POTENTIATION OF THE NASOPHARYNGEAL CARCINOMA CELL LINES BY MARITOCLAX TO ABT-263 IN 2-DIMENSIONAL AND 3-DIMENSIONAL CELL CULTURE METHODS

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

2D	2-dimensional
3D	3-dimensional
Akt	Protein kinase B
APAF-1	Apoptotic protease activating factor 1
ApoG2	Apogossypolone
BAD	BCL-2 antagonist of cell death
BART	*Bam*HI-A region rightward transcript
BAK	BCL-2 antagonist killer 1
BAX	BCL-2 associated X protein
BCL-2	B-cell lymphoma-2
BCL-XL	B-cell lymphoma-extra large
BCL-w	B-cell lymphoma-2 like protein
BCL-2 ASO	Bcl-2 oligodeoxynucleotide antisense
BFL-1/A1	B-cell related protein A1
BH	BCL-2 homology
BID	BCL-2 interacting domain death agonist
BIK	BCL-2 interacting killer
BIM	BCL-2 interacting mediator of cell death
BMF	BCL-2 modifying factor
BOK	BCL-2 related ovarian killer
CTAR	C-terminal activating region
DF	Death factor
DR	Death receptor

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EBER	EBV-encoded small RNA
EBNA	EBV-determined nuclear antigen
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMEM	Eagle's Minimum Essential Medium
FADD	Fas-associated death domain
FASL	Fas ligand
FBS	Fetal bovine serum
FJX1	Four-jointed box 1
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme-A
HRK	Harakiri
IHC	Immunohistochemistry
IMRT	Intensity-modulated radiotherapy
JAK	Janus kinase
JNK	Jun N-terminal kinase
LMP	Latent membrane protein
MAPK	Mitogen-activated protein kinases
MCL-1	Myeloid cell leukaemia 1
MOMP	Mitochondrial outer membrane permeabilization
mTOR	Mammalian target for Rapamycin
$NaHCO_3$	Sodium bicarbonate
NF-κ B	Nuclear factor κ -B

NPC	Nasopharyngeal carcinoma
NOXA	Phorbol-12-myristate-13-acetate-induced protein-1 (NOXA)
OMM	Outer mitochondrial membrane
PI3K	Phosphoinositol-3-kinase
PUMA	p53-upregulated modulator of apoptosis
RPMI	Rosewell Park Memorial Institute 1640
SDS	Sodium dodecyl sulphate
STAT	Signal transducers and activators of transcription
tBID	Truncated BID
TM	Transmembrane
TNF	Tumour necrosis factor
TRAIL	TNF related apoptosis-inducing ligand
ULA	Ultralow-attachment
VEGF	Vascular endothelial growth factor
VDAC	Voltage-dependent anion channel
WHO	World Health Organization

POTENSIASI SEL KANSER NASOFARINKS OLEH MARITOCLAX KEPADA ABT-263 DALAM KAEDAH SEL KULTUR 2-DIMENSI DAN 3-DIMENSI

ABSTRAK

Malaysia mempunyai kes kanser nasofarinks, lebih dikenali sebagai kanser pangkal hidung, yang tertinggi di dunia. Kanser nasofarinks merupakan sejenis kanser yang boleh diubati jika dirawat pada peringkat awal. Namun demikian, rawatan yang sedia ada amat terhad untuk merawat kanser nasofarinks yang berulangan atau barah yang dikesan pada pringkat lewat. Matlamat kajian ini adalah untuk mensasar protein famili BCL-2 yang memainkan peranan penting dalam apoptosis. Beberapa protein dalam famili ini terutamanya protein anti-apoptotik diekspres pada tahap yang tinggi dalam tisu NPC dan oleh itu menjadi sasaran utama terapeutik. Kepentingan klinikal dalam mensasar protein ini meningkat dengan kejayaan pembangunan mimetik BH3, yang mensasar protein anti-apoptotik secara spesifik. Perencat molekul kecil BH3 yang dikenali sebagai ABT-263 boleh merencatkan protein anti-apoptotik BCL-2, BCL-XL dan BCL-w, telah menunjukkan hasil rawatan yang memberansangkan dalam kanser hematopoitik dan kanser paru-paru sel kecil tetapi tidak berkesan dalam tumor pepejal, termasuk kanser nasofarinks. Hal ini disebabkan oleh pengekspresan protein MCL-1 dalam tumor pepejal yang menyebabkan sel kanser resistan terhadap rawatan ABT-263. Justeru itu, pengawalan ekspresi protein MCL-1 dalam tumor pepejal dengan menggunakan drug yang dapat menurunkan tahap MCL-1 atau meneutralkan MCL-1 dijangka mampu meningkatkan kesensitifan sel kanser kepada ABT-263. Di sini saya melaporkan bahawa pengurangan tahap MCL-1 dalam kedua-dua titisan sel kanser nasofarinks, iaitu HK-1 dan C666-1 oleh Maritoclax, bergantung

kepada konsentrasi drug dan tempoh rawatan. Hasil daripada gabungan antara Maritoclax dan ABT-263 ke atas dua jenis titisan sel kanser nasofarinks telah menunjukkan kesan sinergistik. Kajian potensi drug juga menunjukkan kedua-dua titisan sel HK-1 dan C666-1 dapat disensitifkan oleh Maritoclax kepada ABT-263 tetapi potensi drug pada arah yang bertentangan didapati lemah. Hasil kajian yang sama turut dijalankan dengan menggunakan kaedah kultur sel 3D. Kaedah kultur sel 3D telah digunakkan kerana ia menyifatkan persekitaran mikro dan arkitektur tumor *in vivo*. Maritoclax dapat meningkatkan kesensitifan sferoid 3D HK-1 kepada ABT-263 tetapi kesensitifan sferoid kepada Maritoclax oleh ABT-263 didapati lemah. Gabungan dengan Maritoclax juga mensensitifkan gugusan longgar C666-1 kepada ABT-263. Lebih penting, kedua-dua HK-1 dan C666-1 tidak menunjukkan resistan terhadap kombinasi Maritoclax dan ABT-263. Hasil kajian awal mendapati bahawa Simvastatin dapat mensensitifkan HK-1 dan C666-1 kepada ABT-263. Walaupun ABT-263 tidak dapat meningkatkan kesensitifan sel HK-1 kepada Maritoclax, ia agak berkesan dalam mensensitikan sel C666-1 kepada Maritoclax tetapi dalam tetingkap yang sempit. Secara keseluruhan, kajian menunjukkan bahawa kombinasi drug yang dikaji, mempunyai potensi sebagai strategi rawatan kanser nasofarinks. Kajian yang lebih lanjut dengan menggunakan sferoid 3D dan model preklinikal diperlukan untuk membongkar sepenuhnya prospek kombinasi ini.

POTENTIATION OF THE NASOPHARYNGEAL CARCINOMA CELL LINES BY MARITOCLAX TO ABT-263 IN 2-DIMENSIONAL AND 3-DIMENSIONAL CELL CULTURE METHODS

ABSTRACT

Malaysia has one of the highest incidences of Nasopharyngeal carcinoma (NPC) in the world. The cancer is remarkably curable at early stages but treatment options become limited when patients develop a recurrence or diagnosed late, leaving them with very little hope to combat the cancer. The goal of this project is to pharmacologically target the BCL-2 family proteins, the critical regulators of the apoptosis pathway. Some members of the anti-apoptotic proteins are highly expressed in NPC tissues and hence have become attractive therapeutic targets. Clinical interest in targeting these proteins increased with the successful development of BH3-mimetics, which selectively target the anti-apoptotic proteins. BH3-mimetic ABT-263, which selectively inhibits BCL-2, BCL-XL and BCL-w, showed impressive single agent activity in hematopoietic tumours and small cell lung carcinoma (SCLC) but the effect of this drug as monotherapy was disappointing in most solid tumours, including NPC. Anti-apoptotic protein MCL-1 was characterised as the resistance factor of ABT-263 in solid tumours and hence, chemotherapeutic agents or targeted therapies that can reduce the level or neutralise MCL-1 are predicted to synergise with ABT-263. Here I report that Maritoclax reduced the level of MCL-1 in a dose- and time-dependant manner. Combination of Maritoclax and ABT-263 exhibited synergistic effects on the NPC cell lines HK1 and C666-1. Drug potentiation studies revealed that Maritoclax sensitised both HK1 and C666-1 cells to ABT-263 but potentiation was weaker in the opposite direction. Similar results were obtained when the combination was tested in

the 3D cell culture model. The 3D cell culture model was employed as it recapitulates tumour microenvironment and architecture more closely to tumour *in vivo*. Maritoclax potentiated the 3D HK-1 spheroids to ABT-263 but potentiation of the HK-1 spheroids to Maritoxlax by ABT-263 was weak. Combination with Maritoclax markedly sensitised the C666-1 loose aggregates to ABT-263. More notably, both HK-1 and C666-1 cells did not develop resistance to the combination rapidly. Preliminary data demonstrated that Simvastatin sensitised the HK-1 and the C666-1 cells to ABT-263 but potentiation in the reverse direction was modest in the HK-1 cells. Surprisingly, ABT-263 sensitised the C666-1 cells to Simvastatin but this sensitisation occurred in a narrow window. Collectively, the findings illustrate that the combinations investigated could be potential treatment strategies for NPC but further studies in 3D and preclinical models are warranted to fully unravel the prospects of the duos.

CHAPTER 1

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is an aggressive and deadly cancer. NPC is the sixth most common cancer in Southeast Asia with Malaysia reporting one of the highest national incidences (Jemal *et al*., 2011). NPC is curable with radical radiotherapy when detected early but the high probability of distant metastases reduces patient survival (Wee *et al*., 2010; Zhang *et al*., 2013). The median survival time for patients who are suffering from advanced NPC is only 5-11 months (Lee *et al*., 2012a; Zhang *et al*., 2013). Treating patients with a recurrence or advanced NPC is often a challenge as these patients rapidly develop resistance to chemo- and radiotherapy. Thus, novel and improved treatment strategies are urgently needed to curb this disease.

Cancer cells evade apoptosis through various strategies and one such strategy is to up-regulate the anti-apoptotic proteins (e.g. BCL-2, BCL-XL) (Hanahan and Weinberg, 2011). Increase in the level of these proteins in cancer has made them ideal targets for therapy and interest to target these proteins mounted with the generation of BH3-mimetics such as the first generation ABT-737 and now ABT-263 (Navitoclax). ABT-737/263 mimic pro-apoptotic protein BAD and specifically inhibits anti-apoptotic proteins BCL-2, BCL-XL and BCL-w (Oltersdorf *et al*., 2005; Tse *et al*., 2008). The mimetics exhibited encouraging outcome in haematological tumours and small cell lung cancer (SCLC) and now in various phases of clinical trials (Delbridge *et al*., 2016). The impressive outcome observed in haematological tumours and small

cell lung cancer (SCLC) was not mimicked in the solid tumours. Anti-apoptotic protein MCL-1 was reported to confer resistance to ABT-737/263 in solid tumours (van Delft *et al*., 2006; Tse *et al.,* 2008) and hence combining ABT-263 with drugs which either neutralise MCL-1 or induce MCL-1 antagonist NOXA have been shown to reverse the acquired resistance and sensitise cells to ABT-737/263.

Preliminary data in the laboratory revealed that NPC cell lines HK-1 and C666-1 were resistant to single agent treatment of ABT-263 except at unrealistically high-concentrations. The purpose of this study is to sensitise the NPC cell lines to ABT-263 by combining ABT-263 with Maritoclax or Simvastatin. Maritoclax was reported to inhibit MCL-1 in leukaemia cells (Doi *et al*., 2012; Doi *et al*., 2014) and on the other hand Simvastatin was reported to induce BH3-only proteins NOXA and PUMA, which neutralise MCL-1 (Ghavami *et al*., 2010). The NPC cells C666-1 and HK-1 were tested for sensitivity to ABT-263 and Maritoclax or Simvastatin first as single agents and later as combinations at various concentrations using 2-dimensional (2D) drug sensitivity assay. Drug potentiation or drug sensitisation studies were carried out to determine if uptake of one drug will enhance the sensitivity of the cells to another drug. Drug potentiation effects are distinct possibilities that must be considered to fully unravel the potential of the drug combinations. The 2D assays were followed by testing the sensitivity of the combinations in 3-dimentional (3D) NPC spheroids as 3D model epitomises tumour microenvironment and architecture more closely to tumours *in vivo* (Beaumont *et al*., 2013).

1.1 Objectives

Objective 1: To investigate the sensitivity of the NPC cell lines HK-1 and C666-1 to combination with Maritoclax or Simvastatin with BH3 mimetic ABT-263 in activating the intrinsic apoptosis pathway.

Objective 2: To investigate the potentiation of the NPC cell lines to ABT-263 by Maritoclax or Simvastatin and *vice versa*.

Objective 3: To establish 3D spheroids from NPC cell lines HK-1 and C666-1 and test the sensitivity of the spheroids to combination of Maritoclax and ABT-263.

CHAPTER 2

LITERATURE REVIEW

2.1 Nasopharyngeal Carcinoma – a distinct head and neck cancer

Nasopharyngeal carcinoma (NPC) affects the nasopharynx, which is situated on the upper region of the pharynx (Probst *et al*., 2005) (Figure 2.1). More precisely, NPC manifests beneath the nasopharyngeal mucosa or within the pharyngeal recess, which is also known as the Fossa of Rosenmüller (Hoe, 1989; Sham *et al*., 1990).

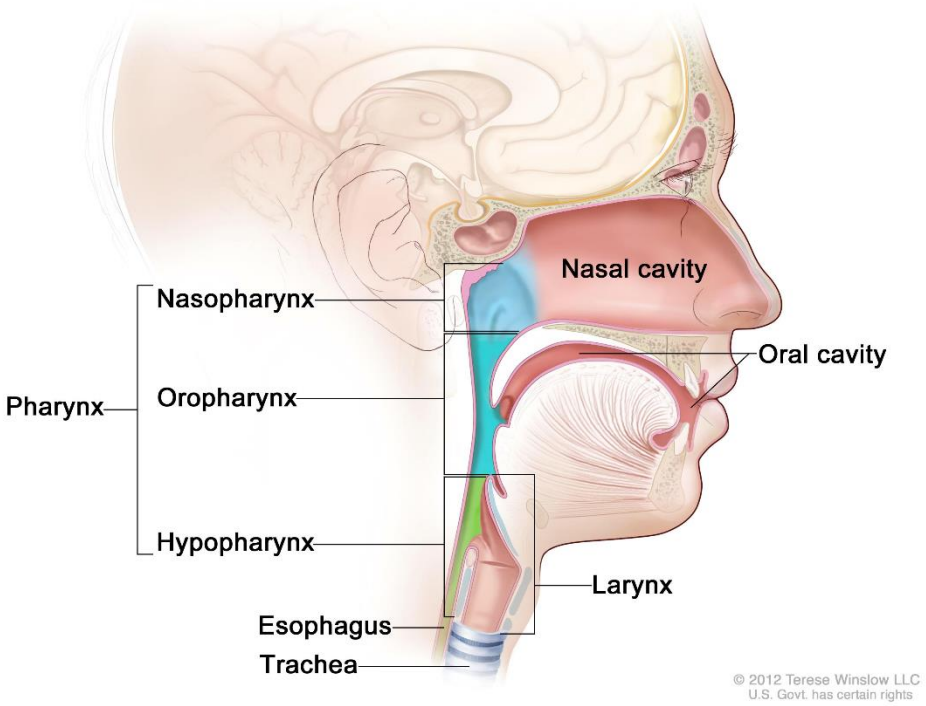


Figure source: www.cancer.gov

Figure 2.1: Anatomy of the human pharynx. The pharynx is divided into three regions. Nasopharynx is situated on the uppermost part of the pharynx, followed by the oropharynx and hypopharynx.

NPC has remarkable distinct regional and racial prevalence. The cancer is less prevalent among the Caucasians but is rampant among the southern Chinese, Inuits of Alaska and native Greenlanders (Nielsen *et al*., 1977; Parkin *et al*., 1992). In Southern China, high NPC incidence rates were recorded particularly in the province of Guangdong, Guangxi, Hainan, Jiangxi, Hunan and Fujian (Zhang *et al*., 2007). Cantonese inhabiting these regions were reported to have the highest rate of NPC incidence (Zhang *et al*., 2007). Interestingly, incidence rate of NPC in Northern China is low (Wei *et al*., 2014).

The incidence of NPC continues to be high among Chinese emigrants to Alaska, Greenland, Canada and North America but the incidences are low among ethnic Chinese who are born in North America (Buell, 1974; Torre *et al*., 2015). High incidences of NPC have also been reported in Hong Kong, Taiwan, Philippines, Thailand, India and Africa (Hsu *et al*., 2006; Torre *et al*., 2015). The distinct geographic and ethnic distribution of NPC demonstrate the multifactorial etiology of the cancer, including environmental factors which may include exposure to nitrosamines and consumption of salted and pickled food (Zou *et al*., 1994), genetic susceptibility and Epstein-Barr virus (EBV) infection (Yu *et al*., 1981; Brennan, 2006; Li *et al*., 2007).

Incidence and mortality rates of NPC in Southeast Asia was the highest (Torre *et al*., 2015) compared to other countries with high NPC incidence and mortality rates (Torre *et al*., 2015). Among the Southeast Asian countries, high incidence of NPC was recorded in countries namely Malaysia, Indonesia and Singapore (Table 2.1). The incidence and mortality rates of NPC were two to three times higher in males compared to females (Jemal *et al*., 2011) (Table 2.1).

Table 2.1: Countries with the highest incidence rates of Nasopharyngeal carcinoma in Southeast Asia.

Nations	Incidence rate (Age-standardized rate per 100,000)		Mortality rate (Age-standardized rate per 100,000)	
	Male	Female	Male	Female
Malaysia	10.6	3.9	3.9	1.2
Singapore	9.7	3.2	4.4	1.3
Indonesia	8.3	3.0	5.0	1.6
Vietnam	7.7	3.4	4.8	2.0
Brunei	7.6	1.5	3.4	0.5

Adapted from: Jemal *et al.,* 2011

NPC was ranked as the fourth most common cancer in Malaysia and the third most common cancer affecting Malaysian males (Omar and Tamin, 2011) (Figures 2.2 and 2.3). The incidences were higher in males residing in Johor, Sabah and Sarawak (Figure 2.4). The ethnic Chinese recorded the highest NPC incidence compared to the Malays and the Indians in Peninsular Malaysia whereas the Bidayuh population recorded the highest NPC incidences in Sarawak compared to the Chinese and the Malays (Devi *et al*., 2004). Interestingly, the incidences of NPC among ethnic Indians are low (Devi *et al*., 2004).

In Singapore, NPC is the eighth most common cancer. Similar to Malaysia, NPC is common among Chinese compared to the other races in Singapore (Lee *et al*., 2014). Whilst in Indonesia, NPC is the fourth most common cancer after cervical and breast cancers and melanoma. NPC was more common in some local natives of Indonesia compared to the others. The cancer was more widespread among the Javanese followed by the Sundanase and the Sumatranese natives (Adham *et al*., 2012).

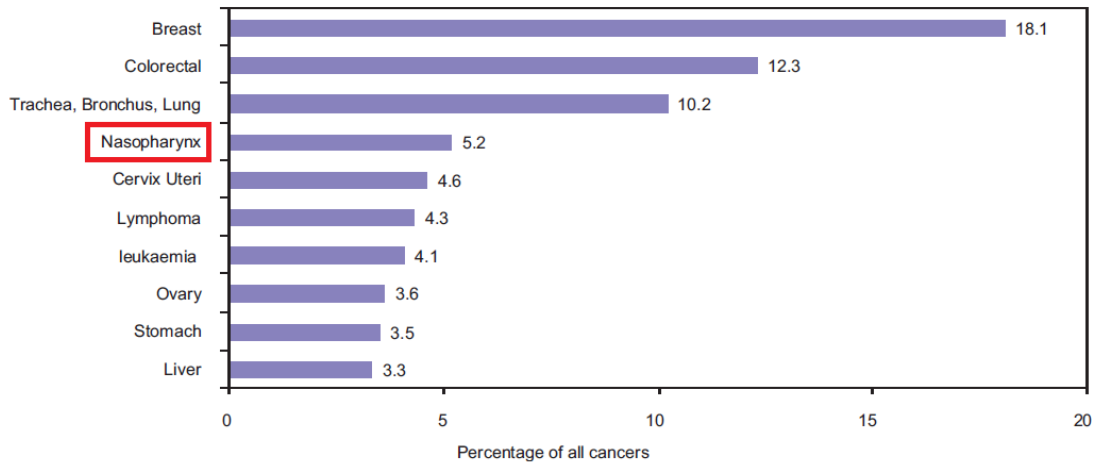


Figure source: National Cancer Registry Report 2007, Ministry of Health, Malaysia

Figure 2.2: The ten most frequent cancers in Malaysia. NPC (red box) is ranked number four after breast, colorectal and trachea, bronchus and lung carcinoma.

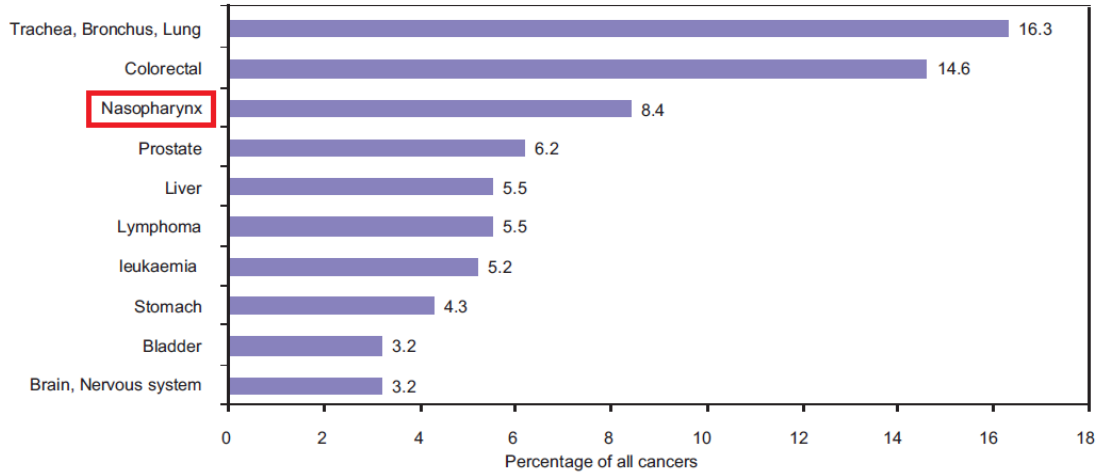
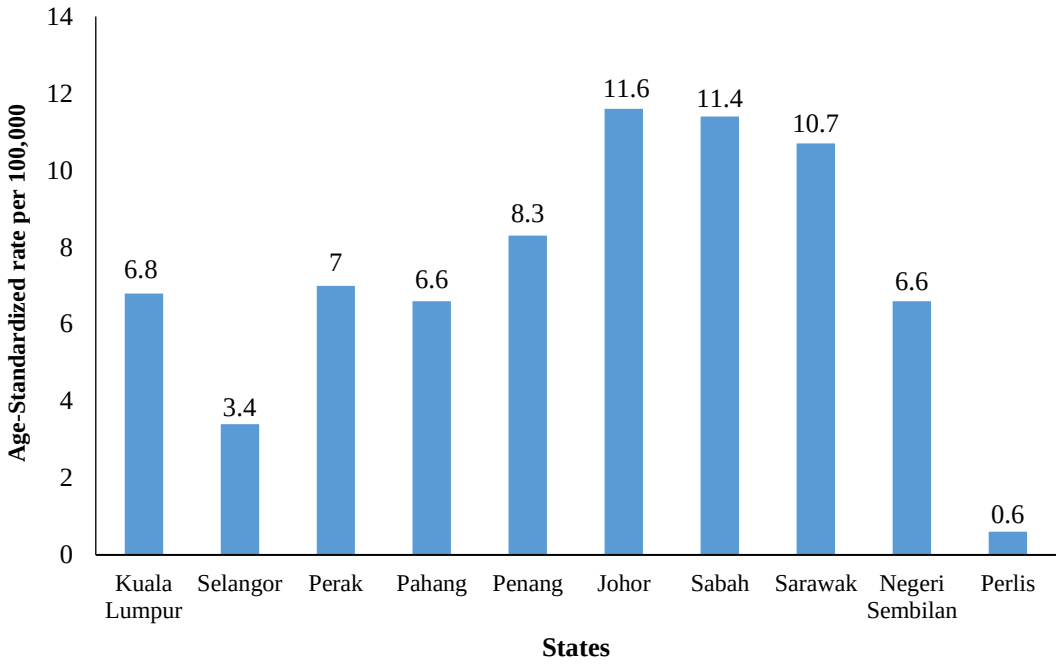


Figure source: National Cancer Registry Report 2007, Ministry of Health, Malaysia

Figure 2.3: The ten most common cancers affecting males in Malaysia. NPC (red box) is ranked third after trachea, bronchus and lung carcinoma and colorectal carcinoma.



Adapted from: National Cancer Registry Report 2007, Ministry of Health, Malaysia

Figure 2.4: Incidence rates of NPC in males in selected states in Malaysia. ***Note:*** *No data was reported for Kelantan, Kedah, Terengganu and Malacca as NPC was not the top five most common malignancies in these states.*

Reports from diverse sources demonstrate that incidences of NPC are the highest in Southeast Asia, particularly in Malaysia. NPC is highly sensitive to radical radiotherapy if diagnosed early but due to its trivial clinical symptoms, NPC is usually detected at late stages (stage III and IV) (Pua *et al.*, 2008). Patients who are diagnosed at late stages and patients who are suffering from a recurrence do not respond well to radio- and chemotherapy. Sadly, due to limited second line treatments, patients often do not survive the cancer. Hence, novel and improved treatment strategies are necessary to improve patient survival. This will be further elaborated in section 2.4.

2.2 Classification of Nasopharyngeal Carcinoma

The World Health Organization (WHO), in 1978, classified NPC into three histological subtypes: keratinizing squamous cell carcinoma (WHO type 1) – distinguished by well differentiated cells which produce keratin, nonkeratinizing carcinoma (WHO type 2) – contains cells which varies in cell differentiation but lack keratin and undifferentiated carcinoma (WHO type 3) (Table 2.2) – distinguished by poorly differentiated cells which exists in many different types of cells namely clear cell, spindle cell and anaplastic cell (Shanmugaratnam and Sobin, 1978) . The WHO classification was amended in 1991. In this amendment, the keratinizing squamous cell carcinoma was retained while WHO types 2 and 3 were merged under one category known as "nonkeratinizing squamous cell carcinoma". This category was further subdivided into differentiated and undifferentiated nonkeratinizing carcinoma (Shanmugaratnam, 1991) (Table 2.2). The amended classification eliminated the use of numerical designation of WHO types 1, 2 and 3. The present WHO classification of NPC still follows the 1991 classification with addition of another category: basaloid squamous cell carcinoma (Barnes, 2005) (Table 2.2). Although NPC classification is amended, most research papers and books do not embrace the amended classification but rather continue using the WHO numerical system to report on the histologic subtypes of NPC.

Table 2.2: Chronology of WHO classification of NPC

WHO Classification of NPC		
1978	**1991**	**Present**
Type 1 - Keratinizing squamous-cell carcinoma	Keratinizing squamous-cell carcinoma	Keratinizing squamous-cell carcinoma
Type 2 - Nonkeratinizing squamous-cell carcinoma	Nonkeratinizing squamous-cell carcinoma Differentiated Undifferentiated	Nonkeratinizing squamous-cell carcinoma Differentiated Undifferentiated
Type 3 - Undifferentiated carcinoma		Basaloid squamous-cell carcinoma

Adapted from: Barnes, 2005

Interestingly, the histologic subtypes of NPC vary with different geographical areas. NPC type III is common in both low NPC-incidence and high NPC-incidence areas. North America, which represents a low-incidence area and Southern China, which represents a high-incidence area comprise of 65% and >95% of NPC type III respectively. Meanwhile, incidences of NPC types I and II are higher in low-prevalence areas such as Northern America with 25% and 12% respectively. On the other hand, in Southern China, the occurrence of NPC types I and II is less than 5% (Tao and Chan, 2007).

NPC types II and III are strongly associated with Epstein-Barr virus (EBV) infection whereas EBV infection is normally absent in NPC type I (Marks *et al*., 1998). However, another study reported that all NPCs regardless of their histologic subtype contain EBV infection (Vasef *et al*., 1997). In Malaysia, a multi-centre NPC study conducted by the Malaysian Nasopharyngeal Carcinoma Study Group reported that 97% of new NPC cases were either histologic subtype II or III. Only six patients suffered from WHO NPC type I (Pua *et al*., 2008).

2.3 The Epstein-Barr virus (EBV) infection

Tony Epstein and Yvonne Barr first described Epstein-Barr virus in 1964. EBV is a human gamma-herpes virus which has a double-stranded DNA in an enclosed envelope (Baer *et al*., 1984). It has a large genome which is approximately 180kb and consists of about 80 open reading frames (Baer *et al*., 1984). Although EBV infects over 95% of humans worldwide, it remains harmless until the equilibrium between the host and the virus is tipped (Cohen, 2000). EBV is associated with a number of malignant carcinomas namely Hodgkin's lymphoma, Burkitt's lymphoma, oral hairy leukoplakia, gastric carcinoma and NPC (Young and Rickinson, 2004). EBV infection is normally not detected in normal epithelium of individuals at high risk of developing NPC and low-grade pre-invasive lesions but deletion of regions of chromosome 3p and 9p are detected in these individuals indicating that genetic events occur early in NPC pathogenesis. These early genetic events are believed to lead to subsequent EBV infection (Lo and Huang, 2002). EBV infection renders cells with growth and survival benefits leading to NPC manifestation (see paragraph below). Additional genetic and epigenetic alterations for example inactivation of the tumour suppressor genes *CDKN2A* (Cyclin-Dependent Kinase Inhibitor 2A), *RASSF1A* (Ras association domain family 1 isoform A) and induction of anti-apoptotic gene *BCL-2* lead to high-grade pre-invasive lesions before the cancer metastasizes to distant organs (Barnes, 2005; Young and Rickinson, 2004).

The transforming capabilities of EBV is rendered by a set of latent genes known as the latent membrane proteins (LMP-1, LMP-2A and LMP-2B), EBV-determined nuclear antigens (EBNA) namely EBNA-leader protein (EBNA-LP), EBNA-1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C two EBV-encoded small RNA, (EBER-1 and EBER-2) and the non-transcript BART (*Bam*HI-A region rightward

transcript) RNAs (Werner *et al*., 2007). LMP-1 is present in 80-90% of NPC tissues and is the principal oncoprotein of NPC (Wang *et al*., 1985). LMP-1 is an integral protein with six transmembrane domains and a carboxy terminus which contains two signaling domains known as the C-terminal activating regions 1 and 2 (CTAR 1 and 2). The CTAR regions directly activate a number of signaling pathways namely the nuclear factor κ-B (NF-κB), mitogen-activated protein kinases (MAPK), phosphoinositol-3-kinase (PI3K), Janus kinase/signal transducers and activators of transcription (JAK/STAT) and the Jun N-terminal kinase (JNK) and p38 MAPK (JNK/p38) (Tsao *et al*., 2002). Activation of these upstream signalling pathways by LMP-1 will subsequently activate downstream proteins of several pathways. Activation of these proteins will lead to aberrant cell proliferation, cell transformation, increase in angiogenesis, metastasis and evasion of apoptosis (Tulalamba & Janvilisri, 2012). Roles of the other EBV oncoproteins are extensively reviewed in Young and Rickinson, 2004 (Young and Rickinson, 2004).

2.4 Treating Nasopharyngeal Carcinoma – where is the problem?

Unlike other head and neck cancers, NPC is highly radio- and chemosensitive and patients who are diagnosed early respond well to these treatment modalities. The 5-year survival rate for stage I and stage II patients' range from 72 to 90% (Chang *et al*., 2004) but unfortunately this is not the case for patients' who are diagnosed late and this is where the problem begins. The 5-year survival rate for stage III and IV patients are ~55% and 30%, respectively, mostly attributed to locoregional recurrence and metastasis (Chang *et al*., 2004). Majority of NPC cases are also diagnosed late due to trivial clinical symptoms (e.g. headache, nasal and aural obstructions) and patients seeking treatment late after the onset of symptoms (Pua *et al*., 2008). Prognosis for

patients suffering from a recurrence or advance disease worsens and therefore they respond poorly to the existing treatment modalities (pers. comm. Dr. Shazril Imran Shaukat, Clinical Oncologist, IPPT, USM). In light of this, novel or improved treatment strategies for NPC are required. Deeper understanding of the molecular mechanisms of NPC has led to testing of targeted therapies for NPC management.

The Epidermal Growth Factor Receptor (EGFR) is expressed in >80% advanced NPC patients and hence a promising therapeutic target (Chua *et al*., 2004). A number of EGFR inhibitors namely Cetuximab, Gefitinib and Erlotinib have been tested in clinical trials for NPC. In a multi-centre phase II study, recurrent or metastatic NPC patients who previously failed platinum-based therapy was tested for combination of Cetuximab and Carboplatin. The combination demonstrated median overall survival of 7.8 months and was not toxic to patients (Chan *et al*., 2005). Another phase II study which evaluated the combination of Cetuximab with concurrent Cisplatin and intensity-modulated radiotherapy (IMRT), in locoregionally advanced NPC patients, reported 2-year progression free-survival of 86.5% (Ma *et al*., 2012). Unlike Cetuximab, clinical trials outcome for Gefitinib and Erlotinib were not promising. A single centre phase II study assessed the safety and tolerability of Gefitinib on 19 recurrent or metastatic NPC patients who failed platinum-based treatment. The study concluded that Gefitinib was not toxic but displayed a poor response rate (Chua *et al*., 2008). Similar outcome was obtained when Gefitinib was tested on 16 metastatic or locoregionally recurrent NPC patients (Ma *et al*., 2008). A phase II trial evaluated the efficacy of Erlotinib as a second-line treatment for recurrent and/or metastatic NPC patients who have undergone treatment with Gemcitabine-platinum chemotherapy. The outcome was not welcoming as Erlotinib was not effective in these patients (You *et al*., 2012). Collectively, most of these trials were

phase II trials, which focused on assessing safety, and tolerability of the EGFR inhibitors and only involved a single centre and a small sample size. Larger multi-centre randomised studies may be needed in the future, to further investigate the prospects of the EGFR inhibitors for NPC treatment.

The Vascular Endothelial Growth Factor (VEGF) is expressed in >60% NPC biopsies (Hui *et al*., 2011a) and was significantly associated with lymph node (Wakisaka *et al*., 1999) and distant metastasis (Guang-Wu *et al*., 2000). Clinical trials with broad spectrum small molecule inhibitors namely Sorafenib and Sunitinib only demonstrated a modest clinical effect on NPC patients. Sorafenib is an oral inhibitor which inhibits serine/threonine kinases C-Raf and B-Raf and the VEGF receptor (VEGFR) -2 and VEGFR-3. Single agent efficacy and safety of Sorafenib was tested on recurrent and/or metastatic NPC patients in phase II clinical study. Sorafenib was well tolerated by patients but it only demonstrated a modest effect (Elser *et al*., 2007). In another phase II study, patients with a metastatic/recurrent NPC achieved median overall survival of 11.8 months after treatment with combination of Sorafenib, Cisplatin and 5-Flurouracil (5-FU). Further randomized trials are needed to compare the combination tested with combination of Cisplatin and 5-FU (Xue *et al*., 2013). Like Sorafenib, Sunitinib also has broad spectrum activity against many molecules such as EGFRs, PDGFRs, c-kit, RET and VEGFR1-3. Preclinical studies showed that Sunitinib displayed concentration-dependent growth inhibition of NPC cell lines but only weakly enhanced the growth inhibition of Cisplatin and Docetaxel. The drug also exhibited significant growth inhibition of NPC xenografts as a single agent but caused severe toxicity in mice when it was concurrently administered with Docetaxel (Hui *et al*., 2011c). A phase II study by the same group, reported that Sunitinib displayed a modest effect in NPC patients with progressive disease after prior platinum-based

chemotherapy. Moreover, development of hemorrhagic complications in patients was a concern in this study (Hui *et al*., 2011b). Bevacuzimab (Avastin®, Genentech, Inc.) showed more promising results compared to the other VEGF inhibitors tested for NPC. A multicentre phase II clinical study tested the feasibility and safety of combination of Bevacuzimab with standard chemoradiation for locoregionally advanced NPC patients. Bevacuzimab was well tolerated and addition of this drug to the standard chemoradiation delayed the progression of NPC to the distant sites (Lee *et al*., 2012b).

The PI3K/AKT/mTOR signaling pathway, which controls key events of cell growth, survival and invasion is frequently abrogated in NPC. In recent years, efficacy of small molecule inhibitors targeting specific molecules of this pathway have been tested for NPC treatment using *in vitro* and *in vivo* models. Phosphorylated AKT (p-AKT) is commonly expressed in NPC tissues and therefore targeting this protein could be a promising treatment approach (Loong *et al*., 2008; Yip *et al*., 2008; Huang *et al*., 2009; Chen *et al*., 2010). PF-04691502, a potent dual PI3K/mTOR inhibitor, demonstrated anti-proliferative activity on NPC cell lines in 2D and 3D cell culture models and also displayed anti-tumour activity in the CNE-2 xenograft model. Combination of PF-04691502 with Cisplatin or Paclitaxel was not encouraging but PF-04691502 showed single agent activity on Cisplatin resistant NPC cell lines (Wong *et al*., 2013). The PIK3CA molecule is another attractive therapeutic target for NPC treatment. Amplification of PIK3CA was reported in 40-70% of NPC tissues and hot spot mutations of this molecule was reported in low frequencies in NPC cell lines and tissues (Hui *et al*., 2002; Or *et al*., 2006). BYL719 is a selective inhibitor of PIK3CA. The drug showed anti-proliferative activity on NPC cells in 2D and 3D cell culture models. Furthermore, the drug demonstrated synergistic effects when combined with MEK inhibitor (AZD6244) (MEK is a member of the MAPK signaling pathway –

another pathway which is frequently deregulated in NPC) in 3D NPC cell culture (Wong *et al*., 2015). A phase II multi-institutional study investigated the activity of AKT inhibitor MK-2206 on 21 recurrent or metastatic NPC patients. The study was however terminated as limited clinical activity was observed with this drug (Ma *et al*., 2015).

Recently, a number of immunomodulatory agents exhibited favourable response in clinical trials, especially the anti-PD-1 (programmed death 1) and anti-PD-L1 (programmed death ligand 1) antibodies (Robert *et al*., 2011; Brahmer *et al*., 2012). The PD-1 receptor inhibitor Keytruda (pembrolizumab, Merck) was approved by the Food and Drug Administration (FDA), in September 2014, for the treatment of melanoma and has shown impressive results for the treatment of other cancers as well (Guha, 2014). The PD-L1 inhibitors are still in clinical development. The PD-1 receptor is expressed in T and B cells whereas its ligand PD-L1 is expressed in many cells in the body including cancer cells. Interaction between the receptor and its ligand weakens the immune system (He *et al*., 2015). Although the PD-1/PD-L1 inhibitors are vigorously tested in many cancers their efficacy in NPC are underexplored. In NPC, up-regulation of PD-L1 is associated with EBV associated LMP-1 and IFN-γ pathways (Fang *et al*., 2014). Immunohistochemistry staining was conducted to detect expression of PD-L1 in 139 NPC patient samples. Findings revealed that patients with high expression of PD-L1 have a poorer disease-free-survival (DFS) compared to patients with low expression of PD-L1 (median DFS 39.6 months' vs 65.2 months'). Hence, targeting patients with high expression of PD-L1 with PD-1/PD-L1 antibodies may be a promising approach for NPC treatment (Fang *et al*., 2014).

A goal of this work is to target the BCL-2 family proteins, the critical regulators of the intrinsic apoptosis pathway (Delbridge *et al*., 2016). Some members of this

family (e.g. BCL-2, BCL-XL and MCL-1) are deregulated in cancer, making them as ideal targets for therapy. The interest to target these molecules for therapy elevated with the development of BH3 mimetics such as ABT-737 and ABT-263 (Navitoclax, AbbVie), which specifically inhibit these proteins. Efforts to target the anti-apoptotic proteins for NPC treatment have been discouraging thus far and therefore sparked our interest to investigate the effect of the BH3 mimetics on NPC cells. These points will be further elaborated in the following sections, which will first introduce to the apoptosis pathways, the BCL-2 family proteins and lastly approaches to target these proteins for NPC therapy.

2.5 Apoptosis Pathways

Apoptosis is a form of programmed cell death for neat disposal of aged, damaged or diseased cells (Kerr *et al*., 1972). Apoptosis plays a major role in normal developmental processes, including eliminating cells during tissue morphogenesis and in the elimination of inactive and self-activating cells during lymphopoiesis (Joaquin and Gollapudi, 2001). It is also essential for sustaining normal homeostasis for cell populations and abrogation of apoptosis pathways can lead to manifestation of cancer or degenerative disease (Tait and Green, 2010). Cells activate apoptosis following diverse stress signals namely activation of oncogenes, ultraviolet radiation, growth-factor deprivation and DNA damage caused by chemotherapeutic agents (Mohana-Kumaran *et al*., 2014). Apoptosis can be initiated through the intrinsic (also known as the mitochondrial) pathway or the extrinsic (also known as death receptor) pathway.

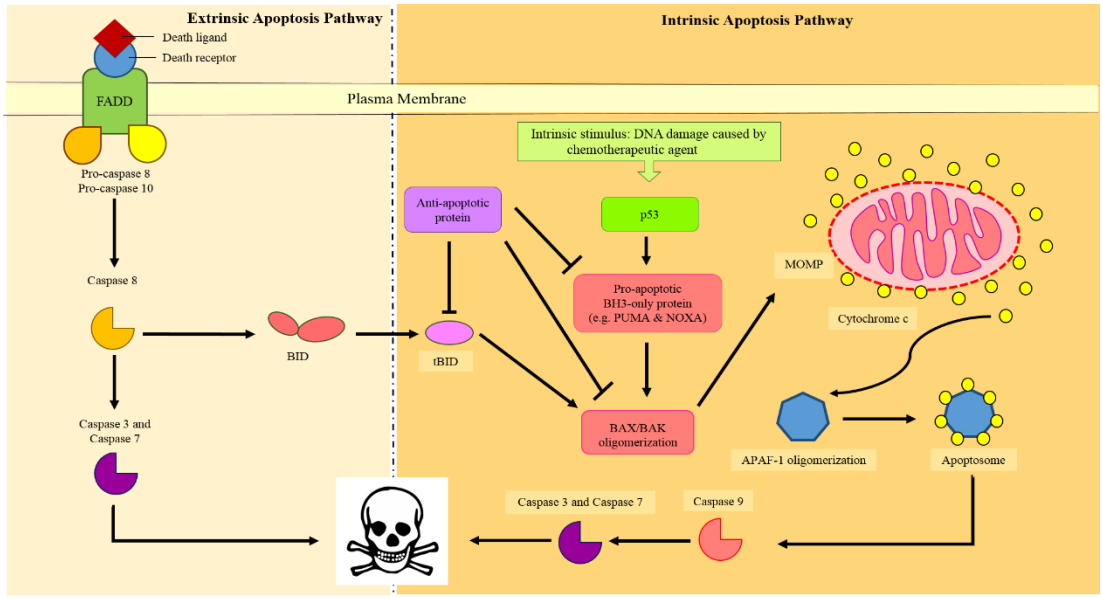


Figure 2.5: The Apoptosis Pathways. The intrinsic apoptosis pathway can be activated by various stress signals. For example, DNA damage (stress signal) caused by chemotherapeutic agents is sensed by p53. P53 translocate to the nucleus and induces genes of pro-apoptotic proteins NOXA and PUMA. Pro-apoptotic proteins in turn neutralise the anti-apoptotic proteins. The anti-apoptotic proteins inhibit the activation of apoptosis by either binding to the BH3-only proteins or activated BAX and BAK. Neutralization of the anti-apoptotic proteins by the BH3-only proteins leads

to oligomerisation of effector pro-apoptotic proteins BAX and BAK. BAX and BAK induces Mitochondrial Outer Membrane Permeabilization (MOMP) which in turn releases cytochrome c into the cytoplasm. Cytochrome c together with APAF-1 form the apoptosome and activate caspase-9. Caspase-9 in turn activates the executioner caspases -3, -6 and -7 which leads to initiation of apoptosis in the cells. The extrinsic apoptosis pathway is activated by the death ligands and their respective death receptors. This leads to recruitment of FADD (FAS-associated death domain protein) and caspases-8 and -10. Activated caspases-8 and -10 activate the executioner caspases -3, -6 and -7 which leads to activation of apoptosis. The intrinsic and the extrinsic pathway crosstalk through the actions of caspase-8. Caspase-8 activates BH3-only protein BID to truncated BID (tBID). Truncated BID directly activates BAX and BAK and thereafter the steps leading to activation of apoptosis follows the intrinsic apoptosis pathway. Solid lines: direct inhibition or activation, red boxes: BH3-only pro-apoptotic proteins, purple boxes: anti-apoptotic proteins and skull: activation of apoptosis in the cells.

2.6 The Extrinsic Apoptosis Pathway

The extrinsic apoptosis pathway will only be briefly introduced, as it is not the focus of this study. The extrinsic pathway is activated when ligands namely FASL, TNF and TRAIL activate their respective death receptors (DR). The DRs are situated at the plasma membrane and the members include the tumour necrosis factor (TNF) – related apoptosis-inducing ligand (TRAIL) receptor 1 (TRAILR1, also known as DR4 and TNFRSF10A), TRAIL2 (also known as DR5 and TNFRSF10B), FAS (also known as CD95 and APO1) and TNF receptor I (TNFR1, also known as TNFRSF1A) (Ichim and Tait, 2016). These death receptors contain a cytosolic "death domain" and through this death domain, they interact with the other cytosolic proteins. Once the ligands bind to the death receptors, the receptors recruit the cytosolic FADD (FAS-associated death domain protein) and caspases-8 and -10 (Figure 2.5). Activation of caspase-8 leads to activation of the executioner caspases such as caspases-3 and -7. The executioner caspases then initiate the proteolytic mechanics of apoptosis in cells (Ichim and Tait, 2016) (Figure 2.5). It is important for our purpose that the extrinsic pathway can initiate the intrinsic apoptosis pathway. The crosstalk between the two

pathways is performed through the actions of caspase 8. Caspase 8 truncates and activates BH3-only protein BID of the intrinsic apoptosis pathway to truncated form BID (tBID) (Li *et al*., 1998; Luo *et al*., 1998) (Figure 2.5). Thereafter the pathway leading to activation of apoptosis follows the intrinsic pathway (see the next section).

2.7 The Intrinsic Apoptosis Pathway

The intrinsic pathway can be activated following diverse stress signals such as hypoxia, nutrient deprivation and DNA damage which is caused by chemotherapeutic agents (Mohana-Kumaran *et al*., 2014). For example, cell senses DNA damage due to treatment with chemotherapeutic agents via the p53 tumour suppressor protein (Junttila and Evan, 2009). Once the stress is sensed, p53 is stabilised and accumulated in the cell. Next, p53 translocate to the nucleus and induces the expression of genes, which are involved in the intrinsic pathway namely pro-apoptotic proteins NOXA and PUMA (Mohana-Kumaran *et al*., 2014) (Figure 2.5). Activation of these proteins neutralise the anti-apoptotic proteins. Inactivation of the anti-apoptotic proteins leads to translocation of BAX from the cytoplasm to the outer mitochondrial membrane (OMM). At the OMM, BAX oligomerises with BAK to induce mitochondrial outer membrane permeabilisation (MOMP) leading to release of mitochondrial cytochrome c (Mohana-Kumaran *et al*., 2014) (Figure 2.5). Cytochrome c interacts with the monomeric cytosolic protein APAF-1 (apoptotic-protease-activating factor-1), initiating the formation of apoptosome, which activates initiator caspase-9 (Figure 2.5). Activated caspase-9 activates caspases-3, 6 and -7, which in turn activate apoptosis (Ichim and Tait, 2016) (Figure 2.5).