MCL-1..	103
Figure 4.21. (A) Distance between the CB of F7 and residue 21(B) The interactions between F7 and proteins and secondary structure of NOXA·BH3 in two complex.	105

List of Tables

Table 2.1. Binding free energy for w.t BAX·BH3 and mutant BAX·BH3. Each term contribute to binding free energy is listed, including intermolecular van der Waals energy, Coulomb energy, reaction field energy and non-electrostatic solvation free energy (kcal/mol).	32
Table 3.1. The peptide sequences of BIM, BAX, BIK, NOXA and an in-silico designed peptide. The sequences are numbered form 1 to 26	43
Table 3.2. List of binding free energy from literature and calculated by Sietraj for each MD trajectory (kcal/mol)	49
Table 3.3. Average binding free energy components of each complex (kcal/mol)	51
Table 3.4. Energy components of wild-type and mutant peptides.	58
Table 4.1. Disassociate constant of 36-mer BH3 peptides in binding with antiapoptotic BCL-2 proteins.	75
Table 4.2. Sequence alignment of BAD and NOXA BH3 peptides	79
Table 4.3. Free energy components for complex BCL-xL/BAD·BH3 and MCL-1/BAD·BH3 (kcal/mol)	82
Table 4.4. Free energy components for complex BCL-xL/NOXA·BH3 and MCL-1/NOXA·BH3 (kcal/mol)	95

List of Abbreviations

BCL-2	B Cell Lymphoma-2
OMM	Outer Mitochondrial Membrane
MAC	Mitochondrial Apoptosis-induced Channel
APAF-1	Apoptotic Protease Activation Factor-1
BH	BCL-2 Homology
BCL-xL	B-cell Lymphoma-extra Large
MCL-1	Myeloid Cell Leukaemia 1
BAK	BCL-2 Antagonist Killer 1
BAX	BCL-2 Associated X protein
BID	BCL-2-interacting domain death agonist
BIM	BCL-2-interacting mediator of cell death
PUMA	p53 Upregulated Modulator of Apoptosis
BAD	BCL-2 Antagonist of cell Death
OMMP	Outer Mitochondrial Membrane Permeabilisation
BC groove	BH3 and C-terminus-binding groove
TM	Carboxy-terminal transmembrane
IUPs	Intrinsically Unstructured Proteins
MD	Molecular Dynamics
ITC	Isothermal Titration Calorimetry
PDB	Protein Data Bank
RMSD	Root-Mean-Square Deviation
FEP	Free Energy Perturbation

TI	Thermodynamic Integration
EDS	Energy Dispersive Spectroscopy
BAR	Bennett Acceptance Ratio
PMF	Potential of Mean Force
DF	Distance Field

Chapter 1

Introduction

1.1 BCL-2 family and cell death

The interest in the investigation of cell death began more than 150 years ago and has been heftily growing in the past decades. In animals, the dominant mode of cell death is gene-controlled apoptosis. Apoptosis plays a vital role in sustainable development and tissue homeostasis in multicellular organism by removing unwanted or damaged cells. Cellular stress like cell shrinkage, DNA fragmentation or cytosine withdrawal will trigger the ‘apoptotic bodies’ to deliver the cell death signal to downstream and dying cells. Disruption of this programmed process will result in a variety of diseases, for example, cancer, viral infections and neurodegenerative disorders[1, 2].

A significant group of proteins involved in apoptosis is B cell lymphoma-2 (BCL-2) family. BCL-2 family proteins are mainly involved in the mitochondrial pathway of apoptosis(Figure 1.1)[3]. Permeabilisation of outer mitochondrial membrane (OMM) is controlled by the complex interactions between BCL-2 family members[4]. In response, a mitochondrial apoptosis-induced channel (MAC) will form and apoptogenic factors (e.g. cytochrome c)[5], which are previously located in the inner membrane of mitochondrial, will diffuse into the cytosol. The highly soluble protein cytochrome c will then bind with a scaffold protein named apoptotic protease activation factor-1

(APAF-1) and promotes the activation of caspase-9[6, 7]. Caspase-9 mediates the activity of the effector caspases (e.g. caspase-3 and caspase-7) and trigger cell death.

Figure 1.1. Mitochondrial Pathway of Apoptosis: Diverse cell death stimulus activate BCL-2 family-regulated mitochondrial pathway. Once receive stimulus from upstream, BH3-only proteins will inhibit antiapoptotic proteins and activate effector proteins. Effector proteins, BAX and BAK will then permeabilize mitochondrial membrane and release cytochrome c which activate APAF1. The downstream caspase 9 will then be activated and deliver signal to effector caspases, thus apoptosis is promoted. (Adapted) [3]

1.2 Subgroups of BCL-2 family

Proteins of BCL-2 share four BCL-2 homology (BH) domains referred to as BH1, BH2, BH3 and BH4. Each family member contains at least one of these conserved motifs. Based on the sequence homology and protein function, the family can be grouped into three subfamilies (Figure 1.2).

Figure 1.2. Three subclasses of BCL-2 family. BCL-2 family proteins are functionally distributed into antiapoptotic proteins and proapoptotic proteins. Proapoptotic proteins can further be classified into effector proteins and BH3-only proteins according to the BCL-2 homology domains defined by sequence similarities. The schematic also show three dimensional structures corresponding to the homology domain. Antiapoptotic proteins and effector proteins share similar structures comprising nine α helices while BH3-only proteins, only contain one BH domain are disordered when it is free.

BCL-2 itself, B-cell lymphoma-extra large (BCL-xL), Bcl-2-like protein 2 (BCL-w), myeloid cell leukaemia 1 (MCL-1), Bcl-2-like protein 10 (Bcl-B) and BCL-2-related gene A1 (A1) are the key players of antiapoptotic proteins in BCL-2 family and they share all four BH domains. Anchoring in the OMM, these BCL-2 proteins modulate the OMM integrity by blocking proapoptotic proteins in the family leading to the interruption of programmed cell death.

One group of proapoptotic proteins called effector proteins compromises BCL-2 antagonist killer 1 (BAK) and BCL-2 associated X protein (BAX). Similar to antiapoptotic proteins, BAK and BAX have sequence homology in four conserved BH regions. However, the location of these two proteins differs. BAK anchor on the outer membrane[8] while BAX is primarily released into the cytosol until cell death message was transmitted. Upon activation, BAX will undergo a conformational change and accumulates on the mitochondrial membrane[9, 10]. After being activated, both BAK and BAX monomers will undergo oligomerization and open a channel for the cytochrome c. Activation and oligomerization of effectors plays a central role in the BCL-2 family regulated apoptosis.

The other group of proapoptotic proteins are called BH3-only proteins that shared only the BH3 domain with other family members. They carry out their proapoptotic function in two different ways. BCL-2-interacting domain death agonist (BID), BCL-2-interacting mediator of cell death (BIM) and perhaps p53 upregulated modulator of apoptosis (PUMA) can directly associate and activate BAX and BAK[11-14]. These

proteins also have the ability to bind and neutralise antiapoptotic proteins so that proapoptotic proteins are released preventing blockage. These proteins are called ‘activators’. The other BH3-only proteins that lack the ability to trigger effector proteins directly are designated as ‘sensitizers’. ‘Sensitizers’ like BCL-2 antagonist of cell death (BAD) and Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) only have the ability to interact with antiapoptotic proteins[15, 16] and allow proapoptotic proteins to trigger apoptosis[11, 17].

Figure 1.3. Activation models addressing how interactions between BCL-2 family proteins regulate oligomerization of effector proteins. BCL-2, BAX are used to represent antiapoptotic proteins and effector proteins respectively. BAD represents ‘sensitizers’ of BH3-only proteins while BIM represents ‘activators’.

Three subgroups of BCL-2 family interact with each other and promote the outer mitochondrial membrane permeabilisation (OMMP) following the release of cytochrome c. The core of the BCL-2 regulated apoptosis is the activation of effector proteins BAX and BAK and two models are proposed to illustrate the activation process.(Figure 1.3). In the direct activation model, certain BH3-only proteins designed as ‘activators’ are blocked by antiapoptotic proteins. Once the programmed cell death is initiated, ‘sensitizers’ occupy the binding pockets of antiapoptotic proteins and displaced the ‘activators’ which will then directly activate BAX and BAK. In the indirect activation model, BAX and BAK can be activated in some other ways like increasing pH or temperature. However in this model, effectors that are initially inhibited by antiapoptotic BCL-2 proteins determined that the key step to start apoptosis is to free BAX and BAK from antiapoptotic proteins. BH3-only proteins play a role in competing with effector proteins and neutralising antiapoptotic proteins so that effector proteins can be released. However, studies on the role of BIM revealed that BIM has the ability to activate effector proteins and sequester antiapoptotic proteins[18]. These observations suggest that both direct and indirect models of activation may work together to ensure effectors could execute their vital role in apoptosis.

1.3 Structural biology of BCL-2 family

The complex interactions between BCL-2 family members are closely related to their three-dimensional structures. X-ray and NMR studies on BCL-2 family showed that antiapoptotic proteins and effector proteins are folded globular proteins while BH3-only

proteins are intrinsically disordered[19]. The corresponding relationship between these helices and sequence homology domains is shown in Figure 1.2. The globular proteins shared a similar structure with a bundle of α helices around a central helix named $\alpha 5$ [20] (Figure 1.4). The α -helices 2 to 5 depict a hydrophobic groove called BCL-2 family BH3 and C-terminus-binding groove (BC groove). The BC groove plays a crucial role in protein interactions by accepting BH3 domain from other proteins. For unactivated BAX and BAK, The BC groove is filled by its carboxy-terminal transmembrane (TM) helix $\alpha 9$ [10]. The BC grooves of pro-survival proteins are normally exposed to engage with proapoptotic proteins. Antiapoptotic BCL-2 protein members also contain a TM domain (i.e $\alpha 9$) that fixes the protein to mitochondria.

Figure 1.4. Three dimensional structure of BCL-xL (PDB code: 1MAZ). The protein compromise eight α helices with $\alpha 5$ in the central (highlight in purple).

BH3-only proteins are primarily intrinsically unstructured proteins (IUPs) except BID. Although these proteins only share similar BH3 homology domain with the globular proteins, BID comprised of eight helices of which two helices are surrounded by six other helices[21]. This special structure buries key BH3 residues so that BID is kept in the inactive state[22]. For other disordered BH3-only proteins, their BH3 domain only form an amphipathic helix when they bind to other BCL-2 proteins.

Recent studies revealed that the process of BAX and BAK activation is accompanied with conformational changes[23]. With $\alpha 9$ helix anchoring on the MOM, the BC groove of effector proteins is free to bind with the BH3 domain of BH3-only protein, BID. Together with the membrane environment, BID will trigger the core domain ($\alpha 1$ - $\alpha 5$) to dissociate from the latch domain ($\alpha 6$ - $\alpha 8$). Also, the cavity between $\alpha 2$, $\alpha 5$ and $\alpha 8$ will dislodge $\alpha 2$ (BH3 domain). The released BH3 domain will then compete with BID to bind to neighboring BAX. The released BH3 domain will attempt to displace BID and bind to the groove of BAX. The BH3-in-groove symmetric BAX dimer will then promote the formation of the MAC pore which required more in-depth study for greater understanding of the process involved in MAC pore formation.

Figure 1.5. The conformational change of BAX along with activation and dimerisation. Core domains comprise $\alpha 1$ - $\alpha 5$ and the latch domain contains $\alpha 6$ - $\alpha 8$. (Adapted)[23]

1.4 BCL-2 family deregulation in cancer

BCL-2 family members control a complex process which differs a lot between different types of tissue and various cell fate stimuli. For example, gene-knockout study on the BCL-xL of mice showed that BCL-xL-deficient mice die in the embryonic stage because of the divergence from normal activity in the neuronal and hematopoietic system[24]. The knockout of the BCL-2 gene caused the retardation of embryonic development and regression of thymus and spleen resulting in polycystic kidney disease[25]. MCL-1-deficiency have significant impact on the survival of hematopoietic stem cells (HSC) and B/T lymphocytes[26-29]. It can also cause heart disease when ablated in cardiomyocytes[30, 31]. Such a functional deviation also exists between BH3-only proteins. BIM, which has affinity for both antiapoptotic proteins and effector proteins, is essential for the transfer of certain cytotoxic stimuli (e.g. calcium ion flux,

cytokine deprivation) and regulates lymphoid homeostasis [32-34]. Cytotoxic stimuli that triggers NOXA and PUMA is the p53 pathway.

Defect in the expression levels of BCL-2 family members deregulates the mitochondrial apoptotic pathway, thus leading to various diseases especially cancer. Triggers for cancer may vary, but all cancer cells show a reduction in apoptosis compared to normal cells. One way cancer cell regulates BCL-2 family members is by disrupting the interaction between BH3-only proteins and their upstream regulators or by deleting the BH3-only gene[35, 36], thus reducing the activation of BH3-only proteins[37]. Mutating the critical members of the BCL-2 family and effector proteins could also reduce apoptosis thus permitting the aggressive propagation of tumour cells[38, 39]. The most potent and well-accepted mechanism which best explains the inhibition of apoptosis in cancerous cells is the overexpression of antiapoptotic proteins. Increased levels of antiapoptotic proteins terminated the apoptotic process by blocking proapoptotic proteins including effectors and BH3-only proteins[40]. The overexpression of BCL-2 was first found in human follicular lymphomas as a result of t(14;18)(q32;q21) chromosomal translocation[41]. Following this finding, the high expression level of BCL-2 was found in numerous cancers like small-cell lung carcinomas[42, 43], melanoma[44], B-chronic lymphocytic leukaemia[45] and breast cancers[46-48]. Similarly, tumours are also found relating to the high level of other antiapoptotic proteins like BCL-xL and MCL-1[49].

The key in killing tumour cells caused by the over-expression of BCL-2 family members is to release the proapoptotic proteins from the over-expressed antiapoptotic proteins. One way to achieve this is to use allele-specific oligonucleotide (ASO) to suppress the expression level of antiapoptotic BCL-2 members[50, 51]. One example is Oblimersen which is in Phase III clinical trials for the treatment of melanoma and myeloma leukemia, and is in Phase II trials of lymphoma, and cancers of the prostate, colon and breast[52-55]. However, Oblimersen did not get the Food and Drug Administration (FDA) approval in the United States because it did not show significant survival improvement in the treatment of lymphocytic leukaemia[56] and melanoma[57]. Another problem ASOs have in the treatment of cancer is that they lack stability and binding specificity.

Compared with ASO inhibitors, a more efficient and more direct approach to treat the over-expression of antiapoptotic BCL-2 proteins is to mimic the activity of the BH3 domain derived from proapoptotic proteins[53]. This strategy was inspired by the finding of the three dimensional structures of BCL-xL monomer[58] and BCL-xL in complex with BAK BH3 peptide[59]. Additional BH3-mimic inhibitors occupy the BC groove of antiapoptotic proteins so that proapoptotic proteins can be released to complete the apoptotic pathway. This cancer therapy plot has been demonstrated by the development of a small molecule ABT-737[60]. Acting as a single agent, ABT-737 and its orally bioavailable derivative ABT-263[61] mainly target on and bind tightly to antiapoptotic protein BCL-xL and BCL-w and have significant anti-tumour activity in chronic lymphocytic leukaemia (CLL)[62, 63]. The study of CLL states that ABT-737

promotes cell death and kills cancer cells by disrupting the peptide-protein complex comprising of BH3-only proteins and antiapoptotic proteins[64, 65]. Moreover, preclinical studies lately indicated that the combination of ABT-737 or ABT-263 with other conventional treatment[66, 67] could increase the efficiency of killing several other tumours including melanoma, small-cell lung cancer and breast cancer[68-70]. Following this, a BCL-2-specific BH3-mimic compound ABT-199 was developed[71]. ABT-199 showed better tolerance than ABT-737 in the treatment of CLL[72] and clinical trial result is encouraging. Some other molecules like WEHI-539[73] are also designed or in the initial clinical trial stage. Although BH3-mimics have achieved some promising result, obstacles still exist. It seems combining BH3-mimics with additional anti-cancer drugs that only have effects on tumours would be a better strategy to selectively kill cancer cells without affecting normal cells[74].

1.5 Molecular dynamics simulation

Molecular dynamics (MD) simulation is the most widely used computational tool for understanding the particle motions, molecular structures and function of macromolecules. The first interest in exploring proteins using computers is in 1975 when MD simulation was used to explore the conformational change of bovine pancreatic trypsin inhibitor[75, 76], a small-size and stable protein. Although this MD simulation is conducted in vacuum and last for only 9.2ps, folding of bovine pancreatic trypsin inhibitor from open-chain conformation to globular protein give a clue that proteins are dynamic systems. Later on, more studies on proteins using MD simulation

demonstrated the possibility and capability of MD simulation in inferring the internal motion of biological systems[77, 78]. MD simulation has progressed significantly over the past decades and with increasing computer power, more complex biological systems and more complicated motional phenomena could be examined[79]. A wide range of applications can be listed, some of which includes the examination of fluorescence depolarisation of tryptophan residues[80], structure refinement[81, 82] and threshold for ligand binding with heme proteins[83].

The 2013 Nobel Prize in Chemistry was awarded to work done in computational chemistry, emphasizing the importance of computational chemistry as a critical counterpart of experimental biology[84]. Martin Karplus, Michael Levitt and Arieh Warshel introduce quantum mechanics into classical MD simulation for probing chemical process in proteins. By implementing this, the particles in protein having interactions with ligands performed accurate calculation using quantum mechanics while other atoms of protein are calculated with molecular mechanics which are simpler and faster[85-87]. This achievement, to a certain degree, remedy the limitation of traditional MD simulation. This definitely broaden the application of MD simulation for exploring the biological system.

With progressively improved molecular modelling in recent days, a lot of computational tools are set up to fill the gap between experiments and simulations. One example is the implementation of MD simulations in molecular recognition. Through modern simulations and modelling, computational tools can speed up the understanding of

ligand-protein binding and discovering a ligand that binds to a specific protein[88]. In this thesis, MD simulations are applied to peptide-protein binding of BCL-2 family systems to explore the interactions between BH3 peptides and antiapoptotic BCL-2 proteins. In Chapter 2 and Chapter3, the impact of peptide length, sequence and helical stability of BH3 peptides from proapoptotic BCL-2 family members in complex with antiapoptotic protein BCL-2 is studied using MD simulations and computational analysis tools. The determinants of selectivity of BH3 peptides to antiapoptotic proteins and protein-peptide recognition are discussed in Chapter 4 by analysing the interactions between selected peptides and proteins which is based on MD simulations of different peptide-protein systems.

1.6 Principle of molecular dynamics simulations

In classical molecular dynamics simulations, atoms and bonds of biological systems are regarded as classical objects and follow classical and statistical mechanics. The execution of MD simulations for interacting particles is implemented by Newton's equation of motion[89-91]:

$$\mathbf{F}_i = m_i \frac{d^2 \mathbf{r}_i(t)}{dt^2} \quad (1.1)$$

In the equation 1.1, $\mathbf{F}_i$ represents at time t , the force acting on i^{th} particle and m_i is the mass of i^{th} particle. Position of i^{th} particle at time t is shown in $\mathbf{r}_i(t)$ and $\mathbf{r}_i(t) = (x_i, y_i, z_i)$. By applying the Newton's equation of motion, the equation that defines the position of i^{th} particles at time $(t + \Delta t)$ are defined as follows:

$$\mathbf{r}_i(t + \Delta t) = 2\mathbf{r}_i(t) - \mathbf{r}_i(t - \Delta t) + \frac{\mathbf{F}_i(t)}{m_i} \Delta t^2 \quad (1.2)$$

Force field models describes the interactions between particles or atoms of many-body system by the potential energy of N interacting atoms $U(\mathbf{r}_1, \dots, \mathbf{r}_N)$ which is determined by a function of the Cartesian coordinates of the atoms r_i , thus the force acting on atom i^{th} is defined with equation:

$$\mathbf{F}_i = -\nabla_i U(\mathbf{r}_1, \mathbf{r}_2, \mathbf{r}_3, \dots, \mathbf{r}_N) \quad (1.3)$$

One problem with determine the potential energy is defining the realistic energy surface. A variety of atomic force fields are developed based on empirical potentials or based on experimental data to describe the physical and chemical characteristics of biological systems such as proteins, DNA and nonbiological molecules like water or organic solvents. In the classic force fields, the function form of potential energy is including bonded and non-bonded energy as in function 1.4. Further, the potential energy is calculated according to equation as in 1.5[92, 93]:

$$E_{total} = E_{bonded} + E_{nonbonded} \quad (1.4)$$

$$\begin{aligned} E_{Total} = & \sum_{bonds} k_r (r - r_0)^2 + \sum_{angles} k_\theta (\theta - \theta_0)^2 + \sum_{torsions} \frac{V_n}{2} [1 + \cos(n\phi - \gamma)] \\ & + \sum_{i < j} 4\epsilon \left[\left(\frac{\sigma_{ij}}{r_{ij}} \right)^{12} - \left(\frac{\sigma_{ij}}{r_{ij}} \right)^6 \right] + \sum_{i < j} k \frac{q_i q_j}{r_{ij}} \end{aligned} \quad (1.5)$$

The bonded interactions between atoms that bond together through covalent bonds are defined by the summation of the first three terms while the last two terms stand for the non bonded energy between atoms that are not bonded chemically and separated by a distance $r_{ij} = |\mathbf{r}_i - \mathbf{r}_j|$. An illustration of related terms are shown in Figure 1.5.

Figure 1.6. Illustration of terms related to potential energy calculated by equation 1.5 (Adapted)[94].

The deviations of bond length and bond angle from equilibrium state r_0 and θ_0 to bond length r and θ are respectively, together with two energy constant k_r and k_θ , are used to define the energy associate with bond length and angle in the first two terms. The third term (i.e. energy associating with chemical bond torisions) is described by periodic energy terms including the equilibrium dihedral angle γ , value of n determined periodicity and potential energy barrier V_n . The last two terms describe the van der Waals interactions and Coulomb electrostatic potential respectively. The Lennard-Jones 6-12 equation which is determined by the distance r_{ij} between atom i and j as well as depth of potential energy ϵ , is used to describe the van der Waals interactions. The Coulomb electrostatic potential is closely related to the charge of paired atoms q_i , q_j and distance between charges r_{ij} .

1.7 Binding free energy

Studying the underlying free energy behaviour of protein-ligand binding is of amount importance in computer-aided drug design. Error in the force field and inefficient sampling often lead to an inaccurate estimate of binding free energy. A wide variety of methods has been created or improved with high efficiency and accuracy. Different approaches can be chosen depending on available computational resources and the desired accuracy.

Methods like Free Energy Perturbation (FEP)[95] and Thermodynamic Integration (TI) [96] are methods based on the calculation along a thermodynamic pathway and are often used to distinguish the difference of binding free energies between two compounds. These methods, which are commonly used in the calculation of relative binding affinity, are accurate and rigorous in estimating the binding free energy. However, the accuracy of this kind of methods mostly depends on the sufficient sampling[97] which leads to an expensive cost of computer resource and high time consuming. Approaches that share the similar mechanism are λ -dynamics[98], Energy Dispersive Spectroscopy (EDS)[99] and Bennett Acceptance Ratio (BAR)[100].

Potential of Mean Force (PMF) methods calculate the energy changes of the system as a function of a reaction coordinates[96]. This kind of methods need an accurate reaction coordinate should convey the reaction mechanism, provide reasonable free energy surfaces, and facilitate calculations of the rate constant. So PMF methods are performed together with umbrella sampling to make up its defect of adequately sampling the system space. Distance Field (DF) has been developed recently to depict the reaction coordinate for the calculation of reversible protein–ligand binding[101]. Different from FEP and TI, PMF methods calculate absolute binding free energy.

End-point methods such as MM_PBSA[102, 103] and MM_GBSA[102, 104] evaluate the binding free energy in a more rough way by considering only two end states. Free energies are decomposed into enthalpy energies and entropy energies. Enthalpy energies are including the internal energy of solute, electrostatic interactions, van der Waals

interactions, the polar and nonpolar energy of solvation. The entropic part is limited to conformational entropy which can be evaluated by normal mode module. Both explicit and implicit solvent models have been developed offering a varying information[105]. End-point methods are suitable for calculating the system that ligand change from unbounded state to bounded state. However, a comparison of estimated values to experimental data is essential when performing the methods.

In this thesis, our targeting system is BH3 peptides in binding to BCL-2 proteins. Considering the system is a ligand-protein complex and estimating the computational costs, the end-point method MM_PBSA is used to explore the free energy behaviour of the peptide-protein system.

1.8 Outline of thesis

With the brief introduction of BCL-2 family and MD simulation, the content in this thesis is organised into five chapters:

In Chapter 2, we focus on the complex BCL-2/BAX·BH3 and study the influence of the BAX·BH3 peptide length on binding affinity to antiapoptotic protein BCL-2. Through MD simulations of 26-mer BAX·BH3 and 21-mer BAX·BH3 complexed with BCL-2,, the role of five truncated residues at the C-terminus of BAX·BH3 was revealed by comparing the contribution of each amino acids in the peptide.

Following Chapter 2, Chapter 3 also studied the binding of BCL-2 to BH3 peptides. In this chapter, the binding mechanism of not only BAX·BH3 but also other three BH3 peptides, BIM·BH3, BIK·BH3, NOXA·BH3 which have varying binding affinities with BCL-2 were discussed. By comparing these four BH3 peptides, information at the molecular level were provided for future BCL-2 targeted BH3-mimic design. Also, hot spots of BCL-2 were revealed.

In Chapter 4, we focus on the selectivity of BH3-only proteins to antiapoptotic proteins of the BCL-2 family. To explore the cause of selectivity, this study selects BAD and NOXA which only bind to BCL-xL and MCL-1 respectively. Based on the MD simulation and binding free energy decomposition, detailed interactions between proteins and peptides were probed and determinants that lead to the recognition of peptide and protein were proposed.

Chapter 5 provides a summary of all the studies on BCL-2 proteins that have been discussed in the thesis.

Chapter 2

Influence of BH3 peptide length in binding with antiapoptotic protein BCL-2

2.1 Introduction

BCL-2 family is a collection of proteins that regulate the mitochondrial pathway by manipulating the integrity of the OMM. The pathway, initiated by mitochondrial outer membrane permeabilization (MOMP), allows the downstream proapoptotic proteins to cross the mitochondrial membrane, diffuse into the cytosol and then trigger the apoptosis[4].

BCL-2 family members are distinguished as sharing at least one of four conserved BCL-2 homology (BH1-4) and consist of three distinct subclasses[106, 107]. One subclass is antiapoptotic proteins that composed of BCL-2, BCL-xL, BCL-w, MCL-1, A1 and BCL-B. These proteins possess BH1 to BH4 domains and promote cell survival by blocking the proapoptotic proteins[108]. The proapoptotic proteins are divided into two subclasses, namely the effector proteins and BH3-only proteins. BH3-only proteins such as BIM, BAD, PUMA and NOXA, sense the pro-death signal and convey the signal to the effector proteins through activation[15, 109]. The effector proteins, BAX and BAK, share sequence homology in BH1-4 regions. Upon death stimulus, BAX and

BAK will undergo oligomerization to form MAC pores within OMM and promote MOMP[9, 110, 111].

Although the interactions between these three subclasses have not been fully understood, it has been widely acknowledged that the coordination between antiapoptotic and proapoptotic proteins is through heterodimerization. Three dimensional structures showed that this heterodimerization was accomplished by binding the BH3 region of proapoptotic proteins to a hydrophobic cleft on antiapoptotic protein called BC groove[58, 112]. Antiapoptotic proteins are used to be thought of only having the ability to sequester BH3-only proteins and having no ability to regulate downstream effector proteins, BAX and BAK[109]. However, recent studies by mutation and other methods give strong evidence that antiapoptotic proteins are able to block the activity of effectors by engaging their BH3 domains[16, 113].

In normal cells, the interplay of antiapoptotic and proapoptotic proteins attains an equilibrium and deliver cell-death signal normally. However, in cancer cells, antiapoptotic proteins are overexpressed leading to the imbalance of these two subgroups of proteins. This event blocks the activation of BAX and BAK and inhibits apoptosis[32, 109]. Thus looking into how BH3 peptides from effector proteins bind to antiapoptotic proteins and developing BH3-mimics that can replace BAX or BAK may be a potential anticancer strategy.

BCL-2 protein is one of the antiapoptotic proteins and its three-dimensional structure has been determined[114]. Like other globular structures in this family, BCL-2 constitute of a bundle of α -helices with a BC groove on the surface. Overexpression of BCL-2 has been identified to be the main cause of follicular lymphoma and other types of tumour[53]. These BCL-2 related cancers are considered having little to do with inhibiting effector protein because early studies on BCL-2 indicated that it binds weakly with BAX or BAK[114, 115]. Nevertheless, recently released crystal structure of BCL-2 in binding with BH3 peptides of BAX strongly supports that BCL-2 may have ability to engage and inhibit BAX[116]. Isothermal titration calorimetry (ITC) also confirmed that BCL-2 can bind to BH3 peptides of BAX tightly, exhibiting the disassociation constant of 15.1 nM[116]. ITC study on this complex also supported the similarity in binding affinity of the 31-mer BAX·BH3 (residue 52-81) (K_D = 35.3 nM) and 36-mer BAX·BH3 (residue 49-84) to BCL-2, while the 26-mer peptide (residue 52-77) has a significantly poor binding affinity (K_D = 610 nM) with BCL-2. This data gives us a clue that the five residues (residue 78-82) at the C-terminus of the BAX peptide plays an important role in the binding of BAX to BCL-2[116]. (Figure 2.1) Although experiments have established this affinity difference, there is still no detailed information on how these five C-terminus residues contribute to the binding affinity and this may provide an insight on the future of BCL-2 targeted drug design.

In this study, MD simulations were performed on BCL-2 in binding with both longer BAX·BH3 peptide (26-mer) and truncated peptide (21-mer) without residue 78-82. Extracting data from the molecular dynamics trajectories, binding affinity calculations

on these two complexes using Sietraj program are in good agreement with experimental data. Alanine scanning was also utilised to compare binding scenario between the two complexes by mutating each residue on BH3 peptides into alanine. Some detailed information were obtained on how the C-terminus residues of BH3 peptides influenced binding to BCL-2.

Figure 2.1. The structure of BCL-2 in binding with BH3 peptides of BAX. Protein BCL-2 is shown in purple ribbon. Longer BAX·BH3 (with residue 78-82) is shown in yellow and shorter BAX·BH3 (without residue 78-82) is shown in pink. Below is the sequence of BAX·BH3.

2.2 Materials and Methods

2.2.1 Preparation of initial structures

The crystal structure of BCL-2 associating with BAX·BH3 was extracted directly from the Protein Data Bank (PDB)[117]. The PDB code is 2XA0[116]. The BAX·BH3 (residues 57-82) and truncated BAX·BH3 (residues 57-77) peptides were prepared from the crystal structure. However, due to a missing loop connecting the $\alpha 1$ and $\alpha 2$ helix in the crystal structure, the C-terminus of $\alpha 1$ and the N-terminus of $\alpha 2$ were capped manually with N-methylamide (NME) and acetylation (ACE) to mimic the backbone of the missing flexible loop. All water molecules are removed. Pre-equilibrated box of TIP3P water[118] were used to solvate all the protein-peptide complexes. The size of the water box was determined to have a minimal distance of 10 Å between the extremities of the water box and the atoms of the complex. Sodium (Na⁺) ions were added to neutralise the net negative charge of the system.

2.2.2 Molecular dynamics simulations

The AMBER03 force field[93] and periodic boundary conditions are employed and molecular dynamics simulations are performed at 300K. Long-range electrostatic interactions were calculated using particle mesh Ewald (PME)[119] and a 12 Å cutoff was used for the van der Waals interactions. The SHAKE algorithm[120] was employed to restrain all bonds involving hydrogen atoms. The minimization step was divided into two parts. In the first step, the water box was energy minimized for 4000 steps using the steepest decent method followed by 1000 steps of conjugate gradient. A restraint of 500

kcal/(mol·Å²) was placed on the protein complex. In the second step, the protein complex and solvent were both minimized for 10000 steps using the steepest decent method followed by conjugate gradient minimization until the energy gradient converges to 0.01 kcal/mol/Å. After minimization, the system was heated from 0K to 300K using Langevin thermostat[121] followed by the production run which lasted for a total of 10ns at constant pressure with a time step of 2fs.

2.2.3 Binding free energy using Sietraj

Sietraj[122, 123] program was used to calculate the binding free energy of BCL-2 to the BH3 peptides. This method was developed by Purisima's group to predict the binding free energy of inhibitors to proteins or proteins to proteins, which was an alternative to the MM_PBSA software. Different from MM_PBSA, Sietraj obtained the binding free energy by calibrating directly the solvation terms with scoring functions[122]. The solvated interaction energy (SIE) method was used to calculate the binding free energy using the following function:

$$\Delta G_{\text{binding}} = \alpha[E_{\text{Coul}}(D_{\text{in}}) + \Delta G_{\text{RF}} + E_{\text{vdw}} + \gamma \cdot \Delta \text{MSA}(\rho)] + C$$

E_{Coul} and E_{vdw} are the intermolecular Coulomb and van der Waals interaction energies in the bound state respectively. ΔG_{RF} represents the change in reaction field energy. The term $\gamma \cdot \Delta \text{MSA}$ represents the change of the molecular surface area upon binding. Four parameters which have been fitted to reproduce the absolute binding free energy of 99

protein-ligand complexes are used in this function. They are the Amber van der Waals radii linear scaling coefficient ($\rho = 1.1$), the solute interior dielectric constant ($D_{in} = 2.25$), the molecular surface area coefficient ($\gamma = 0.0129 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{\AA}^{-2}$), the global proportionality coefficient related to the loss of conformational entropy upon binding ($\alpha = 0.1048$) and a constant ($C = -2.89 \text{ kcal} \cdot \text{mol}^{-1}$). These optimised parameters have been adopted to successfully evaluate the binding affinity of some inhibitor-protein systems[124, 125]. For each simulation, snapshots were extracted from the last 5ns of each molecular dynamics simulation.

2.2.4 Alanine scanning calculation

Alanine scanning calculation is a way to analyze the contribution of side chains from each residue towards the binding free energy by mutating the residue into alanine[126]. In this research, Sietraj program was used to perform alanine scanning on each residue of BH3 peptides and their contributions to the binding free energy were analyzed by calculating the difference in binding free energy before and after mutation ($\Delta\Delta G$) which is given by:

$$\Delta\Delta G_{ala} = \Delta G_{before} - \Delta G_{after}$$

In addition, the polar and non-polar contribution of key residues were estimated to determine the main factor that led to the relatively high contribution.

2.2.5 Hydrogen bond analysis and salt-bridge analysis

Hydrogen bond (H-bond) and salt-bridge are two important factors that strengthen the binding affinity between protein and peptide. The *ptraj* module[127] in AMBER was used to monitor the H-bonds and salt-bridges between BCL-2 and BH3 domains of BAX. All the analyses were targeted on the last 10ns of the MD simulations performed. The criteria for H-bond was set such that the cutoff distance between acceptor and donor atom was 3.5Å and cutoff angle was 120° and only those with occupancies of higher than 60% were considered. Salt-bridges were identified using Visual Molecular Dynamics (VMD) and the stability was analyzed by using *ptraj*. The criteria for salt-bridge was set such that the distance of acceptor and donor atom was within a cutoff distance of 3.2Å.

2.2.6 Helical content analysis

Beside the length and amino acid sequence of the peptide, the stability of helix is another factor that affect the binding affinity of protein and peptide. Helical content is a method to evaluate the formation of α helix. In this analysis, only amino acids with dihedral angles in the range of $(\phi, \psi) = (-60 \pm 20, -40 \pm 20)$ were considered as helical.

2.3 Results and Discussion

2.3.1 MD simulation and binding free energy

A 10ns molecular dynamics simulation was conducted for BCL-2/BAX·BH3 (26-mer) and BCL-2/BAX·BH3 (21-mer). In order to monitor the structure transition of the complexes, root-mean-square deviation (RMSD) of the protein and peptide relative to the starting structure of BCL-2/BAX·BH3 were calculated. RMSD results were plotted against time as show in Figure 2.2. From Figure 2.2, the overall RMSD calculated for both complexes converge fast and equilibrate after 5 ns. So further analyses were conducted using data acquired during the last 5 ns of the simulation.

Figure 2.2. RMSD plotted against time for (A) BCL-2/BAX·BH3 (26-mer) and (B) BCL-2/BAX·BH3 (21-mer) Results for BCL-2 are shown in black and for BH3 peptides are shown in red.

Figure 2.3. Binding free energy of 26-mer BCL-2/BAX·BH3 (Black) and 21-mer BCL-2/BAX·BH3 (Red). (A) Total binding free energy represented as time function (B) Distribution of binding free energy.

To confirm the convergence and stability of the two complexes, total binding free energy was plotted against simulation time as shown in Figure 2.3 (A). As can be seen from the figure, the binding free energy for both complexes exhibited fast convergence and both complexes were more stable during the last 5 ns. From the distribution analysis of binding free energy over the total 10 ns simulation shown in Figure 2.3 (B), the 26-mer BAX·BH3 peptide obviously binds more tightly with BCL-2 than the truncated one which agrees well with experimental observation. Together with previous RMSD calculation, the trajectories extracted from the last 5 ns of the simulation were utilised to calculate the binding free energy using Sietraj for BCL-2 associated with both 26-mer BAX·BH3 peptide and truncated BAX·BH3 peptide. Previous experimental studies established that the missing C-terminus residues (R78-D82) of BAX·BH3 peptide will cause a significant decrease in binding affinity from -10.69 kcal/mol to -8.50 kcal/mol, as determined by K_D (35.3 nM and 610 nM). The Sietraj calculations of our simulation

for BCL-2/BAX·BH3 (26-mer) and BCL-2/BAX·BH3 (21-mer) are -18.35 kcal/mol and -16.35 kcal/mol respectively. As expected, binding free energy (ΔG) of truncated peptide was higher than the untruncated one which means 26-mer BAX·BH3 binds more tightly to BCL-2 than the 21-mer BAX·BH3. Although Sietraj slightly overestimated the absolute binding free energies, parallel to the experimental values, the ΔG of 26-mer BAX·BH3 for BCL-2 was approximately 2 kcal/mol lower than 21-mer BAX·BH3. Since the binding free energy result obtained using Sietraj reached a good agreement with experimental data, the last 5 ns of our simulations were utilised to explore details of how the five truncated C-terminus residues influence the performance of BAX·BH3 peptides.

2.3.2 Residues in charge of binding differences

To better understanding the binding difference, the contribution of each residue of the BAX·BH3 peptides to the binding affinity needs to be found. To this end, alanine scanning was performed for 26-mer BAX·BH3 and 21-mer BAX·BH3 and $\Delta\Delta G$ (kcal/mol) was utilised to indicate the contribution of each residue. Lower $\Delta\Delta G$ means higher contribution. The results for both peptides were aligned in Figure 2.4. To better present the results, amino acids are represented in their one lettered code for peptides and in three lettered code for BCL-2. From the calculation of 26-mer BAX·BH3 presented in Figure 2.4, there were nine residues (L59, L63, R64, I66, D68, E69, L70, M74, R78) with relatively high contribution to binding affinity with BCL-2. To identify their contribution, the polar and non-polar contributions of these nine residues before and

after mutation to alanine were compared.(Table 2.1) Through the comparison of the data, five residues namely L59, L63, I66, L70 and M74 were identified to contribute significantly to the binding affinity through non-polar interactions. This result meets with a common view that residues at these five positions are critical for interaction with antiapoptotic proteins in the BCL-2 family. On the other hand, mutating R64, D68, E69 and R78 into alanine will cause the reduction of polar contribution to the binding affinity which means these four residues are engaged in electrostatic interactions. Interestingly, there is no more than three ionic interactions in previously obtained structures of other antiapoptotic BCL-2 proteins in complex with BH3 peptides. So the high involvement of electrostatic interactions could be a characteristic feature in the binding of BAX·BH3 peptide to BCL-2.

Table 2.1. Binding free energy for w.t BAX·BH3 and mutant BAX·BH3. Each term contribute to binding free energy is listed, including intermolecular van der Waals energy, Coulomb energy, reaction field energy and non-electrostatic solvation free energy (kcal/mol).

	ΔG	E_{vdW}	E_{Coul}	ΔG_{RF}	ΔG_{npsol}	Polar	Non-Polar
w.t.	-18.35	-124.54	-86.82	85.96	-21.81	-0.86	-146.35
L59A	-17.51	-117.72	-86.78	86.30	-21.37	-0.48	-139.09
L63A	-17.51	-117.9	-86.81	86.48	-21.36	-0.33	-139.26
R64A	-17.13	-119.16	-49.05	53.44	-21.26	4.39	-139.55
I66A	-17.52	-118.13	-86.28	86.23	-21.42	-0.05	-139.55

	ΔG	E_{vdW}	E_{Coul}	ΔG_{RF}	ΔG_{npsol}	Polar	Non-Polar
D68A	-17.90	-124.83	-92.52	95.75	-21.68	3.23	-146.51
E69A	-17.24	-126.02	-69.55	80.04	-21.45	10.49	-147.47
L70A	-17.52	-117.13	-86.92	86.28	-21.33	-0.64	-139.06
M74A	-17.61	-118.65	-87.05	86.72	-21.54	-0.33	-140.19
R78A	-17.52	-120.68	-34.68	36.81	-21.08	2.13	-141.76

Alanine scanning results of 21-mer BAX·BH3 and 26-mer BAX·BH3 were aligned as shown in Figure 2.4. According to the alanine scanning results of 26-mer BAX·BH3, it is obvious to see among the five truncated residues, only R78 has a direct interaction with BCL-2 while the remaining four residues have insignificant contribution to the binding affinity. Electrostatic interactions are reduced when R78 is mutate to alanine. To get a deeper understanding of these interactions, salt-bridge and H-bond analyses were carried out. Through the analyses performed, R78 was identified to form H-bond with Glu200 of BCL-2 and the distance of the donor and receptor against simulation time was measured to elucidate the stability of this H-bond.(Figure 2.5B) The H-bond analysis showed that the distance between donor from R78 and receptor from Glu200 was stabilised around 2.5 Å most of the time. This suggest that this stabilising interaction could increase the affinity of the BH3 peptide to BCL-2.

Figure 2.4. Alignment of alanine scanning results of 26-mer and 21-mer BAX·BH3.

Although R78 has been identified as one of the reason why the five truncated residues have a significant influence on the performance of BAX·BH3 peptide in engaging with BCL-2, there are two charged residues namely R64 and D68 which were far from the C-terminus (Figure 2.5A) hence reducing their ability to bind with BCL-2 after the C-terminus were truncated. Similarly, from the binding free energy terms listed in Table 2.1, the polar terms of R64 and D68 decreased a lot after mutation. Following this, H-bond and salt-bridge analyses were conducted on these two residues. Through hydrogen bond scanning, one H-bond was found between R64 and Glu136 and the distance of donor and receptor was analysed. According to Figure 2.5C, the H-bond was strong and stable between R64 of 26-mer peptide and Glu136 of BCL-2 while in BCL-2/BAX·BH3(21-mer) complex, the H-bond was destabilised. Occupancy of this H-bond also dropped from 76.96% to 13.61% after R78 to D82 were truncated. Turning to D68

which is a highly conserved residue in BH3 domain, after looking at the structure using VMD, a salt-bridge was found between D68 and Arg146 of BCL-2. According to the distance analysis shown in Figure 2.5D, the salt-bridge in BCL-2/BAX·BH3(26-mer) complex was stable at the cutoff distance of 3.2Å. However, the same salt-bridge in BCL-2/BAX·BH3(21-mer) complex breaks after 3 ns indicating the importance of the five truncated residues in maintaining the stability of this highly conserved salt-bridge. These observations give a clue that the five C-terminus residues increased the contribution of R64 and D68 indirectly by stabilising their ionic interactions with BCL-2. Electrostatic interactions contribute to binding of ligands to proteins in various ways, i.e. recognising the protein and enhancing the binding. The high involvement of charged residues of BAX·BH3 together with the binding difference induced by charged residues once proves that electrostatic interactions are critical for tight binding of BH3 peptides to BCL-2.

From the comparison between alanine scanning results of BAX·BH3(26-mer) and of truncated BAX·BH3, the binding affinity reduction caused through truncation was mainly due to three polar residues namely R78, R64 and D68. Residue R78 has been identified to directly interact with BCL-2. Why interactions between R64, D68 and BCL-2 are affected and what is the role of the other four truncated residues remain unclear.

Figure 2.5. (A) Three residues of BAX·BH3 in charge of binding difference (B) Hydrogen bond stability of R78 and Glu200 (C) Hydrogen bond stability of R64 and Glu136 (D) Salt-bridge stability of D68 and Arg146

2.3.3 Binding affinity influenced by helical content

BAX·BH3 is an α helix and the helix stability highly impact the binding ability to proteins, it is possible that the four truncated residues of BAX·BH3 which have no direct interaction with BCL-2 can functionally maintain the helix integrity. To support this, helical content analysis was conducted for the common parts (residue 57 to 77) of two BAX·BH3 peptides. From the average helical content data, the missing five C-

terminus residues will cause the helical content to decrease from 92.63% to 79.89%.

Helical content was plotted against time as illustrated in Figure 2.5. From the change in helical content along time, besides the reduction of the helical content, the truncated peptide become less stable during the simulation. This result confirms that besides the direct contribution of R78, the other four residues contribute to the binding affinity by maintaining helix stability. The fluctuation of helix stability can further disrupt electrostatic interactions which normally lies on the surface of complexes.

Figure 2.5. Helical content of the common part of 26-mer BAX·BH3 (Black) and 21-mer BAX·BH3 (Red)

2.4 Conclusion

In this project, the influence of peptide length in the binding of BAX·BH3 peptides to BCL-2 was explored using MD simulations. Sietraj program was used to calculate the binding free energy for both BAX·BH3(26-mer) and BAX·BH3(21-mer). The binding free energy well interpret the experimental data that 26-mer BAX·BH3 peptide exhibited 2 kcal/mol better binding to a BCL-2 protein than 21-mer BAX·BH3 peptide based on their dissociation constant values. Reasons why these five C-terminus residues have such a significant impact on binding were also revealed through alanine scanning analysis. Alanine scanning is a popular and efficient way to understand energetic characteristics of a single residue. But it should be noted that alanine scanning sometimes fail when the non-polar sidechain is buried into binding pocket. However, in our simulation, conserved hydrophobic residues like L63 and I66 showed significant contribution to binding and critical residues we found are same with previous experimental results. The absolute value of alanine scanning may not exactly same with experimental data. The difference is acceptable as the main purpose of performing alanine scanning in this study was to do parallel comparison of peptide BAX·BH3 with different length.

Among the five residues that were absent in the 21-mer peptide, residues 78 on the 26-mer peptide was identified to contribute to the binding free energy through direct electrostatic interaction with protein BCL-2. Residues 79, 80, 81 and 82, although have no direct interaction with protein, contributed to the binding by enhancing the helical stability of the 26-mer peptide. This enhancement was essential in maintaining the electrostatic interactions between BH3 peptide of BAX and the antiapoptotic protein

BCL-2 which in this case improved the stability of ionic interactions between R64/D68 and BCL-2. In the future drug design targeting on BCL-2, especially in the design of peptide therapeutics, the terminus residues should be taken into consideration seriously since they may impact the interactions that far from the terminus.

Chapter 3

Antiapoptotic protein BCL-2 in binding with BH3 peptides of proapoptotic proteins

3.1 Introduction

BCL-2 family plays a critical role in regulating the permeabilisation of OMM which is a critical process in mitochondrial controlled apoptosis[107, 128, 129]. To date, researchers have identified at least 16 family members by distinguishing the BCL-2 homology domains[74]. Compromising proapoptotic and antiapoptotic proteins, the BCL-2 family can be functionally categorised into three groups: antiapoptotic proteins, effector proteins and BH3-only proteins[106, 129]. Antiapoptotic proteins including BCL-2 itself, contain four BH domains and carry out their prosurvival role by inhibiting the activity of proapoptotic proteins including effector proteins and BH3-only proteins. Effector protein members such as BAX and BAK also share BH1-4 domains with other family members and are the key regulator of the BCL-2 regulated apoptosis due to its direct role in permeabilising OMM. BH3-only proteins, although only demonstrate BH3 domain, deliver cell death signal to the other two subgroups of the BCL-2 family. The programmed interactions between these three subfamilies ensure the standard cell death.

On the contrary, the imbalance between them, especially the overexpression of antiapoptotic BCL-2 proteins, will cause various kinds of diseases[130].

Structural studies on BCL-2 family revealed that through heterodimerization antiapoptotic protein and proapoptotic protein modulate their inverse functions[2, 131]. This is corroborated by three-dimensional structure determined by NMR or X-ray crystallography including BCL-xL associated with BIM·BH3[112], BCL-xL with BAD·BH3[132], BCL-xL with BAK·BH3[59] and BCL-2 with BAX·BH3[116]. In these complexes, BC grooves of antiapoptotic proteins which are hydrophobic in nature, interact with the hydrophobic face of the amphipathic α helix formed by BH3 region in proteins that are either effector proteins or BH3-only proteins. Regardless of the fact that the overall heterodimerization mechanism for different proteins is similar, the structural and sequence differences of both receptor proteins and donor proteins will lead to different binding affinity. Mutational studies performed at the BC groove of antiapoptotic proteins have shown that some residues are critical to their binding to proapoptotic proteins and mutation of these crucial residues will cause a reduction in apoptosis inhibition[59, 133, 134]. Similarly, mutations in the BH3 region of proapoptotic proteins, normally on BH3 peptides, can also abolish their binding ability to antiapoptotic proteins and increase cell death ratio[59, 134-136]. With this binding mechanism, small molecules mimicking the BH3 domain of proapoptotic protein could be designed to occupy the BC groove of antiapoptotic proteins so that proapoptotic proteins could be released to induce apoptosis. This scenario has been used in therapy towards cancers induced by overexpression of antiapoptotic proteins. One important

factor when designing BH3 mimics of either short peptides or small molecular moiety is the binding specificity of BH3 peptides to antiapoptotic proteins which decides the templates we use in diverse anticancer strategy. Small molecule ABT-263, which can be used as a single agent or in combination with other anticancer drugs, mainly target on antiapoptotic BCL-2, BCL-xL, and BCL-w which is similar to BAD·BH3[69].

BCL-2 antiapoptotic members have been identified distributed in different tissues. Thus, antiapoptotic members are responsible for different cancer. Take two leading members in antiapoptotic proteins as an example, the expression of BCL-2 is aberrant in follicular lymphoma, leukaemia, breast and lung cancers while the over-expression of BCL-xL is mainly observed in liver cancer, myeloid and T-cell leukaemia[25, 40, 137, 138]. The difference in tissue distribution offers a hint to design small molecules targeting a particular pro-survival member. This scenario is supported by experimental data that showed BH3 peptides from different proapoptotic proteins having significant difference in binding affinities with various pro-survival proteins[15]. In order to search the binding specificity of antiapoptotic proteins to proapoptotic members at the molecular level, some theoretical studies are done around BCL-xL with specific BH3 peptides[139-141]. However, there is limited research on molecular information of the protein BCL-2.

Binding affinity studies on proapoptotic BH3 peptides exhibit a broad range of affinities to the antiapoptotic protein BCL-2. Peptides with dissimilar sequences also interact differently with BCL-2/BIM·BH3, BAX·BH3, BIK·BH3 and NOXA·BH3 binding to

BCL-2 with a binding affinity of 2.44nM, 15.1nM, 234nM and 1620nM respectively. Although experimental research has achieved remarkable advances in identifying the different performance of BH3 peptides, there is still no BCL-2 specified study at the molecular level. In this study, molecular dynamics simulations of BCL-2 in complex with four types of 26-mer peptides namely BIM·BH3, BAX·BH3, BIK·BH3 and NOXA·BH3 were performed to reveal the sequence-dependent determinants of the binding affinity between BCL-2 and BH3 peptides. Table 3.1 shows the sequence alignment of these four BH3 peptides. At the same time, through binding free energy decomposition of BCL-2-BH3 complexes, hotspots on BCL-2 were determined. All these information will give some clues in the future design of “BCL-2 specific drugs”.

Table 3.1. The peptide sequences of BIM, BAX, BIK, NOXA and an in-silico designed peptide. The sequences are numbered form 1 to 26

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
BIM(144-169)	I	R	I	A	Q	E	L	R	R	I	G	D	E	F	N	E	T	Y	T	R	R	V	F	A	N	D
BAX(57-82)	K	K	L	S	E	C	L	R	R	I	G	D	E	L	D	S	N	M	E	L	Q	R	M	I	A	D
BIK(49-74)	N	Q	V	A	L	R	L	A	C	I	G	D	E	M	D	L	C	L	R	S	P	R	L	V	Q	L
NOXA(21-46)	P	E	F	A	A	Q	L	R	K	I	G	D	K	V	Y	C	T	W	S	A	P	D	I	T	V	V
NEWPEP	I	R	L	S	Q	R	L	R	R	I	G	D	E	F	N	L	T	Y	R	L	R	R	M	I	A	D

3.2 Methodology

3.2.1 Preparation of initial structures

Among the four complexes, only the structure of BCL-2 complexed with BAX·BH3 is available in the Protein Data Bank (PDB)[117]. The PDB code is 2XA0[116]. Since no structural information of BCL-2 complexed to BIM·BH3, BIK·BH3 and NOXA·BH3 are available, and their binding mechanism are similar to BAX·BH3, BCL-2/BIK·BH3 and BCL-2/NOXA·BH3 complexes are prepared by mutating the residues in BAX·BH3 to amino acid sequences of BIK·BH3 and NOXA·BH3. Module Prime in Schrodinger was used to build the structure of BCL-2/BIM·BH3 complex. The C-terminus of $\alpha 1$ and the N-terminus of the $\alpha 2$ are capped with an NME and ACE respectively for all four complexes, to mimic the backbone of the missing flexible loop.

With Leap module in Amber10 package, AMBER03 force field[93] was applied to BCL-2 and BH3 peptides. All water molecules in the original crystal structure were removed. All protein-peptide complexes were solvated in a pre-equilibrated TIP3P water box[118], and the size of the box is chosen in a way that the minimal distance between the atoms of the complex and the extremities of the water box is at least 10 Å. Sodium (Na⁺) ions are added to neutralise the net negative charge of the system.

3.2.2 Molecular dynamics simulations

Energy minimisation were conducted to remove bad steric contacts within solvents or protein molecules. The minimisation phase was divided into two steps. Firstly, a restraint of 500 kcal/(mol·Å²) was applied to the protein-peptide complex, and the solvent molecules were minimized for 4000 steps using the steepest decent method and for 1000 steps of conjugate gradient. In the second step, without any restraint, both protein complex and solvent were minimized for 10000 steps using the steepest decent method followed by conjugate gradient minimization until the RMS of energy gradient reaches to 0.01 kcal/mol/Å. After minimization, the system was heated from 0K to 300K using Langevin thermostat[121] followed by the production run which lasted for a total of 10ns at constant pressure with a time step of 2fs. In the MD simulation, the particle mesh Ewald (PME)[119] method was employed to calculate the long-range electrostatic interactions, and a 12 Å cutoff was used for the van der Waals interactions. The SHAKE algorithm[120] was used to restrain all bonds involving hydrogen atoms.

It has been suggested that multiple short simulations can produce better quantitative results than a single long simulation due to the difference in conformational space sampling[142]. To get more precise quantitative results, we run three 10 ns simulations for each complex system with different initial velocities and the trajectories are used in free binding energy calculation and alanine scanning calculation.

3.2.3 Binding free energy using Sietraj

Similar to previous chapter, the total binding free energies of four protein-peptide complexes were evaluate using Sietraj program[122, 123]. The solvated interaction energy (SIE) method is calculated as follows:

$$\Delta G_{\text{binding}} = \alpha(E_{\text{Coul}} + \Delta G_{\text{RF}} + E_{\text{vdw}} + \Delta G_{\text{npol}}) + \text{constant}$$

E_{Coul} and E_{vdw} are the intermolecular Coulomb and van der Waals interaction energies in the bound state respectively. ΔG_{RF} and ΔG_{npol} represent the change in reaction field energy and non-electrostatic solvation free energy between bound and free states respectively. The sum of E_{Coul} and ΔG_{RF} gives the electrostatic contribution to the binding while E_{vdw} and ΔG_{npol} can be grouped as the nonpolar contributor to the binding free energy. α (0.104758) is an empirically determined factor to evaluate the enthalpy-entropy compensation roughly. Constant equals to -2.89 kcal/mol. For each trajectory, every snapshots from the last 5 ns of the simulation were extracted for calculation.

3.2.4 Alanine scanning calculation

To determine the contribution of each residue of the BH3 peptides, alanine scanning calculation is carried out. By mutating the residue into alanine and calculating the binding free energy, contribution of the residue was determined by comparing the binding affinity of the mutated BH3 peptide with the binding affinity of the wild type peptide[126] which follows the equation below:

$$\Delta\Delta G_{\text{ala}} = \Delta G_{\text{before}} - \Delta G_{\text{after}}$$

ΔG_{before} is the binding free energy of wild type peptide and ΔG_{after} represents the binding free energy of peptide after mutation. In this study, alanine scanning was performed using the Sietraj program.

3.2.5 Free energy decomposition

Hot spots of BCL-2 were analysed through free energy decomposition using the MM_PBSA decomposition[143] in the MM_PBSA module. PB approach calculates electrostatic contribution to solvation energy and nonpolar contribution to the free energy of solvation was calculated by LCPO[144]. Each calculation used the snapshots extracted from the last 5 ns of the simulations performed with an interval of 10 steps.

3.2.6 Analysis of molecular dynamics simulations

Hydrogen bond (H-bond) and salt-bridge are two important factors that strengthen the binding affinity between protein and peptide. The *ptraj* module[127] in AMBER was used to monitor the H-bonds and salt-bridges between BCL-2 and BH3 domains of BAX. All the analyses were targeted on the last 10 ns of the MD simulation. The criteria for H-bond was set such that the cutoff distance between acceptor and donor atom was 3.5Å and the cutoff angle was 120° and only those with occupancies of higher than 60%

were considered. Salt-bridges are monitored with Visual Molecular Dynamics (VMD), and the stability was analysed using *ptraj*. The criteria for salt-bridge was set such that the distance between acceptor and donor atom was within a cutoff distance of 3.2Å.

Besides the length and amino acid sequence of the peptide, the stability of helix is another factor that impacts the binding affinity of protein and peptide. Helical content is a method to evaluate the formation of α helix. In this analysis, only amino acids with dihedral angles in the range of $(\phi, \psi) = (-60 \pm 20, -40 \pm 20)$ were considered as helical.

3.3 Results and Discussion

3.3.1 Binding free energy

Since a single long simulation may not be enough to get correct quantitative results with a limitation of sampling of conformational space, three short molecular dynamics simulations (10 ns) were carried out for each complex. Similar to other antiapoptotic BCL-2 proteins, BCL-2 has a broad range of binding affinity with BH3 peptides from different proapoptotic proteins. According to experimental data, binding free energies for BIM·BH3, BAX·BH3, BIK·BH3 and NOXA·BH3, converting from dissociation constant value, are -11.78 kcal/mol, -10.69 kcal/mol, -9.07 kcal/mol and -7.92 kcal/mol respectively[116]. To confirm our simulations, binding free energies for these complexes are calculated using Sietraj and compared with experimental data. (Table 3.2) According to the Sietraj calculation done from the molecular dynamics simulation

trajectories, the binding free energy obtained shows a similar trend to the experimental data from previous work. BIM·BH3 has the strongest association to BCL-2 with a binding free energy of -18.73 kcal/mol. Following BIM·BH3, BAX·BH3 has an average binding free energy of -18.26 kcal/mol which is ~0.5 kcal/mol higher than BIM·BH3. Agreeing with experimental data, BIK·BH3 (-16.34 kcal/mol) and NOXA·BH3 (-15.45 kcal/mol) have relatively high binding free energy. This trend was also illustrated in Figure 3.1 which indicated that data from Sietraj agrees well with experimental results and this help us confirm that multiple short simulations were suitable for our system.

Table 3.2. List of binding free energy from literature and calculated by Sietraj for each MD trajectory (kcal/mol)

	ΔG_{Lit}	AVE	MD1	MD2	MD3
BCL-2/BIM·BH3	-11.78	-18.73	-18.64	-19.13	-18.42
BCL-2/BAX·BH3	-10.69	-18.26	-18.35	-18.27	-18.15
BCL-2/BIK·BH3	-9.07	-16.34	-16.37	-16.20	-16.46
BCL-2/NOXA·BH3	-7.92	-15.45	-14.53	-15.98	-15.84

To get into the reason why these four peptides show a discernible binding difference to BCL-2, Table 4 lists the average binding free energy components corresponding to internal energies (E_{vdw} and E_{Coul}) and solvation energies (ΔG_{RF} and ΔG_{npsol}). The polar

contribution and nonpolar contribution were also determined by summing E_{Coul} and ΔG_{RF} , E_{vdw} and ΔG_{npsol} respectively. The binding free energy component distribution proposed that the nonpolar contributions of four complexes all positively contribute to binding while polar contribution varies. The nonpolar contribution has no big difference for complex BCL-2/BIM·BH3, BCL-2/BAX·BH3 and BCL-2/NOXA·BH3 while BCL-2/BIK·BH3 is ~ 10 kcal/mol higher than the other three complexes. The cause of the altered binding of the BH3 peptides to BCL-2 mainly lies on the E_{Coul} and G_{RF} terms. The total electrostatic contribution was favourable for BCL-2/BIM·BH3 and BCL-2/BAX·BH3, but for BCL-2/BIK·BH3 and BCL-2/NOXA·BH3, electrostatic interactions contribute adversely on the binding affinity. Therefore, it is possible to infer that electrostatic interactions play an major role in the binding of BH3 peptides to BCL-2.

Figure 3.1. Binding free energy from experimental data (shown in blue line) and Sietraj calculation (shown in green line) for four complexes

Table 3.3. Average binding free energy components of each complex (kcal/mol)

	E_{vdw}	E_{Coul}	ΔG_{RF}	ΔG_{npsol}	ΔG_{np}	ΔG_{polar}
BCL-2/ BIM·BH3	-121.81	-119.47	113.10	-22.64	-144.45	-6.37
BCL-2/ BAX·BH3	-122.88	-99.94	96.91	-21.90	-144.78	-3.03
BCL-2/ BIK·BH3	-111.16	-98.34	101.18	-20.09	-131.25	2.84
BCL-2/ NOXA·BH3	-119.55	-20.8	35.46	-20.22	-139.77	14.66

3.3.2 Conserved hydrophobic residues

To better distinguish how amino acid sequence affects the binding affinity, alanine scanning for each residue was done with Sietraj for the four peptides, that is BIM·BH3, BAX·BH3, BIK·BH3 and NOXA·BH3 (Figure 3.2). By mutating each residue to alanine, the contribution of the residue ($\Delta\Delta G_{ala}$) can be seen by comparing the binding free energy before and after mutation. As the value of $\Delta\Delta G_{ala}$ decreases, the residue contributes more strongly to the binding between its peptide and BCL-2. Here, residues with alanine scanning result lower than -0.5 kcal/mol are considered having a high

contribution to the binding affinity. To illustrate the sequence concisely, residues of peptides are numbered from 1 to 26 and are referred to as P1 to P26.

Figure 3.2. Alanine scanning results for four BH3 peptides. Critical hydrophobic residues are highlighted with lines and critical hydrophilic residues are highlighted with dot lines.

As previous described, residue P3, P7, P10, P14 and P18 have been identified as five generally agreed BH3 residues that are critical for hydrophobic interactions with antiapoptotic proteins (Figure 3.3)[59, 112, 145]. From the alanine scanning results in this study, $\Delta\Delta G_{\text{ala}}$ at these five positions show relatively low values indicating that

residues at these five positions contribute significantly to binding regardless of the type of peptide involved. This result further addresses the importance of these five hydrophobic residues. Among these residues, P7 and P10 are highly conserved hydrophobic residues and are present as leucine (Leu) at P7 and isoleucine (Ile) at P10 in all four BH3 peptides. Another common characteristic of these two residues is that structurally they are both buried in the hydrophobic cleft of protein BCL-2 (Figure 3.3). Alanine scanning results for Leu at P7 in different peptides give similar contributions ranging from -0.71 kcal/mol in NOXA·BH3 to -0.85 kcal/mol in BIK·BH3. Similarly, highly conserved isoleucine at P10 contribute to the binding affinity by -0.75 kcal/mol in BIM·BH3 and NOXA·BH3, -0.83 kcal/mol in BAX·BH3 and -0.61 kcal/mol in BIK·BH3. This indicates that conservation of the amino acids at P7 and P10 significantly contribute to the stabilisation of the complex with small variation in discrete peptides. This result successfully meet our expectation that highly conserved residues which are also buried into the hydrophobic cleft of BCL-2 barely affect the performance of BH3 peptides.

Different from P7 and P10, residues at P3 represent entirely different amino acids in various BH3 peptides. Alanine scanning results showed that at this position, phenylalanine (Phe) from NOXA·BH3 has an over 1 kcal/mol contribution, isoleucine from BIM·BH3 and Leu from BAX·BH3 have a ~0.8 kcal/mol contribution to the binding affinity while valine (Val) from BIK·BH3 only contribute 0.42 kcal/mol to binding. Comparing the side chain of these four residues, Val which contributes less in the alanine scanning has a smaller side chain and Phe with its aromatic side chain has

the highest contribution. This obvious difference indicates that residues with larger hydrophobic side chain, especially aromatic residues, improves binding affinity at this site.

Figure 3.3. Five conserved hydrophobic residues of BH3 peptides in complex BCL-2/BAX·BH3. Basic residues of BCL-2 are shown in iceblue, acidic residues are shown in pink, polar residues are in grey, and nonpolar residues of BCL-2 are in white surface.

Similar observation was observed at P14. Amino acids at P14 in the four complexes showed distinct contributions to binding. Alanine scanning result showed that Phe, Leu and Met are more favourable for binding while Val contributes little to the binding. Among Phe, Leu and Met, Phe shows a relatively high contribution (1.06 kcal/mol) while the contribution of Leu and Met are lower (0.83 kcal/mol and 0.82 kcal/mol

respectively). The greater contribution exhibited by Phe may be attributed to its aromatic sidechain which could form π - π stacking with Phe104 in BCL-2 (Figure 3.4A). In comparison to Phe, sidechains of Leu, Met and Val are smaller hence giving us a clue that the presence of bigger hydrophobic sidechain at P14 will be more favourable for binding.

Figure 3.4. (A) Residue located at F14 of BIM·BH3 interacts with Phe104 of BCL-2. (B) π - π stacking between Y18 from BIM·BH3 and Tyr202 from BCL-2. (C) π - π stacking between W18 from NOXA·BH3 and Tyr202 from BCL-2. Residues on BH3 peptides are shown in Licorice and on BCL-2 protein are shown in CPK

From the peptide sequence alignment, amino acids located at P18 are Tyr, Met, Leu and Trp in BIM·BH3, BAX·BH3, BIK·BH3 and NOXA·BH3 respectively. All these amino acids contribute highly to the binding of BH3 peptides to BCL-2 by 1.25 kcal/mol, 0.74 kcal/mol, 0.73 kcal/mol and 1.21 kcal/mol for Tyr, Met, Leu and Trp respectively.

Although all four distinct types of amino acids give an over 0.5 kcal/mol contribution to stabilise the complex, aromatic Tyr and Trp contributed 0.5 kcal/mol more than others due to the presence of aromatic side chains to form π - π stacking with residue Tyr202 in BCL-2 (Figure 3.4BC). This observation agreed with previous molecular study on peptide BIM·BH3 whereby binding of this peptide with different proteins[141] showed favourable π - π stacking between the aromatic ring of P8 and Tyr202.

From all the information we receive from these five conserved hydrophobic residues, with the same residue type, P7 and P10 from different BH3 peptides gave similar contribution. The critical role of P7 in the binding of BH3 peptides to BCL-2 proteins have been recognised by previous experimental studies. Mutation of leucine at this location will cause an interruption of the association of peptide and protein[59, 60, 146]. Various kinds of hydrophobic amino acids are favourable at P3 and P14. However, those with bigger sidechains have better performance in stabilising the complexes. Phenylalanine at both P3 and P14 showed slightly more advantage compared to other amino acids. For P18, amino acids with aromatic ring have higher positive impact on the binding affinity which further affirms that the hydrophobic pockets corresponding to P18 on BCL-2 are more favourable to aromatic amino acids.

Taken together, residues at these positions may be the determinants of peptide selectivity to BCL-2. One thing needs to be noted is that, although a relatively large hydrophobic sidechain is favourable at P3 and P14, steric effect should still be taken into consideration since these residues need to penetrate into the binding pocket in the process of association.

3.3.3 Influence of hydrophilic residues

Previous peptide length study on BCL-2/BAX·BH3 elucidates that P8, P12, P13 and P22 of BAX·BH3 were engaged in electrostatic interactions. Mutations on these residues will lead to varying degrees of decrease in the affinity of BAX·BH3. As can be seen in Figure 3.2, alanine scanning results for residues at these four positions of different peptides contribute differently to binding with BCL-2. Also, at position P6, charged residues have a significant difference in contribution.

Residues located at P6 in BIM·BH3 and BAX·BH3 showed no more than 0.5 kcal/mol contribution while in BIK·BH3, the residue at P6 contributed more than 2 kcal/mol. Looking into the binding free energy components, the internal electrostatic terms of Arg as shown in BIK·BH3 increased from -104.41 kcal/mol to -43.95 kcal/mol after mutating into alanine indicating that internal electrostatic interactions between Arg and BCL-2 play an important role (Table 3.4). What is more, the total polar contribution was also more unfavourable after mutation. The analysis showed that this significant difference was due to the formation of hydrogen bonds between the sidechain of

arginine and residues located at $\alpha 3$ namely backbone oxygen of Tyr108, Ala113 and OD1 of Asp111, OE1 of Gln118.(Figure 3.5A) These H-bonds enhanced the binding between BIK·BH3 and BCL-2.

Table 3.4. Energy components of wild-type and mutant peptides.

		w.t.	Ala-06	Ala-08
BIM	E_{vdW}	-127.35	-125.49	-112.47
	E_{Coul}	-112.68	-140.24	-71.97
	ΔG_{RF}	108.97	136.43	73.11
	ΔG_{npsol}	-23.96	-23.77	-22.98
BAX	E_{vdW}	-124.94	-123.95	-119.16
	E_{Coul}	-86.83	-85.56	-49.05
	ΔG_{RF}	85.97	85.81	53.44
	ΔG_{npsol}	-21.81	-21.82	-21.16
BIK	E_{vdW}	-111.57	-104.92	-
	E_{Coul}	-104.41	-43.95	-
	ΔG_{RF}	107.10	57.88	-
	ΔG_{npsol}	-19.83	-18.63	-
NOXA	E_{vdW}	-115.29	-112.40	-110.92
	E_{Coul}	-28.94	-22.27	15.05
	ΔG_{RF}	38.84	35.28	-2.66
	ΔG_{npsol}	-19.60	-19.34	-18.78

Figure 3.5. (A) The H-bond between P6 and BCL-2 in complex BCL-2/BIK·BH3 (B) Salt-bridge between R8 and Asp140 in complex BCL-2/BIM·BH3(C) The H-bond between R8 and Glu136 in complex BCL-2/BAX·BH3

P8 represents as alanine in BIK·BH3 and arginine in the other three peptides. However, the contribution of arginine in these three peptides is different, and the reason was explored. Results show that the way Arg contribute to the binding can be divided into two cases. In complex BCL-2/BIM·BH3 and BCL-2/NOXA·BH3, there exists a salt-bridge between positively charged atom NH2 of P8 and negatively charged atom OD2 of Asp140 (Figure 3.5B). Instead of a salt-bridge, a strong hydrogen bond was formed

between P8 and the backbone oxygen of Glu136 in complex BCL-2/BAX·BH3 (Figure 3.5C). Based on these two cases, a positive charged residue will be favourable for binding at this position. Mutating Arg in each complex also have also affects nonpolar energies to a certain extent as shown in Table 3.4.

Figure 3.6. (A) The distance between NH2 of P12 and OD2 of Arg146 in four complexes. (B) Distance analysis of OE2 of P13 and NH2 of Arg110 (C) NH1 of P13 and OE1 of Arg107. (D) Distance of NH2 of P22 and OE2 of Glu200 in complex BCL-2/BAX·BH3 and BCL-2/BIK·BH3

P12 is a highly conserved negative-charged residue. Alanine scanning result showed that P12 from BIM·BH3 has the highest affinity contribution, followed by BAX·BH3 and BIK·BH3. As previously identified in peptide length study, there is a strong salt-bridge between P12 and Arg146 in complex BCL-2/BAX·BH3. To determine if this salt-bridge affects the performance of the residue, distances of this salt-bridge for all four complexes are calculated. (Figure 3.6A) As expected, the salt bridge is stable in BCL-2/BIM·BH3 and BCL-2/BAX·BH3. However, in BCL-2/BIK·BH3, the salt bridge exhibited more fluctuation and instability which explain why P12 in this complex contribute the least to binding affinity. Meeting with the negative contribution, this salt-bridge was fully disrupted in the simulation of BCL-2/NOXA·BH3.

P13 represents as negative-charged residue Glu in peptide BIM·BH3, BAX·BH3 and BIK·BH3 but a positive-charged Lys in NOXA·BH3. According to alanine scanning result, P13 has an over 1 kcal/mol contribution in complex BCL-2/BIM·BH3 and BCL-2/BAX·BH3 and negative contribution to BCL-2/NOXA·BH3. This significant difference offers a hint that a positively charged residue will be more favourable to binding. A salt-bridge analysis was carried out and the result points towards the presence of a salt-bridge between P13 and Arg110. Also, a strong hydrogen bond between P13 and Arg107 was observed in BCL-2/BIM·BH3 and BCL-2/BAX·BH3 complexes. It is easy to tell from Figure 3.6B and Figure 3.6C that in BCL-2/BIK·BH3 complex, salt-bridge and hydrogen bond were disrupted during the simulation.

As a non-conserved residue in BCL-2 family, Arg was more favoured at P22. That is why peptide BAX·BH3 and BIK·BH3 contributed more at this position. Based on the preliminary H-bond information, the stability of H-bond between P12 and Glu200 was analysed.(Figure 3.6C) The H-bond in BAX·BH3 is stronger and more durable than the hydrogen bond in BIK·BH3 complex leading to the high contribution of P12.

Distinct from the critical hydrophobic residues, salt-bridge and hydrogen bond played a major role in deciding the binding affinity of BH3 peptides with BCL-2. Arginine at P8 contribute to the binding significantly in all four complexes but in different ways. Residue that was glutamic acid at P6 contribute more to the binding. The negatively charged residue will have higher contribution at P13 by interacting with positively charged residue on BCL-2. The H-bond between P22 and BCL-2 will also help improve the binding affinity. Besides increasing the binding strength, the electrostatic interactions may contribute to the formation of complexes by correcting the orientation of peptide which is also a critical step in peptide-protein binding. This function maybe the reason why the performance of conserved hydrophilic residues have such a significant difference with hydrophobic residues.

3.3.4 Helical content

As we identified in peptide length study, the helical content can influence the binding affinity of the BH3 peptide to BCL-2. Here, we calculated the helical content for peptides andFigure 3.7 showed the calculation result. Looking at the figure, helical

content for BIM·BH3 and BAX·BH3 remained relatively constant during the whole simulation whereas BIK·BH3 and NOXA·BH3 have slight fluctuations. The average helical contents for the four peptides are 82.8% for BIM·BH3 (black), 82.7% for BAX·BH3 (red), 76.1% for BIK·BH3 (pink) and lastly, 71.5% for NOXA·BH3 (green). Figure 3.7 also suggests the denaturation of the helical domain of BIK·BH3 and NOXA·BH3 during the simulation. This implied that since NOXA·BH3 was unable to maintain its helical structure during simulation, the binding affinity between NOXA·BH3 and BCL-2 protein was not as favourable as compared to the other three peptides.

Figure 3.7. Helical Content analysis for four BH3 peptides

Comparing this to the binding affinity values obtained with Sietraj, NOXA·BH3 is

observed to have the weakest binding free energy value. The trend spotted was similar to the binding free energy values, with BIM·BH3 and BAX·BH3 having the strongest affinity out of the four peptides followed by BIK·BH3 and then NOXA·BH3. The helical content of the peptides demonstrated that the ability to maintain its helical structure does have an impact on its binding free energy values which affects the binding affinity of the protein-peptide complex. Hence one way to improve the binding affinity of a peptide to the protein is to improve its helical content.

3.3.5 In-silico design of BH3-like peptide

Based on the information obtained concerning how peptide length and peptide sequence affects binding affinity, a new peptide has been proposed using fragment-based new peptide design. In this method, fragments are selected from known peptide to build a new peptide which is expected to have better binding affinity to the protein. Referring to the sequence alignment of four peptides, at each position, from P1 to P26, the residue that contributes highest among four peptides was selected to form a new peptide. For example, according to our alanine scanning calculation, residue from BIM·BH3 at P1 has the highest contribution compared to other peptides. Thus in the newly designed peptide, Ile was positioned at P1. Most of the residues in the new peptide are chosen following this scheme.

However, as the study also identified helix stability as one of the factor that affects binding affinity, the helix propensity was also taken into consideration. Therefore, for those residues having similar contribution at the same position, their helix propensities determined which residue was selected in the new peptide. According to the contribution study, residues at P3 have no significant difference when binding to BCL-2. So in the new peptide, we choose Lys with higher helix propensity which is more likely to form helix than the other three residues. The previous study on the influence of peptide length indicates that the last five residues mainly play a role in stabilising the helix. So in the new peptide, we keep the five residues in the original crystal structure. Table 3.1 shows the sequence of the new peptide. After the formation of the new complex, Sietraj was employed to analyse the binding of the new peptide to BCL-2 protein. The binding free energy for the new complex is -19.63 kcal/mol.

Figure 3.8. Alanine scanning of the in-silico designed new peptide

Regarding Figure 3.8, the residues that contribute the most to binding affinity are consistent with the previous analysis, namely residues 3, 7, 8, 10, 13, 14, 18 and 22. This suggests that the newly constructed peptide has stronger binding affinity with the BCL-2 protein and the location of the key residues are valuable to the design of novel drug that induces apoptosis.

3.3.6 Hotspots of BCL-2

The decomposition of the binding free energy was performed for BCL-2 in each complex as shown in Figure 3.9. Only residues that contributed more than 1 kcal/mol were considered as being relevant to the binding of peptides and BCL-2. Obviously, in complex BCL-2/BIM·BH3, there were more stronger interactions between the protein and peptide while in complex BCL-2/NOXA·BH3, only a few residues of BCL-2 showed relatively high contribution.

According to the decomposition result, the energetic hot spots of BCL-2 were mainly located at $\alpha 2$, $\alpha 3$, $\alpha 4$, $\alpha 5$ and $\alpha 7$. Among these hot spots, Phe104, Tyr108, Leu137 and Asn143 have relatively high contributions in all four complexes. Located at $\alpha 2$, Phe104 is a conserved residue in BH3 domains of antiapoptotic proteins. Phe104 was also observed to be in contact with three critical hydrophobic residues of the BH3 peptides, namely P7, P10 and P14. Together with Phe104, Tyr108 which is also available in $\alpha 2$ interacts with P10 and P14. Besides this, Tyr108 was also in contact with another critical hydrophobic residue P3. Leu137 was another residue that forms hydrophobic

interaction with residue P7. Although this $\alpha 4$ localised residue is not a conserved residue of BCL-2 family, it shows similarity in some other antiapoptotic proteins like BCL-xL and BCL-w. All these information indicates that interactions between conserved residues of BCL-2 and those four critical hydrophobic residues of BH3 peptides make a significant energetic contribution to the binding of BH3 peptides to BCL-2. To sum all these information, hotspots that are critical to the binding of BCL-2 with BH3 peptides are highly conserved in the antiapoptotic BCL-2 family members. Interaction with these residues is fundamental for binding with BCL-2.

Figure 3.9. Free energy decomposition of BCL-2 in each complex.

On the other hand, several important residues of BCL-2 showed diverse contribution in binding with different BH3 peptides. They are Arg107, Arg110 and Arg146. Localised at $\alpha 2$, Arg107 has an over 5 kcal/mol contribution in complex BCL-2/BIM·BH3 and BCL-2/BAX·BH3 but has only ~1.8 kcal/mol contribution in complex BCL-2/BIK·BH3 and negative contribution in BCL-2/NOXA·BH3. Through hydrogen bond and salt-bridge analysis, H-bond was established between Arg107 and P13 in complex BCL-2/BIM·BH3 and BCL-2/BAX·BH3.

Similarly, Arg110 has an over 7 kcal/mol contribution in complex BIM·BH3 and BAX·BH3, but in the other two complex, no significant contribution was observed. Salt-bridge analysis indicated that Arg110 was favourable to negatively charged residues at P13. From the distance calculation, the salt-bridge was robust and stable in complex BCL-2/BIM·BH3 and BCL-2/BAX·BH3 while in BCL-2/BIK·BH3 the salt-bridge was broken. In NOXA·BH3, P13 is the positively charged residue Lys. The repelling of two same charged residues cause the negative contribution of Arg110 (0.203 kcal/mol) to the total binding affinity.

Another residue that performs differently in each system is Arg146. Arg146 is a highly conserved residue in antiapoptotic BCL-2 family members. Its ionic interaction with P12 which is also a highly conserved residue extensively exists in the binding of proapoptotic proteins to antiapoptotic proteins. From the binding free energy decomposition, the electrostatic contribution of Arg146 was highly favourable to the binding of BIM·BH3 to BCL-2 (-9.34 kcal/mol), following by BAX·BH3 (- 5.54 kcal/

mol and BIK·BH3 (-4.942 kcal/mol). Based on the distance analysis in the Figure 3.6(A), the salt-bridge in BCL-2/BIM·BH3 complex was stable while in BCL-2/NOXA·BH3 complex, the same salt-bridge was disrupted.

3.4 Conclusion

In this chapter, the mechanism of BH3 peptides in binding with antiapoptotic protein BCL-2 was studied by comparing the binding scenario of four complexes namely BCL-2/BIM·BH3, BCL-2/BAX·BH3, BCL-2/BIK·BH3 and BCL-2/NOXA·BH3 which in experimental studies have varying disassociation constants with BCL-2. According to the overall binding affinities calculated by Sietraj, electrostatic interaction was identified as the primary factor that influences the performance of four BH3 peptides while non-polar interactions have the least impact.

To explore the role of each residue on BH3 peptides, alanine scanning was utilised by demonstrating the contribution of each residue in each peptide. By comparing the alanine scanning results for four BH3 peptides, hydrophobic residues at P3, P14 and P18 showed unique contribution because of various kinds of hydrophobic amino acids. Residues with larger hydrophobic sidechain or aromatic sidechain are more favoured at these positions. On the contrary, hydrophilic residues of BH3 peptides showed distinct contribution including P6, P8, P12, P13 and P22. Glutamic acid which formed several hydrogen bonds with residues located at $\alpha 3$ of BCL-2, showed a significant advantage at position P6 when complexed with BIK·BH3. Another position that preferred glutamic

acid was P13 which interacted with BCL-2 through the formation of ionic interactions with Arg110 and Arg 107 of the protein. However, the stability of salt-bridge and hydrogen bond varied in different complexes. Arg at P12, albeit being a highly conserved residue, contributed differently in the four BH3 peptides. Helical content analysis confirmed that helical peptides with higher binding affinities are more stable during the simulation. This positive relation between binding affinity and helical content may be the reason why identical residues at the same position like P12 in different BH3 peptides have different contributions to binding affinity. Also, due to its hydrophilic nature, conserved P12 may play an important role in recognising the protein and correct the orientation of peptide when binding.

Following this picture, a new peptide was designed based on the alanine scanning results. Residues with high contribution to binding affinity at each position are selected, and residue with higher helical propensity was chosen when it comes to residues with similar contribution. The designed peptide showed higher binding affinity to BCL-2 than BIM·BH3 which is the most favourable peptide to BCL-2 in BCL-2 family.

Lastly, through free energy decomposition of BCL-2, hotspots of BCL-2 in binding with BH3 peptides are detected. Phe104, Tyr108, Leu137 and Asn143 were identified as critical hydrophobic hotspots for BH3 peptides which, relatively speaking, contribute more in every complex we studied. On the other hand, polar residues like Arg 107, Arg110 and Arg146 showed distinct contributions when associated with different BH3 peptides.

Chapter 4

The selectivity of BH3 peptides to antiapoptotic proteins BCL-xL and MCL-1

4.1 Introduction

The selective interactions between protein members in the BCL-2 family are critical to the permeability regulation of OMM (Outer Mitochondrial Membrane) thus controlling programmed cell death. Based on conservation of four BH (BCL-2 homology) regions, BCL-2 family members consist of three subclasses. The antiapoptotic protein members namely BCL-xL, BCL-2, MCL-1, BCL-w and Bfl-1 share all four homology domain (BH1 to BH4). The subfamily members including BAX and BAK which play the apoptotic role in cell death regulation also contain four homology regions BH1 to BH4. BH3-only proapoptotic proteins, such as BIM, BIK, Bad, NOXA, PUMA, BID share only the BH3 domain with other BCL-2 family members[106]. The balance and interaction of these structurally related proteins ensure the initiation of the BCL-2 apoptotic pathway. Structural biology illustrates that the interactions between BCL-2 family proteins are based on a conserved mode. That is the BC grooves formed by BH1, BH2 and BH3 domains of globular BCL-2 proteins constitute of a hydrophobic interface to interact with an amphipathic helix formed by BH3 regions[23, 112, 131, 132, 147]. Based on this binding mode, small molecules mimicking BH3 helix are

created to balance the antiapoptotic proteins and proapoptotic proteins in the treatment of various disease [41, 49, 148].

BH3-only proteins, although share little sequence similarity with other family members, convey the upstream cell death signal to initiate apoptosis[149]. In response to various upstream signals, BH3-only proteins achieve their proapoptotic role through two mechanisms[12, 150]. All BH3-only proteins can neutralise the antiapoptotic BCL-2 family proteins by occupying their BC grooves and displace effectors BAX and BAK. Other BH3-only proteins such as BIM, BID and PUMA can directly bind and activate BAX and BAK, which will then form oligomers and permeabilise the mitochondrial outer membrane[11]. Owing to their diverse functions and sequence differences, BH3-only proteins exhibit selectivities to antiapoptotic BCL-2 proteins. BIM, PUMA and tBID can bind and neutralise all antiapoptotic BCL-2 proteins while other BH3-only proteins showed distinct binding affinities. BAD have an affinity to BCL-2, BCL-xL and BCL-w but a subtle affinity to A1 and no affinity to MCL-1 while NOXA can only bind with MCL-1 and A1 (Figure 4.1). In general, the loss of BIM, PUMA and tBID which have an affinity to all antiapoptotic BCL-2 proteins will propel significant apoptotic deviance. Knockout studies on BIM indicate that BH3-only protein BIM is closely related to the apoptosis of the lymphoid and myeloid cell and the regulation of haematopoietic homeostasis[32]. Autoreactive thymocytes are also deleted as a result of ablation of BIM[33, 34]. PUMA receive direct signals from p53 tumour suppressor and other apoptotic stimuli and affect hematopoietic cell death[151-153]. On the other hand, ablation of individual BH3-only proteins that bind selectively to antiapoptotic

BCL-2 proteins has limited effect on apoptosis, and this may associate with apoptotic regulation in different tissues and various stages of cellular development[154]. Mice without NOXA show resistance to gastrointestinal death induced by X-ray and fibroblast death caused by UV radiation[155, 156] and BAD-deficiency gives minor cell death defects except assisting to the death of the pre-T cell and pro-/pre-B cell lymphoblastic leukaemia/lymphoma[157-159].

Figure 4.1. Some BH3-only proteins can bind and inhibit all prosurvival proteins (tBID, BIM and PUMA) while BAD only has the affinity to BCL-2, BCL-xL and BCL-w. NOXA can only bind with MCL-1 and A1.

Experiments have established the affinity difference by measuring the dissociation constants of BH3 peptides from BH3-only proteins to pro-survival proteins[116]. (Table 4.1) The 36-mer BIM, PUMA and BID peptides bind tightly with all antiapoptotic proteins, exhibiting K_D that is lower than 10nM. 36-mer BH3 peptide from BAD has a K_D value of 17.7 nM, 18.4 nM and 76.9 nM to BCL-2, BCL-xL and BCL-w respectively and no detectable affinity to MCL-1 ($>100 \mu\text{M}$). On the other hand, the dissociation constant of 36-mer NOXA BH3 peptides to MCL-1 and A1 is below 40 nM while to BCL-2 is as high as 1620 nM. There is no detectable binding between NOXA BH3 peptides and BCL-xL. This particular binding characteristic indicates that only specific combinations of these two subfamily groups can ensure normal cell death. This mechanism provides a hint that potent small molecules could be designed targeting all pro-survival proteins or just a specific group of these proteins. Although the importance of specific neutralisation of BH3-only proteins has been well illustrated, the sequence and dynamic determinants that caused the difference has not been well understood. A few studies on small molecules have addressed the selectivity of small BH3-mimic molecules in binding with pro-survival BCL-2 proteins[145, 160, 161] and library screening in combination with SPOT array analysis was used to find the sequence specific determinants of BH3 peptides. Thus far, there are only a few studies conducted on the binding selectivity of BH3-only peptides to antiapoptotic proteins at the molecular level and no dynamic information on the complexes was reported.

In this study, we choose BH3-only proteins that bind specifically to antiapoptotic proteins (i.e. NOXA and BAD) and two pro-survival proteins BCL-xL and MCL-1. MD

simulations were executed for four complexes namely BCL-xL/NOXA·BH3, MCL-1/NOXA·BH3, BCL-xL/BAD·BH3 and MCL-1/BAD·BH3. Since NOXA and BAD have significant affinities to MCL-1 and BCL-xL respectively, and there are only structures of BCL-xL/BAD·BH3 and MCL-1/NOXA·BH3, we build the other two complex structures manually. By comparing the binding poses of BH3 peptides when bound to BCL-xL and MCL-1, key residues of the antiapoptotic proteins that lead to the binding selectivity were identified. Energy decomposition is used to compare the hotspots of antiapoptotic proteins BCL-xL and MCL-1. Additionally, critical residues of BH3-only peptides that affect binding selectivity were also discussed.

Table 4.1. Disassociate constant of 36-mer BH3 peptides in binding with antiapoptotic BCL-2 proteins.

	BCL-2	BCL-xL	BCL-w	MCL-1	A1
BIM (136-171)	2.44	6.67	5.24	2.16	< 1
BID (76-111)	4.57	8.62	8.85	1.55	< 1
PUMA (127-162)	3.04	4.65	5.13	2.62	< 1
BAD (137-172)	17.7	18.4	76.9	> 100,000	813
BIK (41-76)	234	23.6	4.35	658	309
NOXA (13-48)	1620	> 100,000	> 100,000	36.9	14.2

4.2 Methodology

4.2.1 Preparation of initial structures

The crystal structures of BCL-xL/BAD·BH3 and MCL-1/NOXA·BH3 were acquired from the Protein Data Bank (PDB)[117] with PDB code of 2BZW[162] and 2ROD[163] respectively. To better compare the binding, only the common parts of BH3 peptides in these two crystal structures were used. Sequences of the BH3 peptides are shown in Table 4.2. Theoretically, BAD has no affinity to MCL-1 and NOXA does not bind to BCL-xL. Thus there are no structures for these two complexes. To obtain these structures, we mutated BAD·BH3 in complex BCL-xL/BAD·BH3 into the sequence of NOXA and conversely mutated NOXA·BH3 into BAD in complex MCL-1/NOXA·BH3. All the hydrogens and waters in the original structures were removed, and missing atoms were added using leap module in Amber 10. All four complexes were solvated in a pre-equilibrated TIP3P water box[118]. The minimal distance between the surface of proteins and water box was at least 10 Å. Sodium (Na⁺) and chloride (Cl⁻) ions were added to neutralise the charge of the systems. AMBER03 force field[93] was applied on all four complexes.

4.2.2 Multiple molecular simulations

Multiple short simulations (5ns) were used to produce better quantitative results.

Preceding the MD simulations were two steps of minimisation. In the first step, solvent molecules were minimised for 4000 steps and 1000 steps using the steepest descent method and conjugate gradient respectively. The complex was under a restraint of 500 kcal/(mol·Å²). The whole system was then minimised for 10000 steps using steepest descent method and continued with conjugate gradient minimization until the RMS of energy gradient reach to 0.01 kcal/mol/Å. This is subsequently followed by the heating of the system from 0K to 300K using Langevin thermostat[121]. A production run of 5 ns were conducted for each complex. A time step of 2ps was used for simulations conducted with long range electrostatic interactions calculated using particle mesh Ewald method[119] and a 12 Å cutoff for the van der Waals interactions. The SHAKE algorithm[120] was employed to restrain all bonds involving hydrogen atoms.

4.2.3 Binding free energy calculation using MM_PBSA

MM_PBSA (Molecular Mechanics Poisson-Boltzmann Surface Area) is used in calculating the binding free energy of protein-peptide complexes[104]. Calculation of binding free energy using MM_PBSA is evaluated according to the equation:

$$\Delta G_{\text{binding}} = \Delta G^{\text{gas}} + \Delta G^{\text{solv}} - T\Delta S$$

The gas-phase interaction energy (ΔG^{gas}), also known as molecular mechanical energies, is the sum of the internal energy (bond, angle, and dihedral), van der Waals interaction (ΔH_{vdW}) and electrostatic interactions (ΔH_{ele}) which are evaluated by the *Sander* program in Amber. The solvation free energy (ΔG^{solv}) represent the polar contribution (ΔG_{polar}) and nonpolar contribution (ΔG_{npolar}). ΔG_{polar} is calculated by Poisson-Boltzmann (PB) equation[164], and ΔG_{npolar} is determined by the solvent-accessible surface area (SASA) terms with the equation $\Delta G_{\text{npolar}} = \gamma \text{SASA} + \beta$ [165]. The values of γ and β in this equation are set to 0.0072 kcal/(mol·Å²) and 0.0 kcal/mol. ΔS represents the conformational entropy change between bound and free states, which is estimated, in this case, by *nmode* module[166] in Amber.

4.2.4 Free energy decomposition

The contribution of protein residues in each complex to binding free energy was analysed by MM_PBSA decomposition[143] in the MM_PBSA module. Electrostatic contribution to solvation energy was calculated by PB approach and non-polar contribution to the free energy of solvation was calculated using LCPO[144]. Each calculation used snapshots extracted from 5 ns simulations with an interval of 10 steps. Pairwise decomposition was also used to identify crucial interactions between peptides and proteins.

Table 4.2. Sequence alignment of BAD and NOXA BH3 peptides

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
BAD	L	W	A	A	Q	R	Y	G	R	E	L	R	R	M	S	D	E	F	E	G	S	F	K
NOXA	A	E	L	P	P	E	F	A	A	Q	L	R	K	I	G	D	K	V	Y	C	T	W	S

4.3 Results and Discussion

In order to gain insights on the binding selectivity between BH3-only proteins and antiapoptotic proteins, we have chosen two antiapoptotic proteins namely BCL-xL and MCL-1. Among these two proteins, BCL-xL only binds with BH3-only proteins BAD, and MCL-1 only has affinity to NOXA. To get a better overview of these two proteins, we make a similarity comparison of the sequence using Smith-Waterman method[167] and sequences of $\alpha 2$ to $\alpha 8$ were compared (Figure 4.2). As illustrated in Figure 4.2, BCL-xL and MCL-1 share little similarity whereby there are only 36 identical residues and 38 similar residues. Besides the mismatch residues, some residues which are called inserted residues only exist in particular protein. For example, at the joint of $\alpha 4$ and $\alpha 5$, the sequence present in BCL-xL is -DGVNWGR- and in MCL-1 is -DGVTNWGR- where one more residue (Asn) was inserted between valine and tryptophan. Taking the sequence alignment as a reference, the residue number of two proteins are renumbered from 1 to 120 to better illustrate the residues of BCL-xL and MCL-1 (Figure 4.3).

Figure 4.2. Sequence Comparison of BCL-xL and MCL-1. Identical residues are shown in green, similar residues are in pink, mismatch residues are in blue and inserted or deleted residues are in brown.

BCL-xL

MCL-1

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117
Q	A	L	R	E	A	G	D	E	F	E	L	R	Y	R	R	A	F	S	D	L	T	S	Q	L	H	I	T	P	G
E	T	L	R	R	V	G	D	G	V	Q	R	N	H	E	T	A	F	Q	G	M	L	R	K	L	D	I	K	N	E
192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221
31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135		136	137	138	139	140	141	142	143	144	145	146
T	A	Y	Q	S	F	E	Q	V	V	N	E	L	F	R	D	G	V	-	N	W	G	R	I	V	A	F	F	S	F
G	D	V	K	S	F	S	R	V	M	V	H	V	F	K	D	G	V	T	N	W	G	R	I	V	T	L	I	S	F
222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251
61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90
147	148	149	150	151			152	153	154	155	156	157	158			159	160	161	162	163	164	165	166	167	168	169	170	171	172
G	G	A	L	C			V	E	S	V	D	K	E			M	Q	V	L	V	S	R	I	A	S	W	M	A	T
G	A	F	V	A	K	H	L	K	S	V	N	Q	E	S	F	I	E	P	L	A	E	T	I	T	D	V	L	V	R
252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281
91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196						
Y	L	N	D	H	L	E	P	W	I	Q	E	N	G	G	W	D	T	F	V	D	L	Y	G						
T	K	R	D					W	L	V	K	Q	R	G	W	D	G	F	V	E	F	F	H	V	Q	D	L	E	G
282	283	284	285					286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307

Figure 4.3. Sequence alignment of BCL-xL and MCL-1. Sequence and original residue number of BCL-xL are highlighted in green and of MCL-2 are in yellow.

4.3.1 BCL-xL/BAD·BH3 versus MCL-1/BAD·BH3

Binding free energies of BCL-xL/BAD·BH3 and MCL-1/BAD·BH3 were obtained based on three simulations using MM_PBSA method. Table 4.3 list the free energies of each simulation and the free energies were decomposed into terms according to internal electrostatic energy, internal van der Waals energy, reaction field polar energy and hydrophobic contribution to solvation energy. According to the disassociation constant obtained experimentally, peptide BAD·BH3 has high affinity with BCL-xL but barely bind to MCL-1. As expected, the binding affinities calculated by MM_PBSA showed ΔG of the BCL-xL/BAD·BH3 complex to be as low as -40.88 kcal/mol while that of MCL-1/BAD·BH3 complex is as high as -12.72 kcal/mol. Investigating the free energy components of each MD simulation, it can be determined that internal electrostatic interactions contribute positively to stabilise the protein-peptide BCL-xL/BAD·BH3 complex while MCL-1/BAD·BH3 complex contribute negatively. Furthermore, the total polar energy (i.e. the sum of ΔH_{ele} and ΔG_{polar}) of BAD·BH3 when bound to MCL-1 is much higher than when bound to BCL-xL indicating that electrostatic interactions between protein and peptide may have a significant influence on the binding selectivity.

Table 4.3. Free energy components for complex BCL-xL/BAD·BH3 and MCL-1/BAD·BH3 (kcal/mol)

	BCL-xL/BAD·BH3			MCL-1/BAD·BH3		
	MD1	MD2	MD3	MD1	MD2	MD3
ΔG_{PB}	-35.25	-40.88	-37.87	-13.36	-12.96	-12.72
ΔH_{ele}	-419.65	-386.05	-351.34	31.62	38.64	30.99
ΔH_{vdw}	-115.86	-121.24	-119.18	-97.74	-95.38	-105.11
ΔG_{polar}	463.35	429.88	397.07	19.4	7.39	23.8
$\Delta G_{nonpolar}$	-16.75	-17.07	-16.25	-15.22	-14.35	-15.98
$\Delta G_{PB, total}$	-88.91	-94.48	-89.70	-61.94	-63.7	-66.3
$-T\Delta S$	53.65	53.60	51.83	48.58	50.74	53.58

From the overview of the binding free energy calculated using MM_PBSA, both polar and non-polar residues affect the discrepancy between BAD·BH3 in complex with BCL-xL and MCL-1. To get the specific residues that are responsible for the significant difference in binding free energy, per-residue decomposition were conducted for both BCL-xL and MCL-1 (Figure 4.4). Residues which have a decomposition energy lower than -1 kcal/mol were considered having relatively high contribution to the binding. Based on the average binding free energy decompositions of complex BCL-xL/BAD·BH3, hotspots of BCL-xL are identified to be F10, R13, Y14, A17, L25, V39, E42, L43, N50, G52, F60, L112 and Y113. This result agrees well with previous hotspot study done by Moroy's group[168]. On the contrary, there are just a few residues having

a relatively high contribution to the binding. They are M21, L25, V29, H42, V43 and R53. To better identify residues that are in charge of the binding difference, the relative binding free energy contribution of each corresponding residue on BCL-xL and MCL-1, based on the sequence alignment, are calculated by the equation as follows:

$$\Delta\Delta G_{\text{per_res}} = \Delta G_{\text{BCL-xL}} - \Delta G_{\text{MCL-1}}$$

Results for this are shown in Figure 4.5 and a more negative $\Delta\Delta G_{\text{per_res}}$ means that this residue contributes more in BCL-xL than in MCL-1. To better look into the interactions in the peptide-protein complex, we have conducted pairwise decomposition and plot the data into Figure 4.6.

Figure 4.4. Binding free energy decomposition for (A) MD1 of BCL-xL (B) MD2 of BCL-xL (C) MD3 of BCL-xL (D) Average of BCL-xL (a) MD1 of MCL-1 (b) MD1 of MCL-1 (c) MD1 of MCL-1 (d) Average of MCL-1. Residue number follows sequence alignment number.

Figure 4.5. Difference between contribution of each residue on BCL-xL and MCL-1 in associating with BAD·BH3.

Figure 4.6. Pair-wise binding free energy decomposition. More negative means this pair of residues contribute more to binding

The first residue that was most likely to have a large influence in the binding selectivity is residue 53 which is located at $\alpha 5$ and represents as Arg in both BCL-xL and MCL-1 (Figure 4.5). From the per-residue decomposition in Figure 4.5, it is evident that the contribution of residue 53 in complex BCL-xL/BAD·BH3 is around 6 kcal/mol higher than in complex MCL-1/BAD·BH3. Preceding studies on the BCL-2 family have shown that this positively-charged residue can form a conserved salt-bridge with negatively-charged residue Asp on BH3 domain from another protein which is essential for the stability of the peptide-protein complex[169] (Figure 4.7). From the pairwise decomposition performed, the interaction between residue 53 and D16 from complex BCL-xL/BAD·BH3 is quite strong (lower than -10 kcal/mol) while in MCL-1/BAD·BH3, it is higher than -3 kcal/mol (Figure 25). Salt-bridge analysis was also used to find out why this conserved residue Arg53 contribute differently to the binding with the same residue D16 on BH3 peptide (Figure 4.8A). Results showed that the salt-bridge between BCL-xL and BAD·BH3 was stable during the simulation while this salt-bridge in MCL-1/BAD·BH3 complex was disrupted after 1ns. To reveal the reason that lead to the breaking of the salt-bridge, we monitor the simulation and find out the breaking of this salt-bridge is because of residue E19 which is less stable in complex MCL-1/BAD·BH3 and its negatively charge atom OE2 attack the NH2 of Arg53 at 1ns (Figure 4.8B).

Figure 4.7. Salt-bridge between Arg53 of protein and D16 of peptide. The loop between $\alpha 4$ and $\alpha 5$ is highlighted in cyan. Residues on the protein are shown in CPK and residues on peptide are shown in Licorice.

From pairwise decomposition, E19 interacting with residue 50 and 52 could be the key to the instability of E19 in complex MCL-1/BAD·BH3. In BCL-xL/BAD·BH3 complex, there are hydrogen bonds formed among E19, Asn50 and Gly52 in both BCL-xL and MCL-1 and these hydrogen bonds were not present in MCL-1/BAD·BH3 complex. Figure 4.8C and Figure 4.8D showed the hydrogen bond analysis done for BCL-xL and MCL-1 respectively. The identification of these hydrogen bonds also explains why residue 50 and 52 showed over 1 kcal/mol higher contribution in BCL-xL than in MCL-1. These hydrogen bonds in BCL-xL keep E19 in a more steady state while in MCL-1, E19 compete with D16 in interacting with Arg53.

Figure 4.8. (A) Salt-bridge between Arg53 and D16 (B) Distance between NH2 of Arg53 and OE2 of E19 (C) Hydrogen bond between ND2 of Asn 50 and CD of E19 (D) Hydrogen bond between N of Gly52 and CD of E19.

Reasons why conserved residues have such an impact on the binding still have to be found. To the end, backbone analysis on Asn50 and Gly52 were done, and the results showed that the backbone of Asn50 from MCL-1 fluctuate more than the one from BCL-xL. As can be seen in Figure 4.9AB, the population of dihedral angle of Asn50 from BCL-xL is stable at $(-153^\circ, 153^\circ)$ while the one from MCL-1 is less concentrated. To get a thorough understanding of this difference, RMSF (root mean square fluctuation) analysis on the backbone of protein was conducted. As expected, the loop between $\alpha 4$ and $\alpha 5$ which contained Asn50 fluctuate more in MCL-1 than in BCL-xL (Figure 4.9C). To our notice, the sequence of the loop is -DGVNWGR- in BCL-xL and in MCL-1 there insert one more residue representing as -DGVTNWGR-. This gives us an insight that the various performance of the loop in two proteins, especially the residue Asn50, is due to this sequence difference. As a consequence, the stability of salt-bridge between Arg53 and D16 differed in the two proteins. This guess is further proved by the sequence of protein A1 which also barely bind with peptide BAD·BH3. In A1, the sequence of this loop also has one more residue as such -DGIINWGR-. In this way, the sequence of the loop between $\alpha 4$ and $\alpha 5$ could be a form of recognition of antiapoptotic proteins to BH3 peptide BAD.

Figure 4.9. Backbone analysis of residue Asn50 on (A) BCL-xL (B) MCL-1. Colours are used to illustrate the popularity of dihedral angles. Higher popularity is shown in smaller number. (C) RMSF analysis of proteins (BCL-xL in black and MCL-1 in red).

Figure 4.10. (A) SASA of F18 from BH3 peptide in both complex (B) SASA of residue10 on BCL-xL and MCL-1. Interactions between residue10 and F18 in complex (C) BCL-xL/BAD·BH3 (D) MCL-1/BAD·BH3

Figure 4.11. DSSP analysis for BAD·BH3 in complex (A) BCL-xL/BAD·BH3 and (B) MCL-1/BAD·BH3

Another residue that contributes more in complex BCL-xL/BAD·BH3 is residue 10 which is corresponding to Phe97 and Val197 on protein BCL-xL and MCL-1 respectively (Figure 4.10CD). Residue10 locates at the $\alpha 2$ of the protein and form van der Waals interactions with BH3 peptides due to the hydrophobic features. From the pair-wise decomposition, Phe in BCL-xL interacts with L11, M14, S15 and F18 tightly. Together with F18 of BAD·BH3, Phe in BCL-xL has a -2.15 kcal/mol free energy while Val in MCL-1 only interacts with F18 with a 1 kcal/mol contribution. What's more, solvent accessible surface (SASA) analysis on both residue10 and F18 showed that complex MCL-1/BAD·BH3 is more exposed to the solvent which will lead to unstable interactions (Figure 4.10AB). This difference could be a result of more solvent-exposed hotspot on MCL-1 than on BCL-xL corresponding to residue F18 which in agreement with the previous observations[145, 146, 161]. However, on protein A1, it also represents as Phe at this position which means although there exists an interacting

difference between BCL-xL and MCL-1 at this position, it is not a determination to the binding selectivity. Although Val on MCL-1 is not in charge of the selectivity of BH3-only proteins, the loss of this van der Waals interactions, perhaps together with the loss of electrostatic interactions between Arg53 and D16 in complex MCL-1/BAD·BH3, has an impact on the helix stability of C-terminus of the peptide. DSSP analysis was utilised to illustrate the helix stability in Figure 4.11. Apparently, the C-terminus of BH3 peptide in complex MCL-1/BAD·BH3 is denatured from D16 along the simulation.

Residue 42 is another residue that exhibits significant difference in two proteins when interacts with the BAD·BH3 peptide. Representing as Glu and His in BCL-xL and MCL-1 respectively, residue 42 from BCL-xL has a strong interaction with both R12 and Q5 from the BH3 peptide according to pair-wise decomposition. However, in complex MCL-1/BAD·BH3, residue 42 only has interaction with R12 which is weaker than in complex BCL-xL/BAD·BH3. Further analysis showed that Glu in BCL-xL, which acts as hydrogen bond acceptor form a few strong and stable ionic interactions with R12 and Q5 (Figure 4.12A) while His in MCL-1 can only form one hydrogen bond with its backbone oxygen (Figure 4.12B). Sequence alignment of antiapoptotic proteins shows that at the position corresponding to residue 42, BCL-xL, BCL-2 and BCL-w which are selective to BAD all represent as Glu while on the other hand, MCL-1 and A1 which have no affinity to BAD, represents as His and Lys respectively. Although His and Lys are different amino acids, they are all hydrogen bond donor. In this way, glutamic acid or hydrogen bond acceptor at this position could be a key in recognition of BAD·BH3 peptide to antiapoptotic proteins.

Figure 4.12. Ionic interactions between BAD·BH3 and residue 42 from (A) BCL-xL (B) MCL-1.

Figure 4.13. The interactions between Y7 and residue 17, 21 of (A) BCL-xL (B) MCL-1. $\alpha 3$ in each protein is highlighted in iceblue. DSSP result for $\alpha 3$ of (C) BCL-xL and (D) MCL-1

From pair-wise decomposition, residue 17 representing as Ala in both BCL-xL and MCL-1, interacts with polar amino acid Y7 differently in two complex. Ala17 locating at $\alpha 3$ has close interaction with Y7 and they together contribute to the binding over 2 kcal/mol while this interaction vanishes in MCL-1/BAD·BH3. Further analysis found out that in complex BCL-xL/BAD·BH3, the backbone oxygen forms a strong hydrogen bond with Y7 which contribute to the penetrate to the binding pocket of BCL-xL corresponding to Y7. Vanishing this hydrogen bond, Y7 in complex MCL-1/BAD·BH3 also losses van der Waals interaction with residue 21 which is one of the key members that consist the binding site corresponding to Y7 (Figure 13AB). From the sequence alignment, although Ala17 is a conserved residue in BCL-xL and MCL-1, the sequence of other residues is distinctively different at $\alpha 3$. So it is possible that the dynamics of $\alpha 3$ determined the association of Ala17 with Y7 which is supported by the RMSF which shows that residue 15 to 20 on BCL-xL fluctuated more during the simulation (Figure 4.9C). To confirm this, the secondary structure of two proteins were analysed through DSSP as shown in Figure 13CD and result indicated that, different from the well-packed α helix of $\alpha 3$ of MCL-1, the $\alpha 3$ of BCL-xL is more flexible and show less helical content. So the difference in the flexibility of $\alpha 3$ could be the reason why bulky Y7 could penetrate the binding pocket of BCL-xL and form van der Waals interactions with residue 21 while it is more solvent-exposed when binding with MCL-1 and loss interactions with residue 21.

4.3.2 BCL-xL/NOXA·BH3 versus MCL-1/NOXA·BH3

Same with preceding studies, binding free energy calculations for BCL-xL/NOXA·BH3 and MCL-1/NOXA·BH3 were also obtained from three short simulation trajectories for each complex. Binding free energy calculated using MM_PBSA for each trajectory, and free energy components are given in Table 4.4. According to experimental results on affinities, peptide NOXA·BH3, on the contrary to BAD·BH3, is detected only having affinity with MCL-1 but not BCL-xL. As expected, the binding affinity of NOXA·BH3 to MCL-1 is significantly higher than BCL-xL in our simulations. Looking into the free energy components listed in Table 4.3, the internal van der Waals interactions and nonpolar interaction of solvation energy of two complexes are quite similar. On the other hand, the electrostatic contribution differs in two complexes where the internal electrostatic interactions negatively contribute to the association of NOXA·BH3 to BCL-xL. Same with the selectivity of BAD·BH3, the crucial factor that affects the selectivity of NOXA·BH3 is electrostatic interactions as well.

Table 4.4. Free energy components for complex BCL-xL/NOXA·BH3 and MCL-1/NOXA·BH3 (kcal/mol)

BCL-xL/NOXA·BH3				MCL-1/NOXA·BH3		
	MD1	MD2	MD3	MD1	MD2	MD3
ΔG_{PB}	-25.04	-22.87	-24.41	-40.28	-41.37	-44.31

	BCL-xL/NOXA·BH3			MCL-1/NOXA·BH3		
	MD1	MD2	MD3	MD1	MD2	MD3
$\Delta G_{PB, total}$	-78.41	-72.71	-72.97	-91.84	-91.39	-92.70
ΔH_{ele}	23.64	8.88	21.95	-113.31	-116.97	-119.04
ΔH_{vdw}	-122.02	-103.48	-108.38	-112.96	-116.15	-116.45
ΔG_{polar}	36.74	37.41	29.11	151.14	159.25	159.35
$\Delta G_{nonpolar}$	-16.77	-15.52	-15.65	-16.71	-17.52	-16.57
$-T\Delta S$	-53.01	-49.84	-49.56	-51.56	-50.02	-48.39

The contribution of each residue on proteins was discovered by per-residue free energy decomposition as showed in Figure 4.14 and residues with a decomposition energy lower than -1 kcal/mol are considered having signification contribution. Looking through the results of BCL-xL and MCL-1, it is evident that there are more critical residues on MCL-1, especially those residues located at $\alpha 4$, than BCL-xL when binding with peptide NOXA·BH3. To better understanding the contribution difference between two antiapoptotic proteins when integrating with BH3 peptide originate from BH3-only protein NOXA, a relative binding free energy decomposition between equivalent residues which are determined through sequence alignment of two proteins was made following the equation (Figure 4.15):

Figure 4.14. Binding free energy decomposition for (A) MD1 of BCL-xL (B) MD2 of BCL-xL (C) MD3 of BCL-xL (D) Average of BCL-xL (a) MD1 of MCL-1 (b) MD1 of MCL-1 (c) MD1 of MCL-1 (d) Average of MCL-1. Residue number follows sequence alignment number.

Figure 4.15. Difference between the contribution of each residue on BCL-xL and MCL-1 in associating with NOXA·BH3.

Figure 4.16. Pair-wise binding free energy decomposition for complex (A) BCL-xL / NOXA·BH3 and (B) MCL-1/NOXA·BH3

$$\Delta\Delta G_{\text{per_res}} = \Delta G_{\text{MCL-1}} - \Delta G_{\text{BCL-xL}}$$

The lower relative binding free energy means more contribution this residue made in MCL-1 than in BCL-xL. To our notice, the residue with the most significant contribution to the binding difference is Arg53 which is in agreement with the previous comparison of BCL-xL/BAD·BH3 and MCL-1/BAD·BH3. When being integrated with NOXA·BH3, the contribution of R53 in MCL-1 is over 8 kcal/mol than in BCL-xL. Considering the pair-wise binding free energy decomposition plotted in Figure 4.16, Arg53 on MCL-1 together with D16 on the NOXA·BH3 peptide contribute to binding free energy over 10 kcal/mol while on BCL-xL together with D16 contribute much less. Obviously, same with previously identified, the stability of the ionic interactions between these two residues plays an major role in binding and salt-bridge analysis once proved that (Figure 4.17A). Salt-bridge between Arg53 and D16 in complex MCL-1/BAD·BH3 is stronger and more stable during the simulation while on the other hand, in BCL-xL/BAD·BH3 fluctuate more. Looking into the pair-wise decomposition, Arg53 on BCL-xL has a contact with Y19 which together contribute to binding around 1 kcal/mol which show less contribution to binding in complex MCL-1/BAD·BH3 and instead, Y19 interact with Asn50 tightly and together contribute to binding around 1.9 kcal/mol. Figure 4.18BC illustrate the interactions between Arg53, Asn50, D16 and Y19. In complex MCL-1/BAD·BH3, the CH2 of Asn50 form a CH- π bond with the aromatic ring of Y19 and both residues are stable during the simulation. Beside the salt bridge between Arg53 and D16, there exist a hydrogen bond between atom HE of Arg53 and OD1 of D16. What's more, Arg53 form inter-protein ionic interactions with Asp46

which is located at the loop between $\alpha 4$ and $\alpha 5$. The multiple ionic interactions between Arg53, Asp46 and D16 enhance the conserved salt-bridge. In contrast, the interaction between Asn50 and Y19 is not strong which can be told from pair-wise energy decomposition and result in the fluctuation of Y19. As a consequence, Y19 attack the space between Arg53 and D16 and salt-bridge breaks.

Although residues mentioned above are all conserved residues, significant deviation is present in the binding scenarios of two complex. The vital factor in charge of this difference is the sequence of the loop is -DGVNWGR- in BCL-xL and -DGVTNWGR- in MCL-1. This sequence, on the one hand, determines the conformation of the backbone of residue Asn50 which is located at the beginning of $\alpha 5$ and difference of structure and stability of backbone of Asn50 has a large impact on the interaction with Y19. This is supported by the backbone dihedral angles of Asn50 in two complexes as illustrated in Figure 4.18. From the result, the backbone dihedral angle of Asn50 on BCL-xL lies on (-162, 162) and on MCL-1 lies on (78, 126) which figures out the notable difference of backbone conformation of Asn50 in two complexes. On the other hand, the relative longer loop of MCL-1 offers more space for the interaction of Arg53 and Asp83 which in turn stabilise the salt-bridge of Arg53-D16. In complex BCL-xL/NOXA·BH3 the space between Arg53, Asp46 and D16 is relatively small and when unstable Y19 attack the system salt-bridge breaks due to the steric hindrance. As a result of the break of this salt-bridge, water molecules are penetrating into the space between peptide and protein which will further destabilise the complex BCL-xL/NOXA·BH3 (Figure 4.19).

Figure 4.17. (A) Distance analysis of salt-bridge between Arg53 and D16. Illustration of interactions between Arg53, Asn50 of protein and D16, Y19 of peptide in complex (B) BCL-xL/NOXA·BH3 and (C) MCL-1/NOXA·BH3.

Figure 4.18. Backbone analysis of residue Asn50 on (A) BCL-xL (B) MCL-1. (C) RMSF analysis of proteins (BCL-xL in black and MCL-1 in red)

Figure 4.19. With the break of salt-bridge between Arg53 and D16 in complex BCL-xL/NOXA·BH3, water molecules penetrate into the binding site.

Figure 4.20. (A) The salt-bridge and H-bond analysis of E2 in contact with protein MCL-1. (B) DSSP for the BH3 peptide NOXA·BH3 when in complex with MCL-1.

Following Arg53, residue 38 representing as Gln in BCL-xL and Arg in MCL-1 has the second large difference in the contribution. Pair-wise decomposition result showed that Arg38 interacts with E2 tightly and together contribute to binding over 4 kcal/mol. Salt-bridge analysis indicate that residue 38 on MCL-1 interact with the N-terminus residue E2 on NOXA·BH3 through salt-bridge (Figure 20A). Another residue that has strong electrostatic interaction with E2 is residue 35 which representing as Ser in both proteins and contribute over 1 kcal/mol more in MCL-1/NOXA·BH3. Distance analysis showed that salt-bridge formed by E2 and Arg38 as well as the hydrogen bond between E2 and Ser35 are formed at around 3.2ns of the simulation (Figure 20A). Comparing the distance analysis with the DSSP result for peptide NOXA·BH3 (Figure 20B), one interesting thing is that with the formation of ionic interactions, the helix on N-terminus is extended. As the BH3-only proteins are first disordered and forming helix while

binding with antiapoptotic proteins, this result proved that ionic interaction is necessary for this process. Although Arg38 is not an essential residue in the binding with peptide NOXA·BH3, as in protein A1 at this position representing as Gln which is same with BCL-xL, it contributes to the binding affinity and helix formation.

Residue 21 is not a conserved residue and BCL-xL at this position is equipped with Leu and MCL-1 is provided with Met. Pair-wise energy decomposition showed that Met21 on MCL-1, locating at $\alpha 3$, interacts with hydrophobic residue F7 and L11 on NOXA·BH3 tightly through van der Waals interaction while Leu21 on BCL-xL only interacts with F7 weakly ($< 1\text{kcal/mol}$). Distance analysis on the CB of residue 21 and F7 were conducted to indicate hydrophobic interactions and the result indicated that in complex BCL-xL/NOXA·BH3 there is a compulsion between these two residues while in complex MCL-1/NOXA·BH3 the interaction is relatively stable (Figure 4.21A). As shown in Figure, Met21, Val39 and Val48 on MCL-1 formed a hydrophobic pocket which can pack F7 well, especially the long sidechain of Met21 separate F7 from L11. On the contrary, Leu21 on BCL-xL only interact with L11 and lost affinity to F7 which as a consequence only interact with Val39. To our notice, with losing affinity with Leu21, backbone hydrogen bond between F7 and L11 breaks and N-terminus of BH3 peptide is denatured (Figure 4.21B). Same with previous study on the dynamics of BCL-xL and MCL-1[170], RMSF analysis was conducted on backbone atoms of protein indicate that $\alpha 3$ of BCL-xL fluctuate more during the simulation, especially residue 21. This fluctuation together with smaller hydrophobic sidechain leads to the instability of interactions between Leu21 and F7, L11 in complex BCL-xL/NOXA·BH3 (Figure

4.18C). According to the sequence of antiapoptotic proteins, residue at this position on BCL-2 which also barely interact with BH3-only protein NOXA represents as Met as well which indicate that residue 21 itself can not determine the selectivity of antiapoptotic proteins. However, due to sequence difference, the flexibility of $\alpha 3$ of BCL-xL and MCL-1 differs a lot which has a large impact on the binding pocket corresponding to residue 7 on BH3 peptides.

Figure 4.21. (A) Distance between the CB of F7 and residue 21(B) The interactions between F7 and proteins and secondary structure of NOXA·BH3 in two complex.

4.4 Conclusion

Experimental studies on the integrating of BH3-only proteins and antiapoptotic proteins revealed that BH3 peptides derived from BH3-only protein BAD and NOXA have binding specificity to antiapoptotic proteins representing as BAD only has an affinity with BCL-xL, BCL-2 and BCL-w while NOXA only bind with MCL-1 and A1. In this chapter, the binding selectivity of BH3-only proteins towards antiapoptotic proteins were studied through running MD simulations of antiapoptotic proteins BCL-xL and MCL-1 in complex with BH3-only peptides BAD·BH3 and NOXA·BH3.

In this thesis, through comparing the overall binding free energy components of BAD·BH3 in associating with BCL-xL and MCL-1, both polar and nonpolar interactions have an impact on the binding affinity difference. Sequence difference of the loop between $\alpha 4$ and $\alpha 5$, representing as -DGVNWGR- in BCL-xL and -DGVTNWGR- in MCL-1 is a crucial factor that determines the dynamics of Asn50 which will, in turn, affect the stability of E19 of BAD peptide when associating with antiapoptotic proteins. As a result, the salt-bridge between Arg53 and D16 which is conserved in BCL-2 family and primarily impact the integrating of BH3 peptides to proteins will be affected. Glutamic acid at residue 42 in antiapoptotic proteins could be another ionic recognition point for the BH3 peptide BAD as it can form ionic interactions with R12. Hydrophobic residue 10 is not a determinant for binding selectivity, but losing interactions between residue 10 and F18 is unfavourable for helix

formation and lead to a denature of C-terminus of BAD·BH3. Also, the more flexible $\alpha 3$ of BCL-xL allows the bulky Y7 form a hydrogen bond with Ala 17 and well interact with hydrophobic pocket corresponding to Y7. In contrast, the stable and well-packed helix of MCL-1 is not favourable for Y7 and disrupt the interaction of Y7 and residue 21 of MCL-1.

In the comparison of the binding of NOXA·BH3 to different proteins, the difference of loop between $\alpha 4$ and $\alpha 5$ is once proved to have significant impact on the dynamics of Asn50 following which the stability of ionic interaction of Arg53 and D16 was determined. Losing the salt-bridge will on one hand decrease the binding affinity of the BH3 peptides to proteins and on the other hand will open a channel for letting water molecules penetrating into the binding groove and as a result, the complex is destabilised. Also, the flexible $\alpha 3$ of BCL-xL in our simulation especially residue 21 is not favourable to Y7 and residue 21 (Leu) loss interaction with Y7 along the simulation. What's more, ionic interaction studies on the N-terminus of NOXA·BH3 proved that ionic interactions enhance the helix formation of BH3 peptides during the binding to antiapoptotic proteins.

Previous studies on peptide-protein recognition have revealed that electrostatic interactions are the identification of peptide binding site[171]. In our study on the selectivity of BH3-only proteins, the conserved salt-bridge of Arg53 contributed differently which could be a recognition site for BH3-only proteins. The sequence of loop between $\alpha 4$ and $\alpha 5$, representing as -DGVNWGR- in BCL-xL and -DGVTNWGR-

in MCL-1 largely impact the stability of the salt-bridge. As the BH3 domain of BH3-only proteins is forming helix while binding, the identification of this loop and electrostatic integration with Arg53 become essential in the future drug design. Through our study, we emphasise the importance of the dynamic of $\alpha 3$ of BCL-xL and MCL-1 which determines the binding scenario of residue corresponding to Y7 and F7 of BAD and NOXA respectively.

Chapter 5

Summary

In this thesis, the complex interactions between BCL-2 family which play a vital role in mitochondrial regulated apoptosis are investigated through running MD simulations on the core model of BH3 peptides associating with BCL-2 proteins.

In the beginning, we study the impact of the C-terminus residues of BH3 peptides of BAX on the binding with protein BCL-2 which used to be thought of having no affinity to BAX. Through analysing the data we get from MD simulations, among the five C-terminus residues (residue 78-82), residue 78 has direct contact with BCL-2 and stabilise the complex through forming a hydrogen bond. The other four residues, although barely having contact with BCL-2, contribute to the binding indirectly through increasing the helical content and stability of BAX·BH3 which also help stabilise the ionic interactions between peptide and protein. Nowadays, peptides therapeutics is quite promising in the pharmaceutical research and development for their high efficiency, tolerability and safety[172]. A rational design of peptide therapeutics always starts with studying a known crystal structure of the peptide. Thus it is important to explore the BH3 peptides in binding with antiapoptotic proteins in the designing peptide therapeutics targeting on BCL-2 family. Our research on the structure of BH3 peptides of BAX in binding with BCL-2 provides information on the influence of peptide length

on the performance of peptide. So in the future design of peptide that inhibits protein BCL-2, the terminus residues should be carefully considered.

To get a better understanding of the binding picture of antiapoptotic protein BCL-2, in the second study, we choose BH3 peptides that having the various binding affinities with BCL-2 including BIM·BH3, BAX·BH3, BIK·BH3 and NOXA·BH3. Through comparing the alanine scanning results for BH3 peptides, among the five critical hydrophobic residues, P3, P14 and P18 favour more of residues with aromatic sidechain or relatively large hydrophobic sidechain. However, the size of sidechain is not the only criteria when choosing hydrophobic amino acids at these positions. A balance of steric effect and binding affinity should be taken into consideration. Hydrophilic residues including P6, P8, P12, P13 and P22 contribute differently in each BH3 peptides. Glutamic acid is favourable at P6 and P13 which could form strong salt-bridge with BCL-2. Conserved residue P12 from different BH3 peptides, interacting with same residues on BCL-2, has a distinctive contribution to binding. With this result, although residues that critical for the binding is hydrophobic residues, charged residues on BH3 peptides may enhance the binding and are responsible for the different binding affinity to BCL-2. Therefore, including electrostatic interactions in the future drugs that inhibit BCL-2 is essential. This study also emphasises the impact of helical stability of BH3 peptides on the binding. Less stable helix or lower helical content will deduct the electrostatic interactions which in turn decrease the binding affinity. On the other hand, this chapter identifies both hydrophobic and hydrophilic hotspots on BCL-2 including Phe104, Tyr108, Leu137, Asn143 and Arg 107, Arg110 and Arg146. These residues

should be paid more attention in the future therapeutics targeting on BCL-2. Creating reasonable interactions with these residues will increase the affinity of the drug. This chapter also proposes a new peptide using fragment-based new peptide design and our new peptide gives a high binding affinity to BCL-2. It will be nice if we have a chance to test the ability of new peptide in an experimental way.

The last chapter investigates the selectivity of BH3-only proteins to antiapoptotic proteins. In this study, we choose BH3-only proteins that have binding specificity to antiapoptotic proteins (i.e. NOXA and BAD) and two prosurvival proteins BCL-xL and MCL-1. By executing MD simulations on complexes and comparing the binding affinity using free energy decomposition, the importance of loop between $\alpha 4$ and $\alpha 5$ is emphasised since it is a determinant of the stability of conserved salt-bridge Arg53-D16 which could be a recognition site for peptide binding. The electrostatic interactions contribute to the formation of complexes in various ways like enhancing the binding and recognising the proteins. The salt-bridge between Arg53 and D16 plays an recognising role with high possibility. The flexibility difference of $\alpha 3$ between BCL-xL and MCL-1 determines the binding sites for Y7 or F7 which is likewise a crucial factor for peptide selectivity. Also, the important role of electrostatic interactions in peptide helix formation while binding is also revealed. High selectivity is one of the most significant strengths that peptides therapeutics have. Exploring the selectivity of BH3 peptides to antiapoptotic proteins in BCL-2 family provides information on future peptide design with precise targeting. On the other side, considering the determinants

of selectivity when developing small molecules can lead to novel drugs with inhibition to particular antiapoptotic proteins in BCL-2 family.

In recent days, BCL-2 family members have become a novel anti-cancer therapy target since their the abnormal expression will lead to various types of cancer. Through studying the binding mechanism of BH3 peptides in binding with BCL-2 proteins, will offer insights into future drug design targeting on BCL-2 family. Our studies on BCL-2 provide the information on the influence of peptide length, the peptide sequence and helical content on the binding. Also, our identification on the recognition of BH3 peptides to antiapoptotic proteins also gives some insights into the future design of drugs with specifications.

Publications

Antiapoptotic Protein Bcl-2 in binding with Different BH3 Peptides: Study the Influence of BH3 Peptides Length, Sequence and Helix Stability on Binding affinity (In preparation)

The selectivity of BH3-only proteins toward antiapoptotic proteins: study the sequence and dynamic difference of BCL-xL and MCL-1 (In preparation)

References

1. Thompson, C.B., Apoptosis in the pathogenesis and treatment of disease. Science, 1995. **267**(5203): p. 1456.
2. Reed, J.C., *Mechanisms of apoptosis*. The American journal of pathology, 2000. **157**(5): p. 1415-1430.
3. Czabotar, P.E., et al., Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nature reviews Molecular cell biology, 2014. **15**(1): p. 49-63.
4. Green, D.R. and G.I. Evan, *A matter of life and death*. Cancer cell, 2002. **1**(1): p. 19-30.
5. Kluck, R.M., et al., The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science, 1997. **275**(5303): p. 1132-1136.
6. Li, P., et al., Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell, 1997. **91**(4): p. 479-489.
7. Riedl, S.J. and G.S. Salvesen, The apoptosome: signalling platform of cell death. Nature reviews Molecular cell biology, 2007. **8**(5): p. 405-413.
8. Griffiths, G.J., et al., Cell damage-induced conformational changes of the pro-apoptotic protein Bak in vivo precede the onset of apoptosis. The Journal of cell biology, 1999. **144**(5): p. 903-914.
9. Wolter, K.G., et al., Movement of Bax from the cytosol to mitochondria during apoptosis. The Journal of cell biology, 1997. **139**(5): p. 1281-1292.

10. Suzuki, M., R.J. Youle, and N. Tjandra, Structure of Bax: coregulation of dimer formation and intracellular localization. *Cell*, 2000. **103**(4): p. 645-654.
11. Letai, A., et al., Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer cell*, 2002. **2**(3): p. 183-192.
12. Kuwana, T., et al., BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Molecular cell*, 2005. **17**(4): p. 525-535.
13. Certo, M., et al., Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer cell*, 2006. **9**(5): p. 351-365.
14. Gavathiotis, E., et al., BAX activation is initiated at a novel interaction site. *Nature*, 2008. **455**(7216): p. 1076-1081.
15. Chen, L., et al., Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Molecular cell*, 2005. **17**(3): p. 393-403.
16. Willis, S.N., et al., Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science*, 2007. **315**(5813): p. 856-859.
17. Willis, S.N., et al., Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes & development*, 2005. **19**(11): p. 1294-1305.
18. Mérimo, D., et al., The role of BH3-only protein Bim extends beyond inhibiting Bcl-2-like prosurvival proteins. *The Journal of cell biology*, 2009. **186**(3): p. 355-362.

19. Hinds, M., et al., Bim, Bad and Bmf: intrinsically unstructured BH3-only proteins that undergo a localized conformational change upon binding to prosurvival Bcl-2 targets. *Cell Death & Differentiation*, 2007. **14**(1): p. 128-136.
20. Fesikt, W., X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. 1996.
21. McDonnell, J.M., et al., Solution structure of the proapoptotic molecule BID: a structural basis for apoptotic agonists and antagonists. *Cell*, 1999. **96**(5): p. 625-634.
22. Chou, J.J., et al., Solution structure of BID, an intracellular amplifier of apoptotic signaling. *Cell*, 1999. **96**(5): p. 615-624.
23. Czabotar, P.E., et al., Bax crystal structures reveal how BH3 domains activate Bax and nucleate its oligomerization to induce apoptosis. *Cell*, 2013. **152**(3): p. 519-531.
24. Motoyama, N., et al., Massive cell death of immature hematopoietic cells and neurons in Bcl-x-deficient mice. *Science*, 1995. **267**(5203): p. 1506.
25. Veis, D.J., et al., Bcl-2-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell*, 1993. **75**(2): p. 229-240.
26. Opferman, J.T., et al., Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature*, 2003. **426**(6967): p. 671-676.
27. Opferman, J.T., et al., Obligate role of anti-apoptotic MCL-1 in the survival of hematopoietic stem cells. *Science*, 2005. **307**(5712): p. 1101-1104.
28. Vikstrom, I., et al., Mcl-1 is essential for germinal center formation and B cell memory. *Science*, 2010. **330**(6007): p. 1095-1099.

29. Peperzak, V., et al., Mcl-1 is essential for the survival of plasma cells. *Nature immunology*, 2013. **14**(3): p. 290-297.
30. Thomas, R.L., et al., Loss of MCL-1 leads to impaired autophagy and rapid development of heart failure. *Genes & development*, 2013. **27**(12): p. 1365-1377.
31. Wang, X., et al., Deletion of MCL-1 causes lethal cardiac failure and mitochondrial dysfunction. *Genes & development*, 2013. **27**(12): p. 1351-1364.
32. Bouillet, P., et al., Proapoptotic Bcl-2 relative Bim required for certain apoptotic responses, leukocyte homeostasis, and to preclude autoimmunity. *Science*, 1999. **286**(5445): p. 1735-1738.
33. Bouillet, P., et al., BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature*, 2002. **415**(6874): p. 922-926.
34. Enders, A., et al., Loss of the pro-apoptotic BH3-only Bcl-2 family member Bim inhibits BCR stimulation–induced apoptosis and deletion of autoreactive B cells. *The Journal of experimental medicine*, 2003. **198**(7): p. 1119-1126.
35. Egle, A., et al., *Bim is a suppressor of Myc-induced mouse B cell leukemia*. *Proceedings of the National Academy of Sciences of the United States of America*, 2004. **101**(16): p. 6164-6169.
36. Garrison, S.P., et al., Selection against PUMA gene expression in Myc-driven B-cell lymphomagenesis. *Molecular and cellular biology*, 2008. **28**(17): p. 5391-5402.
37. Letai, A.G., Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nature Reviews Cancer*, 2008. **8**(2): p. 121-132.

38. Eischen, C.M., et al., Bax loss impairs Myc-induced apoptosis and circumvents the selection of p53 mutations during Myc-mediated lymphomagenesis. *Molecular and cellular biology*, 2001. **21**(22): p. 7653-7662.
39. Deng, J., et al., BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer cell*, 2007. **12**(2): p. 171-185.
40. Kirkin, V., S. Joos, and M. Zörnig, *The role of Bcl-2 family members in tumorigenesis*. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 2004. **1644**(2): p. 229-249.
41. Tsujimoto, Y., et al., Cloning of the chromosome breakpoint of neoplastic B cells with the t (14; 18) chromosome translocation. *Science*, 1984. **226**(4678): p. 1097-1099.
42. Ben-Ezra, J.M., et al., Small cell carcinomas of the lung express the Bcl-2 protein. *The American journal of pathology*, 1994. **145**(5): p. 1036.
43. Higashiyama, M., et al., High prevalence of bcl-2 oncoprotein expression in small cell lung cancer. *Anticancer research*, 1994. **15**(2): p. 503-505.
44. Leiter, U., et al., Antiapoptotic bcl-2 and bcl-xL in advanced malignant melanoma. *Archives of dermatological research*, 2000. **292**(5): p. 225-232.
45. Schena, M., et al., Growth-and differentiation-associated expression of bcl-2 in B-chronic lymphocytic leukemia cells. *Blood*, 1992. **79**(11): p. 2981-2989.
46. Lipponen, P., et al., Apoptosis suppressing protein bcl-2 is expressed in well-differentiated breast carcinomas with favourable prognosis. *The Journal of pathology*, 1995. **177**(1): p. 49-55.

47. Lê, M.G., et al., c-myc, p53 and bcl-2, apoptosis-related genes in infiltrating breast carcinomas: Evidence of a link between bcl-2 protein over-expression and a lower risk of metastasis and death in operable patients. *International journal of cancer*, 1999. **84**(6): p. 562-567.
48. Nakopoulou, L., et al., bcl-2 protein expression is associated with a prognostically favourable phenotype in breast cancer irrespective of p53 immunostaining. *Histopathology*, 1999. **34**(4): p. 310-319.
49. Beroukhi, R., et al., The landscape of somatic copy-number alteration across human cancers. *Nature*, 2010. **463**(7283): p. 899-905.
50. Reed, J.C., et al., Antisense-mediated inhibition of BCL2 protooncogene expression and leukemic cell growth and survival: comparisons of phosphodiester and phosphorothioate oligodeoxynucleotides. *Cancer research*, 1990. **50**(20): p. 6565-6570.
51. Olie, R.A. and U. Zangemeister-Wittke, Targeting tumor cell resistance to apoptosis induction with antisense oligonucleotides: progress and therapeutic potential. *Drug Resistance Updates*, 2001. **4**(1): p. 9-15.
52. Cummings, J., et al., Apoptosis pathway-targeted drugs—from the bench to the clinic. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, 2004. **1705**(1): p. 53-66.
53. Fesik, S.W., Promoting apoptosis as a strategy for cancer drug discovery. *Nature Reviews Cancer*, 2005. **5**(11): p. 876-885.
54. Gleave, M.E. and B.P. Monia, *Antisense therapy for cancer*. *Nature Reviews Cancer*, 2005. **5**(6): p. 468-479.

55. Pandey, M.K., et al., Targeting Cell Survival Proteins for Cancer Cell Death. Pharmaceuticals, 2016. **9**(1): p. 11.
56. O'Brien, S., et al., 5-year survival in patients with relapsed or refractory chronic lymphocytic leukemia in a randomized, phase III trial of fludarabine plus cyclophosphamide with or without oblimersen. Journal of Clinical Oncology, 2009. **27**(31): p. 5208-5212.
57. Frantz, S., Lessons learnt from Genasense's failure. Nature Reviews Drug Discovery, 2004. **3**(7): p. 542-542.
58. Muchmore, S.W., et al., X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. Nature, 1996. **381**(6580): p. 335-341.
59. Sattler, M., et al., Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science, 1997. **275**(5302): p. 983-986.
60. Oltersdorf, T., et al., An inhibitor of Bcl-2 family proteins induces regression of solid tumours. Nature, 2005. **435**(7042): p. 677-681.
61. Tse, C., et al., ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. Cancer research, 2008. **68**(9): p. 3421-3428.
62. Wilson, W.H., et al., Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. The lancet oncology, 2010. **11**(12): p. 1149-1159.
63. Roberts, A.W., et al., Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. Journal of Clinical Oncology, 2012. **30**(5): p. 488-496.

64. Moore, V.D.G., et al., Chronic lymphocytic leukemia requires BCL2 to sequester prodeath BIM, explaining sensitivity to BCL2 antagonist ABT-737. *The Journal of clinical investigation*, 2007. **117**(1): p. 112-121.
65. Mérimo, D., et al., Bcl-2, Bcl-xL, and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. *Blood*, 2012. **119**(24): p. 5807-5816.
66. Mason, K.D., et al., In vivo efficacy of the Bcl-2 antagonist ABT-737 against aggressive Myc-driven lymphomas. *Proceedings of the National Academy of Sciences*, 2008. **105**(46): p. 17961-17966.
67. Tan, N., et al., Navitoclax enhances the efficacy of taxanes in non-small cell lung cancer models. *Clinical Cancer Research*, 2011. **17**(6): p. 1394-1404.
68. Shoemaker, A.R., et al., Activity of the Bcl-2 family inhibitor ABT-263 in a panel of small cell lung cancer xenograft models. *Clinical Cancer Research*, 2008. **14**(11): p. 3268-3277.
69. Ackler, S., et al., The Bcl-2 inhibitor ABT-263 enhances the response of multiple chemotherapeutic regimens in hematologic tumors in vivo. *Cancer chemotherapy and pharmacology*, 2010. **66**(5): p. 869-880.
70. Oakes, S.R., et al., Sensitization of BCL-2-expressing breast tumors to chemotherapy by the BH3 mimetic ABT-737. *Proceedings of the National Academy of Sciences*, 2012. **109**(8): p. 2766-2771.
71. Souers, A.J., et al., ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nature medicine*, 2013. **19**(2): p. 202-208.

72. Roberts, A.W., et al., Targeting BCL2 with venetoclax in relapsed chronic lymphocytic leukemia. *New England Journal of Medicine*, 2016. **374**(4): p. 311-322.
73. Lessene, G., et al., Structure-guided design of a selective BCL-XL inhibitor. *Nature chemical biology*, 2013. **9**(6): p. 390-397.
74. Delbridge, A.R., et al., Thirty years of BCL-2: translating cell death discoveries into novel cancer therapies. *Nature Reviews Cancer*, 2016. **16**(2): p. 99-109.
75. Levitt, M. and A. Warshel, Computer simulation of protein folding. *Nature*, 1975. **253**(5494): p. 694-698.
76. McCammon, J.A., B.R. Gelin, and M. Karplus, *Dynamics of folded proteins*. *Nature*, 1977. **267**(5612): p. 585-590.
77. Artymiuk, P., et al., Crystallographic studies of the dynamic properties of lysozyme. 1979.
78. Frauenfelder, H., G.A. Petsko, and D. Tsernoglou, Temperature-dependent X-ray diffraction as a probe of protein structural dynamics. 1979.
79. Karplus, M. and J.A. McCammon, Molecular dynamics simulations of biomolecules. *Nature Structural & Molecular Biology*, 2002. **9**(9): p. 646-652.
80. Ichiye, T. and M. Karplus, Fluorescence depolarization of tryptophan residues in proteins: a molecular dynamics study. *Biochemistry*, 1983. **22**(12): p. 2884-2893.
81. Nilsson, L., et al., Structure refinement of oligonucleotides by molecular dynamics with nuclear Overhauser effect interproton distance restraints: application to 5' d (CGTACG) 2. *Journal of molecular biology*, 1986. **188**(3): p. 455-475.
82. Brunger, A.T., J. Kuriyan, and M. Karplus, Crystallographic R factor refinement by molecular dynamics. *Science*, 1987. **235**(4787): p. 458-460.

83. Case, D. and M. Karplus, Dynamics of ligand binding to heme proteins. *Journal of molecular biology*, 1979. **132**(3): p. 343-368.
84. Nussinov, R., The significance of the 2013 Nobel Prize in Chemistry and the challenges ahead. *PLoS Comput Biol*, 2014. **10**(1): p. e1003423.
85. Warshel, A. and M. Levitt, Theoretical studies of enzymic reactions: dielectric, electrostatic and steric stabilization of the carbonium ion in the reaction of lysozyme. *Journal of molecular biology*, 1976. **103**(2): p. 227-249.
86. Field, M.J., P.A. Bash, and M. Karplus, A combined quantum mechanical and molecular mechanical potential for molecular dynamics simulations. *Journal of Computational Chemistry*, 1990. **11**(6): p. 700-733.
87. Warshel, A., *Computer modelling of chemical reactions in enzymes and solutions*. 2013.
88. Gilson, M.K. and H.-X. Zhou, *Calculation of protein-ligand binding affinities*. *Annual review of biophysics and biomolecular structure*, 2007. **36**(1): p. 21.
89. Case, D.A., et al., *Amber 10*. 2008, University of California.
90. Lindahl, E.R., *Molecular dynamics simulations*. *Molecular modeling of proteins*, 2008: p. 3-23.
91. Petrenko, R. and J. Meller, *Molecular dynamics*. eLS, 2010.
92. Cornell, W.D., et al., A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. *Journal of the American Chemical Society*, 1995. **117**(19): p. 5179-5197.

93. Duan, Y., et al., A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations. *Journal of computational chemistry*, 2003. **24**(16): p. 1999-2012.
94. Jensen, F., *Introduction to computational chemistry*. 2013: John Wiley & Sons.
95. Zwanzig, R.W., High-temperature equation of state by a perturbation method. I. nonpolar gases. *The Journal of Chemical Physics*, 1954. **22**(8): p. 1420-1426.
96. Kirkwood, J.G., *Statistical mechanics of fluid mixtures*. *The Journal of Chemical Physics*, 1935. **3**(5): p. 300-313.
97. Christ, C.D. and T. Fox, Accuracy assessment and automation of free energy calculations for drug design. *Journal of chemical information and modeling*, 2013. **54**(1): p. 108-120.
98. van Gunsteren, W.F., P.K. Weiner, and A.J. Wilkinson, *Computer simulation of biomolecular systems: theoretical and experimental applications*. Vol. 3. 2013: Springer Science & Business Media.
99. Christ, C.D. and W.F. van Gunsteren, Enveloping distribution sampling: A method to calculate free energy differences from a single simulation. *The Journal of chemical physics*, 2007. **126**(18): p. 184110.
100. Bennett, C.H., Efficient estimation of free energy differences from Monte Carlo data. *Journal of Computational Physics*, 1976. **22**(2): p. 245-268.
101. de Ruiter, A. and C. Oostenbrink, Protein-Ligand Binding from Distancefield Distances and Hamiltonian Replica Exchange Simulations. *Journal of chemical theory and computation*, 2013. **9**(2): p. 883-892.

102. Srinivasan, J., et al., Continuum solvent studies of the stability of DNA, RNA, and phosphoramidate– DNA helices. *Journal of the American Chemical Society*, 1998. **120**(37): p. 9401-9409.
103. Homeyer, N. and H. Gohlke, Free energy calculations by the molecular mechanics Poisson– Boltzmann surface area method. *Molecular Informatics*, 2012. **31**(2): p. 114-122.
104. Kollman, P.A., et al., Calculating structures and free energies of complex molecules: combining molecular mechanics and continuum models. *Accounts of chemical research*, 2000. **33**(12): p. 889-897.
105. Wagoner, J. and N.A. Baker, Solvation forces on biomolecular structures: a comparison of explicit solvent and Poisson–Boltzmann models. *Journal of computational chemistry*, 2004. **25**(13): p. 1623-1629.
106. Adams, J.M. and S. Cory, The Bcl-2 protein family: arbiters of cell survival. *Science*, 1998. **281**(5381): p. 1322-1326.
107. Adams, J.M. and S. Cory, Life-or-death decisions by the Bcl-2 protein family. *Trends in biochemical sciences*, 2001. **26**(1): p. 61-66.
108. Kvaisakul, M., et al., Vaccinia virus anti-apoptotic F1L is a novel Bcl-2-like domain-swapped dimer that binds a highly selective subset of BH3-containing death ligands. *Cell Death & Differentiation*, 2008. **15**(10): p. 1564-1571.
109. Kim, H., et al., Hierarchical regulation of mitochondrion-dependent apoptosis by BCL-2 subfamilies. *Nature cell biology*, 2006. **8**(12): p. 1348-1358.
110. Li, H., et al., Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*, 1998. **94**(4): p. 491-501.

111. Gross, A., J.M. McDonnell, and S.J. Korsmeyer, BCL-2 family members and the mitochondria in apoptosis. *Genes & development*, 1999. **13**(15): p. 1899-1911.
112. Liu, X., et al., The structure of a Bcl-x L/Bim fragment complex: Implications for Bim function. *Immunity*, 2003. **19**(3): p. 341-352.
113. Fletcher, J.I., et al., Apoptosis is triggered when prosurvival Bcl-2 proteins cannot restrain Bax. *Proceedings of the National Academy of Sciences*, 2008. **105**(47): p. 18081-18087.
114. Petros, A.M., et al., *Solution structure of the antiapoptotic protein bcl-2*. *Proceedings of the National Academy of Sciences*, 2001. **98**(6): p. 3012-3017.
115. Zhai, D., et al., Differential regulation of Bax and Bak by anti-apoptotic Bcl-2 family proteins Bcl-B and Mcl-1. *Journal of Biological Chemistry*, 2008. **283**(15): p. 9580-9586.
116. Ku, B., et al., Evidence that inhibition of BAX activation by BCL-2 involves its tight and preferential interaction with the BH3 domain of BAX. *Cell research*, 2010. **21**(4): p. 627-641.
117. Berman, H.M., et al., *The protein data bank*. *Nucleic acids research*, 2000. **28**(1): p. 235-242.
118. Jorgensen, W.L., et al., Comparison of simple potential functions for simulating liquid water. *The Journal of chemical physics*, 1983. **79**: p. 926.
119. Essmann, U., et al., A smooth particle mesh Ewald method. *Journal of Chemical Physics*, 1995. **103**(19): p. 8577-8593.

120. Ryckaert, J.-P., G. Ciccotti, and H.J. Berendsen, Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of *n*-alkanes. *Journal of Computational Physics*, 1977. **23**(3): p. 327-341.
121. Pastor, R.W., B.R. Brooks, and A. Szabo, An analysis of the accuracy of Langevin and molecular dynamics algorithms. *Molecular Physics*, 1988. **65**(6): p. 1409-1419.
122. Naïm, M., et al., Solvated interaction energy (SIE) for scoring protein-ligand binding affinities. 1. Exploring the parameter space. *Journal of chemical information and modeling*, 2007. **47**(1): p. 122-133.
123. Cui, Q., et al., Molecular Dynamics—Solvated Interaction Energy Studies of Protein–Protein Interactions: The MP1–p14 Scaffolding Complex. *Journal of molecular biology*, 2008. **379**(4): p. 787-802.
124. Rodriguez-Granillo, A., A. Crespo, and P. Wittung-Stafshede, Interdomain interactions modulate collective dynamics of the metal-binding domains in the Wilson disease protein. *The Journal of Physical Chemistry B*, 2010. **114**(5): p. 1836-1848.
125. Yang, B., et al., Computational determination of binding structures and free energies of phosphodiesterase-2 with benzo [1, 4] diazepam-2-one derivatives. *The Journal of Physical Chemistry B*, 2010. **114**(48): p. 16020-16028.
126. Massova, I. and P.A. Kollman, Computational alanine scanning to probe protein-protein interactions: a novel approach to evaluate binding free energies. *Journal of the American Chemical Society*, 1999. **121**(36): p. 8133-8143.
127. Case, D., et al., *AMBER II*. University of California, San Francisco, 2010. **142**.

128. Spierings, D., et al., Connected to death: the (unexpurgated) mitochondrial pathway of apoptosis. *Science*, 2005. **310**(5745): p. 66-67.
129. Skommer, J., D. Wlodkowic, and A. Deptala, Larger than life: mitochondria and the Bcl-2 family. *Leukemia research*, 2007. **31**(3): p. 277-286.
130. Sorenson, C.M., *Bcl-2 family members and disease*. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 2004. **1644**(2): p. 169-177.
131. Petros, A.M., E.T. Olejniczak, and S.W. Fesik, *Structural biology of the Bcl-2 family of proteins*. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, 2004. **1644**(2): p. 83-94.
132. Petros, A.M., et al., Rationale for Bcl-XL/Bad peptide complex formation from structure, mutagenesis, and biophysical studies. *Protein Science*, 2000. **9**(12): p. 2528-2534.
133. Yin, X.-M., Z.N. Oltvai, and S.J. Korsmeyer, BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature*, 1994. **369**(6478): p. 321-323.
134. Day, C.L., et al., Solution structure of prosurvival Mcl-1 and characterization of its binding by proapoptotic BH3-only ligands. *Journal of Biological Chemistry*, 2005. **280**(6): p. 4738-4744.
135. Zha, J., et al., BH3 domain of BAD is required for heterodimerization with BCL-XL and pro-apoptotic activity. *Journal of Biological Chemistry*, 1997. **272**(39): p. 24101-24104.

136. Wang, K., et al., Mutagenesis of the BH3 domain of BAX identifies residues critical for dimerization and killing. *Molecular and Cellular Biology*, 1998. **18**(10): p. 6083-6089.
137. Campos, L., et al., High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood*, 1993. **81**(11): p. 3091-3096.
138. Olopade, O.I., et al., Overexpression of BCL-x protein in primary breast cancer is associated with high tumor grade and nodal metastases. *The cancer journal from Scientific American*, 1996. **3**(4): p. 230-237.
139. Lama, D. and R. Sankararamakrishnan, Anti-apoptotic Bcl-XL protein in complex with BH3 peptides of pro-apoptotic Bak, Bad, and Bim proteins: Comparative molecular dynamics simulations. *Proteins: Structure, Function, and Bioinformatics*, 2008. **73**(2): p. 492-514.
140. Lama, D. and R. Sankararamakrishnan, Molecular dynamics simulations of pro-apoptotic BH3 peptide helices in aqueous medium: relationship between helix stability and their binding affinities to the anti-apoptotic protein Bcl-XL. *Journal of computer-aided molecular design*, 2011. **25**(5): p. 413-426.
141. Delgado-Soler, L., et al., Molecular determinants of Bim (BH3) peptide binding to pro-survival proteins. *Journal of chemical information and modeling*, 2012. **52**(8): p. 2107-2118.
142. Sadiq, S.K., et al., Accurate ensemble molecular dynamics binding free energy ranking of multidrug-resistant HIV-1 proteases. *Journal of chemical information and modeling*, 2010. **50**(5): p. 890-905.

143. Gohlke, H., C. Kiel, and D.A. Case, Insights into protein–protein binding by binding free energy calculation and free energy decomposition for the Ras–Raf and Ras–RalGDS complexes. *Journal of molecular biology*, 2003. **330**(4): p. 891-913.
144. Weiser, J., P.S. Shenkin, and W.C. Still, Approximate atomic surfaces from linear combinations of pairwise overlaps (LCPO). *Journal of Computational Chemistry*, 1999. **20**(2): p. 217-230.
145. Lee, E.F., et al., A novel BH3 ligand that selectively targets Mcl-1 reveals that apoptosis can proceed without Mcl-1 degradation. *The Journal of cell biology*, 2008. **180**(2): p. 341-355.
146. Boersma, M.D., et al., Hydrophile scanning as a complement to alanine scanning for exploring and manipulating protein–protein recognition: application to the Bim BH3 domain. *Protein Science*, 2008. **17**(7): p. 1232-1240.
147. Czabotar, P.E., et al., Structural insights into the degradation of Mcl-1 induced by BH3 domains. *Proceedings of the National Academy of Sciences*, 2007. **104**(15): p. 6217-6222.
148. Bajwa, N., C. Liao, and Z. Nikolovska-Coleska, Inhibitors of the anti-apoptotic Bcl-2 proteins: a patent review. *Expert opinion on therapeutic patents*, 2012. **22**(1): p. 37-55.
149. Huang, D.C. and A. Strasser, BH3-only proteins—essential initiators of apoptotic cell death. *Cell*, 2000. **103**(6): p. 839-842.
150. Llambi, F., et al., A unified model of mammalian BCL-2 protein family interactions at the mitochondria. *Molecular cell*, 2011. **44**(4): p. 517-531.

151. Nakano, K. and K.H. Vousden, PUMA, a novel proapoptotic gene, is induced by p53. *Molecular cell*, 2001. **7**(3): p. 683-694.
152. Yu, J., et al., PUMA induces the rapid apoptosis of colorectal cancer cells. *Molecular cell*, 2001. **7**(3): p. 673-682.
153. Jeffers, J.R., et al., Puma is an essential mediator of p53-dependent and-independent apoptotic pathways. *Cancer cell*, 2003. **4**(4): p. 321-328.
154. Youle, R.J. and A. Strasser, The BCL-2 protein family: opposing activities that mediate cell death. *Nature reviews Molecular cell biology*, 2008. **9**(1): p. 47-59.
155. Shibue, T., et al., Integral role of Noxa in p53-mediated apoptotic response. *Genes & development*, 2003. **17**(18): p. 2233-2238.
156. Naik, E., et al., Ultraviolet radiation triggers apoptosis of fibroblasts and skin keratinocytes mainly via the BH3-only protein Noxa. *The Journal of cell biology*, 2007. **176**(4): p. 415-424.
157. Ranger, A.M., et al., Bad-deficient mice develop diffuse large B cell lymphoma. *Proceedings of the National Academy of Sciences*, 2003. **100**(16): p. 9324-9329.
158. Kelly, P., et al., Individual and overlapping roles of BH3-only proteins Bim and Bad in apoptosis of lymphocytes and platelets and in suppression of thymic lymphoma development. *Cell Death & Differentiation*, 2010. **17**(10): p. 1655-1664.
159. Baumgartner, F., et al., Minor cell-death defects but reduced tumor latency in mice lacking the BH3-only proteins Bad and Bmf. *Oncogene*, 2013. **32**(5): p. 621-630.
160. van Delft, M.F., et al., The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer cell*, 2006. **10**(5): p. 389-399.

161. Lee, E., et al., Crystal structure of ABT-737 complexed with Bcl-xL: implications for selectivity of antagonists of the Bcl-2 family. *Cell Death & Differentiation*, 2007. **14**(9): p. 1711-1713.
162. Ku, B., et al., Structural and biochemical bases for the inhibition of autophagy and apoptosis by viral BCL-2 of murine γ -herpesvirus 68. *PLoS pathogens*, 2008. **4**(2): p. e25.
163. Day, C.L., et al., Structure of the BH3 domains from the p53-inducible BH3-only proteins Noxa and Puma in complex with Mcl-1. *Journal of molecular biology*, 2008. **380**(5): p. 958-971.
164. Luo, R., L. David, and M.K. Gilson, Accelerated Poisson–Boltzmann calculations for static and dynamic systems. *Journal of computational chemistry*, 2002. **23**(13): p. 1244-1253.
165. Sitkoff, D., K.A. Sharp, and B. Honig, Accurate calculation of hydration free energies using macroscopic solvent models. *The Journal of Physical Chemistry*, 1994. **98**(7): p. 1978-1988.
166. Nguyen, D.T. and D.A. Case, On finding stationary states on large-molecule potential energy surfaces. *The Journal of Physical Chemistry*, 1985. **89**(19): p. 4020-4026.
167. Smith, T.F. and M.S. Waterman, Identification of common molecular subsequences. *Journal of molecular biology*, 1981. **147**(1): p. 195-197.
168. Moroy, G., et al., Molecular Basis for Bcl-2 Homology 3 Domain Recognition in the Bcl-2 Protein Family IDENTIFICATION OF CONSERVED HOT SPOT INTERACTIONS. *Journal of Biological Chemistry*, 2009. **284**(26): p. 17499-17511.

169. Lessene, G., P.E. Czabotar, and P.M. Colman, *BCL-2 family antagonists for cancer therapy*. Nature Reviews Drug Discovery, 2008. **7**(12): p. 989-1000.
170. Yang, C.-Y. and S. Wang, Analysis of flexibility and hotspots in bcl-xl and mcl-1 proteins for the design of selective small-molecule inhibitors. ACS Medicinal Chemistry Letters, 2012. **3**(4): p. 308-312.
171. Dagliyan, O., et al., Structural and dynamic determinants of protein-peptide recognition. Structure, 2011. **19**(12): p. 1837-1845.
172. Fosgerau, K. and T. Hoffmann, Peptide therapeutics: current status and future directions. Drug discovery today, 2015. **20**(1): p. 122-128.