EFFECTS OF *Catharanthus roseus* AQUEOUS EXTRACT ON JURKAT CELLS AND NORMAL PERIPHERAL BLOOD MONONUCLEAR CELLS

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by

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LIST OF ABBREVIATIONS

APAF-1	Apoptotic protease-activating factor 1
AICD	Activation-induced cell death
AIF	Apoptosis inducing factor
ANT	Adenine nucleotide translocase
APC	Antigen presenting cells
AP-1	Activator protein 1
APS	Ammonium Persulfate
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
BH	Bcl-2 homology domain
Bad	Bcl-2-antagonist of cell death
Bid	BH3-interacting domain
Bik	Bcl-2-interacting killer
Bim	Bcl-2-interacting mediator
C_t	Threshold cycle
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
DISC	Death inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DW	Dry weight
ELISA	Enzyme-linked immunosorbent assay
Endo G	Endonuclease G
FADD	Fas-associated death domain
FITC	Fluorescin isothiocyanate
FSC	Forward scatter
HPLC	High performance liquid chromatography
IAP	Inhibitor of apoptotic protein
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JC-1	5,5V ,6,6V -tetrachloro-1,1V ,3,3V -tetraethylbenzimidazolylcarbocyanine iodide
mAb	monoclonal antibodies
MAPK	Mitogen activated protein kinase
MCA	Methylcholanthrene
MHC	Major histocompatibity complex
MMP	Mitochondrial membrane potential
MTS	3-(4,5-dimethylthiazol-2-yl)-5(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NK	Natural Killer

NO	Nitric oxide
OD	Optical density
PARP	poly ADP ribose polymerase
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PHA	Phytohaemagglutinin
PI	Propidium iodide
PKC	Protein kinase C
PMS	Phenazine methosulfate
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
Rb	Retinoblastoma
RIN	RNA integrity number
RLU	Relative light unit
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT	Reverse transcription
SAGE	Serial analysis of gene expression
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMAC	Second mitochondria-derived activator of caspases
SpA	Staphylococcus protein A
SSC	Side scatter
TEMED	Tetramethylethylenediamine
TCR	T-cell receptor
TGF	Transforming growth factor
TLC	Thin layer chromatography
TLR	Toll-like receptor
TRADD	TNER-associated death domain
TRAIL	TNF-related apoptosis inducing ligand
TNFR	Tumor necrosis factor receptor
UV	Ultraviolet
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor

KESAN-KESAN EKSTRAK AKUES *Catharanthus roseus* KE ATAS SEL JURKAT DAN SEL DARAH PERIFERAL MONONUKLEAR NORMAL

ABSTRAK

Objektif utama kajian terbaharu ini ialah untuk menilai kesan-kesan ekstrak akues daun *C.roseus* ke atas sel Jurkat (T-sel leukemik) dan sel darah periferal mononuklear normal.

Analitikal kromatografi cecair prestasi tinggi (HPLC) telah digunakan untuk mendapatkan kepekatan vinblastine dalam ekstrak *C.roseus* manakala pengenalpastian sebatian terpilih telah ditentukan dengan membandingkan spektrum UV setiap kompaun dengan standard sebatian yang sebelum ini dikenal pasti dalam *C.roseus*. Data analisis menunjukkan kepekatan vinblastine adalah 1636 ± 0.2 μg g^{-1} ekstrak akueus, bersamaan dengan 445.6 ± 0.06 μg g^{-1} mg daun kering. Selain itu, empat alkaloid telah dikenal pasti sebagai serpentine, vindoline, catharanthine dan vindoline.

Kesan-kesan toksik ekstrak ke atas sel Jurkat dan sel darah periferal mononuklear normal telah ditentukan menerusi *MTS* [3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] *assay*. Ekstrak *C.roseus* telah merencat pertumbuhan sel Jurkat secara ketara, tetapi juga telah merangsang pertumbuhan sel mononuklear normal. Kesan toksik ke atas sel Jurkat telah ditunjukkan dengan nilai-nilai kepekatan rencatan separuh maksimum (IC_{50}) 2.55 μg/ml dan 2.38 μg/ml, masing-masing pada 48 dan 72 jam. Pertumbuhan maksimal sel darah periferal mononuklear normal didapati apabila diubati dengan kepekatan 1000 μg/ml ekstrak *C.roseus* pada masa pengeraman pelbagai untuk penderma darah yang berbeza.

Analisa aliran sitometri mendedahkan bahawa ekstak *C.roseus* telah menyebabkan perencatan fasa S ke atas sel Jurkat manakala induksi apoptosis telah disahkan oleh pengesanan fragmentasi DNA dan perubahan *phosphatidylserine* dalam cara yang bergantung kepada masa.

Penilaian caspase 3/7, pembebasan cytochrome c dan potensi membran mitokondria (MMP) mendedahkan mekanisma apoptosis sel Jurkat yang dirawat oleh *C.roseus* ekstrak adalah melalui laluan mitokondria yang melibatkan pembebasan cytochrome c dari mitokondria ke cytosol dan pengaktifan caspase 3/7. Pembebasan cytochrome c telah dikaitkan dengan kekurangan MMP.

Ungkapan gen berkaitan onkologi manusia telah ditayangkan dengan menggunakan pemprosesan tinggi RT-PCR teknik. Analisis gen ungkapan sel Jurkat yang dirawat oleh *C.roseus* ekstrak menunjukkan bahawa 36 gen kebezaan selia gen daripada 1023 gen. Daripada jumlah ini, 20 gen telah meningkat dan 16 menurun ekspresi. Gen kebezaan terlibat dalam apoptosis dan kitaran sel.

Kesan proliferatif pada sel darah periferal mononuklear normal yang dirawat oleh ekstrak *C.roseus* terus dinilai dengan imunofenotip menggunakan antibodi monoklonal terpilih dan diikuti oleh pengukuran kuantitatif komplemen (C3, C4) dan immunoglobulin (IgG, IgA, IgM). Induksi proliferasi sel boleh diperhatikan terutamanya $CD4^+$, menghasilkan nisbah CD4/CD8 yang lebih tinggi berbanding dengan kawalan. Penghasilan ketara bagi IgG, IgA dan C3 telah didokumenkan.

Data-data ini menunjukkan bahawa ekstrak akues *C.roseus* menunjukkan kesan yang terpilih antara sel-sel normal dan Jurkat. Ini menunjukkan potensi kegunaannya sebagai

rawatan leukemik dan pembantu tambahan di mana imun aktiviti telah ditindas semasa kemoterapi.

EFFECTS OF *Catharanthus roseus* AQUEOUS EXTRACT ON JURKAT CELLS AND NORMAL PERIPHERAL BLOOD MONONUCLEAR CELLS

ABSTRACT

The objective of the present study was to evaluate the effects of crude aqueous extract of *C.roseus* leaves on Jurkat cell line (leukemic T-cells) and normal peripheral blood mononuclear cells (PBMCs).

Analytical high performance liquid chromatography (HPLC) was used to obtain the concentration of vinblastine in the *C.roseus* extract while the identification of selective compounds was determined by comparing the UV spectra of each compound with standards of compounds previously identified in *C.roseus*. The analytical data indicated the concentration of vinblastine was 1636 ± 0.2 μg g^{-1} of aqueous extract, equivalent to 445.6 ± 0.06 μg g^{-1} of dried leaves. Additionally, four of the alkaloids were identified as serpentine, vindoline, catharanthine and vindoline.

The cytotoxicity effects of the extract on Jurkat cells and normal PBMCs were first examined by MTS [3-(4,5-dimethylthiazol-2-yl)-5(3- carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. The *C.roseus* extract was found significantly inhibited the proliferation of the Jurkat cells and also induced the proliferation of normal PBMCs. The cytotoxicity effect on Jurkat cells was demonstrated with IC_{50} values of 2.55 μg/ml and 2.38 μg/ml at 48 h and 72 h, respectively. Maximal cell proliferation was observed in PBMCs incubated with 1000 μg/ml of the *C.roseus* extract at various incubation times for different blood donor.

Flow cytometry analysis revealed that the *C.roseus* extract caused S-phase Jurkat cells arrest while induction of apoptosis was confirmed by the detection of DNA fragmentation and translocated phosphatidylserine in a time-dependent manner.

Examination of the caspase 3/7, cytochrome c release and mitochondrial membrane potential (MMP) revealed apoptosis mechanism in *C.roseus*-treated Jurkat cells was mediated *via* mitochondrial pathway involving the release of cytochrome c from mitochondria to cytosol and activation of caspase 3/7. The cytochrome c release was correlated with the depletion of MMP.

The expression of human oncology-related genes was screened by using high throughput RT-PCR technique. The gene expression analysis of the *C.roseus*-treated Jurkat cells showed that 36 genes were differentially expressed out of 1023 genes. Of these, 20 genes were upregulated and 16 were downregulated. The differentially expressed genes were involved in apoptosis and cell cycle progression.

The proliferative effects on normal PBMCs treated with the *C.roseus* extract were further assessed by immunophenotyping using selective monoclonal antibodies followed by quantitative measurement of complements (C3, C4) and immunoglobulins (IgG, IgA, IgM). The induction of the cell proliferation could be observed especially $CD4^+$, indicating higher CD4/CD8 ratio as compared to control. A significant production of IgG, IgA and C3 was documented.

These data indicated that the *C.roseus* extract showed selective effects between normal and Jurkat cells, suggesting its potential use as leukemic treatment and adjuvant supplement, whose immune activities were suppressed during chemotherapies.

CHAPTER 1

INTRODUCTION

1.1 Research Background

Leukemia is one of the most common cancers in the world (Agostini et al., 2011). A report provided by the International Agency for Research on Cancer (IARC) stated that in 2008, leukemia has been listed as amongst the top fifteen most common cancers in the world, contributing to 2.8% of incidence rate and 3.4% of mortality rate for all cancer types, in both men and women. In Malaysia, this disease has been listed as the tenth commonest cancer, with 4.3% incidence rate and 5.8% mortality rate in both sexes. According to the cancer statistic reported by Surveillance Epidemiology and End Result (SEER), National Cancer Institute, it is estimated that 26,830 men and 20,320 women from the United State population that will be diagnosed with leukemia and 23,540 men and women will die of leukemia in 2012, equivalent of 1 in 74 men and women will be diagnosed with leukemia during their lifetime (Howlader et al., 2012).

The pathogenesis of leukemia arises from malignant transformation of hematopoietic precursor cells (Lutterbach, 2000) and associated with deregulation of transcription factors (Pandolfi, 2001), resulting in disruption of stem cell differentiation and cell proliferation (Look, 1997; Steffen et al., 2005; Tenen, 2003; Warner et al., 2004). The disruption may alter the normal physiological properties of the cells that include cell cycle control, programmed cell death (apoptosis) and DNA repair (Hiddemanna et al., 2005; Lutterbach, 2000). Since the alteration of apoptosis is mostly seen in all cancer types especially in leukemia (Accordi et al., 2010;

Heidari et al., 2010; Wemeau et al., 2010) and carcinoma (Bhatnagar et al., 2010), many cytotoxic drugs targeting this mechanism have gained prominence in medical oncology (Esposti 2010; Reis et al., 2010; Rigaud et al., 2010). The examples of common chemotherapeutic drugs for acute myeloid leukemia (AML) include nucleoside analogs (cytosine arabinoside [Ara-C]) and anthracyclines (idarubicin, daunorubicin). These drugs inhibit the proliferation of cancer cells through the impairment of DNA replication and induction of apoptosis (Guzman et al., 2005). Previous studies have reported on the mechanism of other conventional cytotoxic drug, all-trans retinoic acid (ATRA) that is used as a treatment for acute promyelocytic leukemia. The mechanism of ATRA is mainly through the activation of ubiquitin-proteosome and caspase system, leading to the induction of apoptosis (Nervi et al., 1998; Zhu et al., 1999). In addition, a study conducted by Liu et al. (2000) has revealed 169 genes modulated by ATRA that are involved in various signaling pathways including cell cycle regulation and apoptosis.

However, one downside of conventional cancer therapies is the side effects. Although modern chemotherapy results in very high remission induction rates, yet there are many cases of patients experiencing relapse after receiving chemotherapy. For instance, the chemotherapy performed on acute lymphocytic leukemia (ALL) patients usually gives successful recovery rates but almost 25% of children and 70% of adult experienced relapse after the treatment (Rezaei et al. 2012). This may be due to the quiescent state of leukemic stem cells (LSCs), allowing some of the malignant stem cells to be resistant towards the standard chemotherapy and eventually contribute to relapse (Guzman et al., 2005). Moreover, although these drugs are used to target tumour cells, most of them can also induce genotoxic, carcinogenic and

teratogenic effects in non-tumour cells (Chung et al., 1998; Philip 2005). These side effects limit the application of chemotherapeutic agents despite their high efficacy in the killing of target malignant cells.

Hence, the search for alternative or complementary drugs that are effective on cancer cells through the induction of apoptosis while showing minimal toxicity to normal cells and/or benefit the immune response is an active area of research (Alonso-Castro et al., 2012). Many of these investigations involve plant-based, folkloric medicine from various societies around over the world. A report from World Health Organisation (WHO, 1996) stated that about 80% of the world population is wholly or partially dependent on plant-based therapies. Moreover, a previous report stated that around two thirds of current medicines are derived from plants, or are synthetics derived from lead compounds discovered in plants (Rollinger, Langer, & Stuppner, 2006).

One of the intensively investigated medicinal plants is *Catharanthus roseus* (*C.roseus*) (L) G. Don, formerly *Vinca rosea* L. (Apocynaceae) that is commonly known as Madagascar periwinkle. It belongs to a taxonomical group of plants that contains more than 130 different pharmacologically active terpenoid indole alkaloids (TIAs) (Blasko & Cordell, 1990; Costa et al., 2008). Several authors reported how this medicinal plant is highly consumed, worldwide, by means of decocts and infusions for various applications, such as diabetes mellitus, rheumatism, fever or arrest of bleeding. The hot water extracts of the roots are also used for the treatment of stomach problems, heart diseases and as antigalactagogue and cholagogue agent (Ross, 2003). In addition, the leaves of this species are also chewed, in some places,

as a way to suppress the sensations of hunger and fatigue (van der Heijden et al., 2004). The anticancer alkaloids present in this plant namely vinblastine and vincristine have been proven to induce mechanism of apoptosis through caspase-3 activation (Berry et al., 2001). Previous studies showed that the induction of apoptosis initiated by these compounds are associated with cell cycle arrest, particularly metaphase arrest by vinblastine (Berry et al., 2001) and G_2/M phase arrest by vincristine (Wang et al., 1998). These alkaloids achieved a very important role for the treatment of Hodgkin's and non-Hodgkins's lymphomas, acute lymphoblastic leukaemia, neuroblastoma and breast carcinoma (Dong et al., 1995). As the induction of apoptosis is a highly desirable goal of strategy for cancer control, it is thus important to evaluate potential apoptotic inducer from plants, either in the form of crude extracts or as isolated compounds (Taraphdar et al., 2001).

However, the problem arises from vinblastine and vincristine is the toxicity effects. These two alkaloids have similarity in structure, only the substitution of a formyl group for a methyl group in vincristine, in comparison with vinblastine. The dose-limiting toxicity for vincristine is neurotoxicity while bone marrow toxicity limits the use of vinblastine (Lobert, Ingram & Correia, 1999). Even though many further studies have been conducted to reduce the toxicity effects through the modification of their structure and production of analogues of vinblastine and vincristine (Ishikawa et al., 2009; Kuboyama et al., 2004), yet the safety effects on normal cells remain unclear. Therefore, the study of the safety sides of *C.roseus* extract is getting more important. Moreover, only few data are available that reported the effects of this extract on normal cells. Differential effects of the extract between cancer cells and normal cells could lead to innovative strategies in cancer control. To

study new therapeutic immunomodulatory approaches, in present study, the cytotoxicity effects of crude *C.roseus* aqueous extract were compared between leukemic Jurkat T-cell line and normal peripheral blood mononuclear cells (PBMCs). Investigation of the cell proliferation effect of the natural product on normal immune cells is considered as a preliminary study for the discovery of new immunomodulator from herbs, and human PBMCs is commonly used as a target cell for this study (Kumar et al., 2004; Swamy & Tan, 2000). Thus, an investigation of cytotoxicity effects on normal PBMCs may expand the knowledge of the possible biological effects of this crude extract consumed in folkloric medicine.

1.2 Objectives of the Study

The *C.roseus* aqueous extract that were usually prepared by decoction or hot water extract has been historically used to treat a wide assortment of diseases in various developed and developing countries. Ironically, only few scientific reviews reported on the effects of this crude aqueous extract on cells, especially on the normal cells. The objectives of the present study are as follow:

1. To quantify concentration of vinblastine, one of the anticancer compounds in *C.roseus* aqueous extract
2. To compare the growth inhibitory effects of the *C.roseus* aqueous extract on normal PBMCs and Jurkat cells
3. To identify the type of cell death in *C.roseus*-treated Jurkat cells
4. To determine the proliferation of T-helper ($CD4^+$) and T-cytotoxic ($CD8^+$) cells in *C.roseus*-treated PBMCs
5. To evaluate the effects of the *C.roseus* aqueous extract on the production of immunoglobulins and complements

6. To screen the differential expression of genes in *C.roseus*-treated Jurkat cells associated with apoptosis and cell cycle progression

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer Surveillance

Carcinogenesis implies adaptation of cancer cells to an adverse environment. The immune surveillance hypothesis originally describes the ability of the immune system to recognize and destroy malignant cells. However, in certain circumstances, the cancer cells are capable to modify the immune system to avoid destruction (Tham & Abastado, 2011). Further discoveries on immune surveillance led to the formation of a concept of "cancer immunoediting" that further clarified the hypothesis of cancer immunosurveillance. Three distinct stages of cancer immunoediting include elimination, equilibrium and escape (Dunn, Fecci & Curry, 2012), as shown in Figure 2.1.

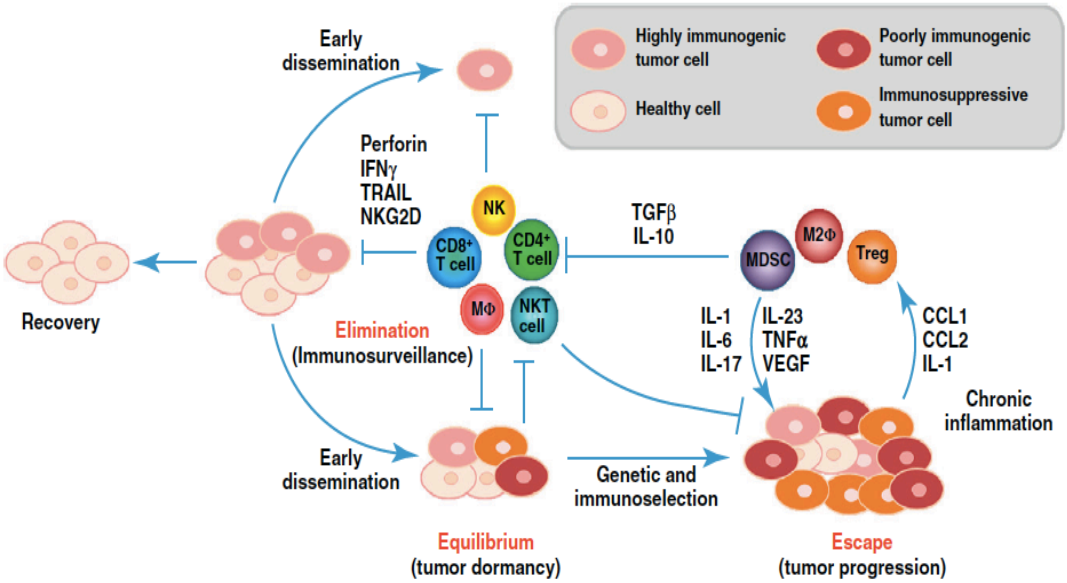

Figure 2.1. Phases involved in immunoediting. Taken from "Escape of tumor immune surveillance and metastasis" by M. Tham and J.P. Abastado (2012). *Drug Discovery Today: Disease Models*, 8(2-3), p. 82.

2.1.1 Elimination

In this phase, tumor-specific antigens trigger innate and adaptive immune response, which leads to the destruction and early dissemination of cancer cells, especially high immunogenic cancer cells (Figure 2.1). Infiltration of immune cells into tumors is a well-documented observation especially in solid tumors (Chowa, Mollerb & Smytha, 2012). There are many mechanisms involved to eliminate the cancer cells, for instance the recognition of stress-induced ligands by lymphocyte activation receptor will alert the neighboring cells to early transformation (Guerra et al., 2008). Another mechanism can be through the release of danger signals by either transformed or dying tumor cells that will initiate an activation of immune system (Sims et al., 2009). On the other hand, in the absence of signals, the early transformed cancer cells could not be recognized by the immune cells and will enter the next phase called equilibrium phase.

2.1.2 Equilibrium

The equilibrium phase is defined as a latent period in cancer development in which the survived cancer cells persist in a dynamic interaction with immunity within the cancer microenvironment (Dunn et al, 2012). Koebel et al. (2007) showed that when a low dose of carcinogen MCA administered into immunocompetent mice, small masses were formed at the injection site at a long-term period of time. Meanwhile, when the carcinogen MCA administered into immunocompetent mice with absence of either T cells or IFN, the masses were formed rapidly. The equilibrium phase involves immune selection pressure resulting a formation of tumor cells with reduced immunogenicity. Since equilibrium phase is a continuous process between elimination of tumor cells and emergence of resistant tumor cells, thus this phase is considered as the longest phase in immunoediting (Chowa, 2012). The

cancer cells survive in equilibrium phase by reducing their immunogenicity and/or induce immunosuppression. The cellular components of immune system affected by reduced immunogenicity and immunosuppression include Natural Killer (NK) cells, macrophages, $CD8^+$ T- cells and $CD4^+$ T- cells, as shown in Figure 2.1. It has been shown that in CD8-depleted animals, the pathological examination of their lungs revealed a higher number of proliferating cancer cells, suggesting $CD8^+$ cells control metastatic dormancy through its secretion of cytostatic cytokines and direct cytotoxicity that will inhibit the proliferation of cancer cells. In this phase, the number of immunosuppressive and poorly immunogenic cancer cells begins to accumulate (Tham & Abastado, 2011). Overall, there are three possibilities of outcomes as a result of the interaction between the tumor cells and immunity which are; 1) the tumor cells may persist in this phase without progression, 2) the tumor cells may outstrip immune pressure, 3) the tumor cells may be clinically manifested in escape phase (Dunn et al, 2012).

2.1.3 Escape

In this phase, the tumor cells progress and overcome the host through either intrinsic or extrinsic changes. Intrinsic changes occur at tumor cell level by reducing the ability of a tumor cell to be recognized or killed, whereas extrinsic changes involve the microenvironment by attenuating the effector capacity of the immune system (Dunn et al., 2012). Reduction of immunogenicity such as loss of major histocompatibility complex (MHC) class I protein of tumor cells can lower immune recognition by tumor-specific T cells or other recognition pathways. Additionally, an expression of anti-apoptotic molecules may cause the tumor cells become resistant to immune system, allowing them to survive. On the other hand, a complex

immunosuppressive network within the tumor environment creates a crosstalk between the environment and tumor cells. One of the possible mechanisms that lead to immunosuppressive is the release of several factors from immune cells such as vascular endothelial growth factor (VEGF), transforming growth factor (TGF), prostaglandin E2 and interleukin-10 (IL-10) (Chow et al., 2012), as shown in Figure 2.1.

2.2 Plant-derived Anticancer Compounds

The application of plants has proved to be an important natural source to develop an effective anticancer therapy for several years. Approximately 30 compounds that are derived from plant have been successfully isolated and are currently under clinical trials (Nirmala, Samundeeswari & Sankar, 2011). The secondary metabolites have proved to be an excellent reservoir of new medical compounds from various types of plant for examples, *Catharanthus roseus, Podophyllum* species*, Taxus brevifolia, Camptotheca acuminate, Betula alba, Cephalotaxus* species*, Erythroxylum pervillei, Curcuma longa, Ipomoeca batatas* and *Centaurea schischkinii* (Davis & Kuttan, 2000). According to Nirmala et al. (2011), the major plant-derived anticancerous compounds has been classified into four: 1) vinca alkaloids; 2) epipodophyllatoxin lignans; 3) taxane diterpenoids and 4) camptothecin quinolone alkaloid derivatives.

2.2.1 Major Groups of Natural Anticancer Compounds

Vinca alkaloids are the most important class of anticancer drugs. The mechanism of action includes the inhibition of cell proliferation through mitotic arrest that eventually leads to the mechanism of programmed cell death, apoptosis. The major naturally occurring active anticancer compounds are known as vinblastine

and vincristine also present in the *C.roseus*. The effectiveness of these compounds has been developed by producing semi-synthetic analogues such as vinorelbine and vindesine. These novel vinca alkaloids are currently under phase II clinical trials (Okouneva et al., 2003; Simeons et al., 2008).

The second group of anticancer compounds, which is known as podophyllatoxin, comprises of compounds isolated from *Podophyllum* species. So far, there were two semi-synthetic analogs derived from the isomer of podophyllatoxin, Epipodophyllotoxin namely Etoposide and Teniposide. These two semi-synthetic compounds are believed to exhibit effective effects for the treatment of lymphomas, bronchial and testicular cancers (Shoeb, 2006).

The third group is known as taxanes and the major compounds are known as paclitaxel (Taxol®) and Docetaxel (Taxotere®). Paclitaxel is the major compound extracted from the bark of the Pacific Yew, *Taxus brevifolia Nutt.* (Taxaceae) while the Docetaxel is the semi-synthetic derivative of paclitaxel (Nirmala et al., 2011). Many previous reports have emphasized that these compounds are well known as an effective microtubule inhibitor for cancer chemotherapy. Moreover, these drugs are usually applied for the treatment of ovarian cancers, squamous cancers of the head and neck, as well as for non-small cell lung cancer, small-cell lung cancer, and other types of cancers (Singla, Garg & Aggarwal 2002).

Another major group of natural compounds used in cancer chemotherapy is camptothecin extracted from the barks and stem of the Chinese ornamental tree, *Camptotheca acuminate*. The analogues of this compound were previously produced

due to the poor solubility and severe toxicity effects of camptothecin. The examples of the analoques include topotecan, irinotecan, 9-aminocamptothecin, lurtotecan and rubitecan. The anticancer mechanism of these compounds is through the inhibition of DNA Topoisomerase I leading to the disruption of the DNA functions and transcription (Srivastava et al., 2005).

Many other plant-derived anticancer compounds also have anticancer properties through various mechanisms, for instance, berbamine that was isolated from *Berberis amarensis* and usually used for the treatment of chronic myeloid leukemia. This compound inhibited the proliferation of cancer cells via caspase-3-dependent apoptosis (Kim et al., 2007). Other active compounds that also act through the induction of apoptosis are Silvestrol that was derived from *Aglaia foveolata* (Kinghom et al., 2009) has been used for prostate, breast and lung cancers while PG490-88 from *Tripterygium wilfordii* (Liu et al., 2011) is widely used to treat prostate cancer.

2.2.2 *Catharanthus roseus*

This plant is commonly known as periwinkle or kemunting cina (in Malay) and belongs to Apocynaceae family. This plant belongs to a small genus of 8 species and most of the species originate from Madagascar. Figure 2.2 illustrates the various species of *C.roseus* plant. The Madagascar periwinkle has been cultivated as an ornament throughout the tropics and occasionally in the subtropics. This plant can be characterized by an erect or decumbent, deciduous undershrub that can grow between 30 to 200 cm height and usually with white latex. The length of the roots is usually up to 70 cm long while the stems are often woody at base. The leaves are

Figure 2.2. Various species of Madagascar periwinkle *C.roseus*. Taken from "Know the medical herb: *Catharanthus roseus* (Vinca rosea)" by K. Y. Loh (2008). *Malaysian Family Physician*, 3(2), p. 123.

decussate, glossy, elliptical to obovate or narrowly obovate, wedge-shaped while the colour of the leaves are dark green (Aslam et al., 2010; Padua et al., 1999). Some of the leaves are oblique at base, apex is obtuse or acute with a mucronate tip. Meanwhile, the flowers are actinomorphic, bisexual, 5-merous and subsessile. The sepals are slightly connate at base and green in colour. Meanwhile, the petal is salver-shaped, and the colour of the petal can vary, including pink, rose-purple or white with a purple, red, pink, pale yellow or white centre with tube 2-3 cm long and widening near the top (Padua et al., 1999).

C. roseus is synonym with its characteristic alkaloids (Pereira et al., 2010). Even though it has been reported that this plant contains more than 130 alkaloids,

only some of these are commercially available such as serpentine, ajmalicine, vincristine and 3', 4'-anhydrovinblastine. The first two alkaloids are prescribed for antihypertensive and another report has mentioned that serpentine also has been used as sedative. The vinblastine (vincaleucoblastine) and vincristine (leucristine) are commonly used as anticancer for various types of cancers (van der Heijden et al., 2004). Aslam et al. (2010) reported that vinblastine sulphate with its brand drug name Velban has been used for the treatment of Hodgkin's disease, lymphocarcoma, choriocarcinoma, neuroblastoma, breast carcinoma, lungs and other organs in acute and chronic leukemia while vincristine sulphate with its brand drug name, Oncovin has been scientifically proven can treat acute leukaemia in children, Hodgkin's disease, Wilkins's tumor, neuroblastoma and reticulum cell sarcoma. Vinblastine and vincristine are known as secondary metabolites and considered as the end products of this plant extract.

Moreover, the production of the vinblastine and vincristine is still dependent on the wild type plant because they remain impossible to be synthesized *in vitro* (Exposito et al., 2009). These alkaloids are shown to be tissue-specific, which require both aerial and root parts of a plant to be synthesized (De Luca & Laflamme, 2001). The yield of the compounds extracted from the plant is usually very low and therefore, difficult for purification. These limitations make the compounds become highly valuable and expensive (Costa et al., 2008; Fett-Neto, DiCosmo, Reynolds, & Sakata, 1992; Noble, 1990). This is due to the complexity and unique bisindole alkaloid structure, which are difficult to produce synthetically. Previous study reported that this is partly due to the difficulty to obtain correct conformation of stereogenic centers, which are crucially important for their activity. For instance, in

vincristine the configuration at the C-16′ stereogenic centre is S while at C-14′ it is R and S at C-20′. The inversion of C-16 configuration from S to R results in a complete loss of activity as does the C-14′ conversion from R to S (Kuehne & Marko, 1990). The chemical structures of the major active compounds are illustrated in Figure 2.3.

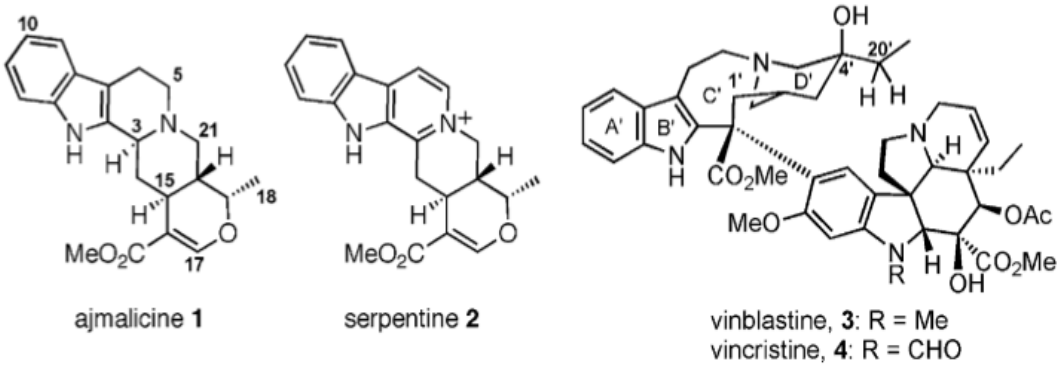

Figure 2.3. The chemical structures of selected terpenoid indole alkaloids derived from *C.roseus* plant. Ajmalicine (1) used as antihypertensive agent, serpentine (2) dor sedative, vinblastine (3) and vincristine (4) used as anticancer agents. Adapted from "Rapid Identification of Enzyme Variants for Reengineered Alkaloid Biosynthesis in Periwinkle" by P. Bernhardt, E. McCoy and S. E. O'Connor, 2007, *Chemistry & Biology*, 14, p889.

Apart from the clinical application of some important alkaloids in *C.roseus* plant, the medicinal purposes of its wide application as a folk remedy should be highlighted. The extracts are usually prepared as a decoction or hot water extract of either the entire plant, leaves, roots or aerial parts (Aslam et al., 2010). An extensive study has previously summarized the traditional purposes of the extract, that vary in different countries, for example the hot water extract of *C.roseus* has been used for diabetes in Europe (Swanston-Flatt et al., 1989) while in India, the juice from the leaves was used to treat wasp stings (Farnsworth et al., 1961). However, there are not many scientific reviews that clearly emphasized the biological effects of the *C.roseus*

crude extract despite its wide applications as a worldwide folk remedy. The summary of the traditional medicinal purposes of the application of *C.roseus* aqueous extract that were consumed orally is as shown in Table 2.1.

Table 2.1
The Traditional Medicinal Purposes of Crude Aqueous Extract of C.roseus

Country	Method of preparation	Traditional uses	Citation
Australia	Hot water extract of dried leaves	Menorrhagia, diabetes and extract of root bark is taken orally as febrifuge.	Bhandari et al., 1959; Webb, 1984
Brazil	Hot water extract of the entire plant	Diabetes mellitus.	Brandao et al., 1985; De Mello, 1980
China	Hot water extract of the aerial parts	Menstrual regulators.	Fransworth, 1961; Virmani et al., 1978
Cook Island	Decoction of dried leaves	Diabetes, hypertension and cancer	Holdsworth, 1990
Dominica	Hot water extract of leaves	Consumed by pregnant woman to combat primary inertia in childbirth, diabetes	Hodge & Taylor, 1956
England	Hot water extract of dried entire plant	Diabetes	Thompson, 1976
Europe	Decoction of dried leaves	Diabetes	Swanston-Flatt et al., 1989
France	Hot water extract of dried leaves	Antigalactagogue	Fransworth, 1961
French Guina	Hot water extract of dried leaves	Cholagogue	Luu, 1975
India	Hot water extract of dried entire plant	Cancer	Virmani et al., 1978
Jamaica	Hot water extract of dried leaves	Diabetes	Morrison, 1982
Kenya	Hot water extract of dried leaves	Diabetes	Morrison, 1982
Mozambique	Hot water extract of aerial parts	Diabetes, rheumatism	Amico, 1977
North Vietnam	Hot water extract of	Menstrual regulators	Fransworth, 1961; Virmani et al., 1978
Pakistan	Hot water extract of dried ovules	Diabetes	Atta-Ur-Rahman, 1982
Peru	Hot water extract of dried entire plant	Cancers, heart disease and leishmaniasis	Fransworth, 1961
Philippines	Hot water extract of root	Consumed by pregnant women for abortion	Virmani et al., 1978
South Africa	Hot water extract of dried leaves	Menorrhagia and diabetes	Bhandari et al., 1959;
South Vietnam	Hot water extract of entire plant	Antigalactagogue	Fransworth, 1961; Virmani et al., 1978
Taiwan	Decoction of dried entire plant	Diabetes and liver diseases	Fransworth, 1961
Thailand	Hot water extract dried entire plant	Diabetes	Yang et al., 1987

Country	Method of preparation	Traditional uses	Citation
Venda	Hot water extract of dried root	Venereal diseases	Siegel, 1976
West Indies	Hot water extract of leafy stems	Diabetes	Nguywen, 1977

Notes. Adapted and revised from "*Catharanthus roseus* (L.) G. Don. An Important Drug: Its Applications and Production" by J. Aslam, S. H. Khan, Z. H. Siddiqui, Z. Fatima, M. Maqsood, M. A. Bhat, S. A. Nasim, A. Ilah, I. Z. Ahmad**,** S. A. Khan, A. Mujib and M. P. Sharma, 2010, *Pharmacie Globale*, 4(12), p. 2.

2.3 Mechanism of Cell Death

The balance between the rate of cell death and the rate of cell division ensure the integrity and physiological homeostasis of an organ. The mechanism of cell death is important to remove terminally injured or unwanted cells that utilize valuable substrates and nutrients (Coates et al., 2010).

2.3.1 Type I Cell Death

Type I cell death is also known as apoptosis. The term Apoptosis is originated from Greek word (apo – from, ptosis – falling) (Kerr, Wylie & Currie, 1972). Apoptosis can be defined as a genetically programmed mechanism that allows the cell to commit suicide (Duprez et al. 2009; Fulda et al. 2010). Apoptosis is characterized with distinct biochemical and morphological features that play important roles in the maintenance of tissue development, homeostasis and regulation of immune responses. However, the interruption of this mechanism can lead to various disorders including cancer, autoimmune and neurodegenerative diseases. Therefore, the modulation of the cascade pathway of apoptosis is a potential therapeutic approach for the treatment of several human diseases (Agostini et al., 2011).

Apoptosis has been further classified into two major well-studied pathways namely extrinsic or receptor-mediated pathway and intrinsic or mitochondria-mediated pathway, as illustrated in Figure 2.4. The extrinsic pathway is initiated through an engagement between the death receptors present on the cell surface and their respective ligands (Scaffidi et al., 1998). The death receptors are from a sub-group of Tumor Necrosis Factor receptors (TNFR) superfamily, for example TNF, CD95 (Fas) and TNF-related apoptosis inducing ligand (TRAIL) receptors. These tetrameric receptors have an extracellular domain to engage the ligands and an intracellular cytoplasmic domain that is also referred to death domain that is responsible for the death signal transmission (Scott et al., 2009). The signal is transmitted from the surface to the intracellular signaling pathways via an interaction between the death receptors and their specific ligands (Ashkenazi & Dixit, 1998). This interaction would further induce homotypical interactions of the death receptors with Fas-associated death domain (FADD) or with TNER-associated death domain (TRADD) of specific nuclear protein. As a result, a signal complex called death inducing signaling complex (DISC) is formed and activated leading to the recruitment and activation of initiator caspases, procaspase-8 and -10 (Green, 1998, 2005; Portt et al., 2011). The caspase cascade is the most studied signaling pathway in apoptosis. Activated caspase 8 will subsequently activate downstream effector caspases, leading to the onset of apoptosis (Zhang et al., 2010).

The second apoptosis pathway is known as intrinsic or mitochondria-mediated pathway (Figure 2.4). This pathway can be induced by receptor-independent stimuli such as radiation, free radicals, viral infections and serum/growth factor withdrawal (Indran et al., 2011). Following the death trigger,

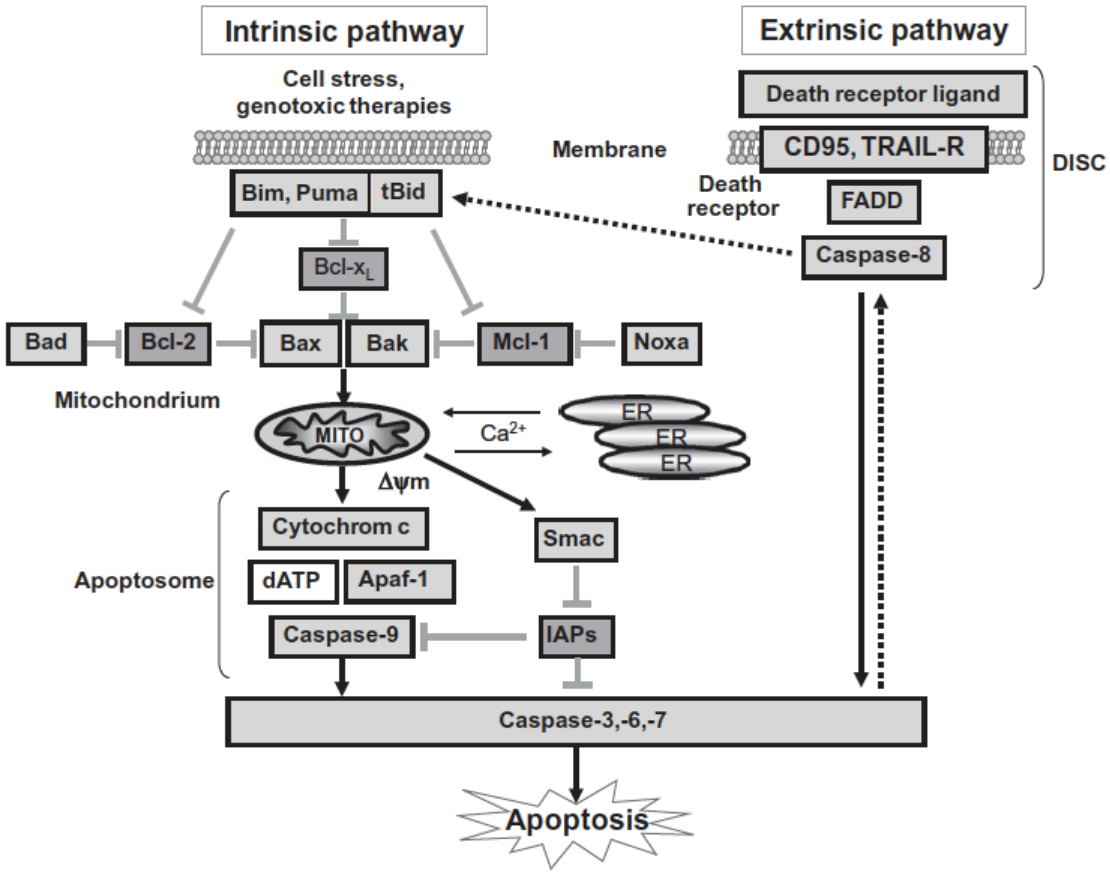

Figure 2.4. Apoptosis signaling pathways. The extrinsic pathway is initiated at the cellular membrane through the binding of death receptor ligands to their respective receptors while the intrinsic pathway involves the depletion of mitochondrial membrane potential and release of pro-apoptotic proteins such as cytochrome c, Smac/DIABLO (second mitochondria-derived activator of caspases). The black arrow indicates activation while the grey line indicates inhibition. Taken from "Targeting Apoptotic Pathway by Celecoxib in Cancer" by V. Jendrossek, 2011, *Cancer Letters*, p.3.

the mitochondrial permeability transition pore open and cause the changes of the permeability in the inner part of the mitochondria (Hengatner, 2000).

The mitochondrial transmembrane potential is lost leading to the release of pro-apopotic proteins that can be classified into two categories. The first group comprises proteins that activate the caspase-dependent pathway. The examples of the proteins are cytochrome c and Smac/DIABLO (second mitochondria-derived activator of caspases). The cytochrome c induces oligomerization of Apaf-1

(apoptotic protease activating factor 1) and this activates the caspase 9. This mechanism further initiates the formation of apoptosome which consists of activated cytochrome c, Apaf-1 and caspase 9. An executioner caspases 3 and 7 are further activated and consequently, the cell dismantles through nuclear fragmentation (Hengatner, 2000; Gross et al., 1999; Slee et al., 1999). Apoptotic progression occurs as the protein of Smac/DIABLO binds to IAPs (inhibitor of apoptosis proteins) to deactivate them and thus, allowing the apoptosis to occur (Scaffidi et al., 1998). The second group of pro-apoptotic proteins consists of apoptosis inducing factor (AIF) and endonuclease G (Endo G). The release of these proteins has been reported to occur during late apoptosis and associated with the translocation of the proteins to the nucleus and DNA fragmentation. Unlike the first group of proteins, the apoptosis mechanism initiated by the second group of pro-apoptotic proteins is caspase-independent (Joza et al., 2001).

Morphologically, apoptotic cells are characterized by chromatin condensation, cleavage of chromosomal DNA into internucleosomal fragments, cell shrinkage, membrane blebbing, formation of apoptotic bodies with plasma membrane breakdown, phospatidylserine (PS) externalization to the outer leaflet of outer membrane, followed by ordered removal by phagocytes (Luthi & Martin, 2007). A typical distinct morphological feature of apoptotic cells is a cleavage of DNA into fragments with a size of 180-200 bp (Compton, 1992).

(a) Apoptotic-regulated Proteins

The mechanism of apoptosis can be regulated by several factors. Apart from the proteins that directly take part as components in the apoptotic pathway such as

death receptors, adaptor proteins, kinases and caspases, the well-known and major regulator is Bcl-2 family that includes at least 20 proteins and share between one and four conserved regions, designated "Bcl-2 homology domains" (BH) (Kuroda & Taniwaki, 2009; Willis et al., 2007). A study reported by Kuroda and Taniwaki (2009) has further broadened the groups into BH1-4, BH1-3 (Bax/Bak-like proteins), BH2-3, BH3-4, and BH-3 only proteins. The proteins that carry these domains have been further classified into pro- and anti-apoptotic, as shown in Figure 2.5.

All the anti-apoptotic members, which are Bcl-2, Bcl-X_L, Bcl-w, A1 and Bcl-B carry four regions of the BH domains except for Mcl-1 that has three regions. Additionally, the pro-apoptotic proteins, the Bax/Bak-like proteins include Bax, Bak, and Bok with three BH domains whereas BH-3 only proteins consist of Bim (Bcl-2-interacting mediator of cell death, also known as Bod), Bad (Bcl-2-antagonist of cell death), Bik (Bcl-2-interacting killer, also known as Nbk or Blk), Bid (BH3-interacting domain death agonist), Hrk (Harakiri, also known as DP5), Noxa, Puma (p53-upregulated modulator of apoptosis, also known as Bbc3) and Bmf (Bcl- 2 modifying factor). The pro-apoptotic members with different groups are activated by different death stimuli and capable of neutralizing the anti-apoptotic proteins (Cory & Adams, 2002; Huang & Strasser, 2000).

A previous study has shown that Bcl-2 and Bcl-X_L protect the cells by interacting with mitochondrial proteins, for instance adenine nucleotide translocase (ANT) and voltage dependent anion channel (VDAC). As a result, mitochondrial pores formation is inhibited, thus protecting membrane integrity and inhibiting release of cytochrome c. Bax, a pro-apoptotic protein is located in the cytosol.

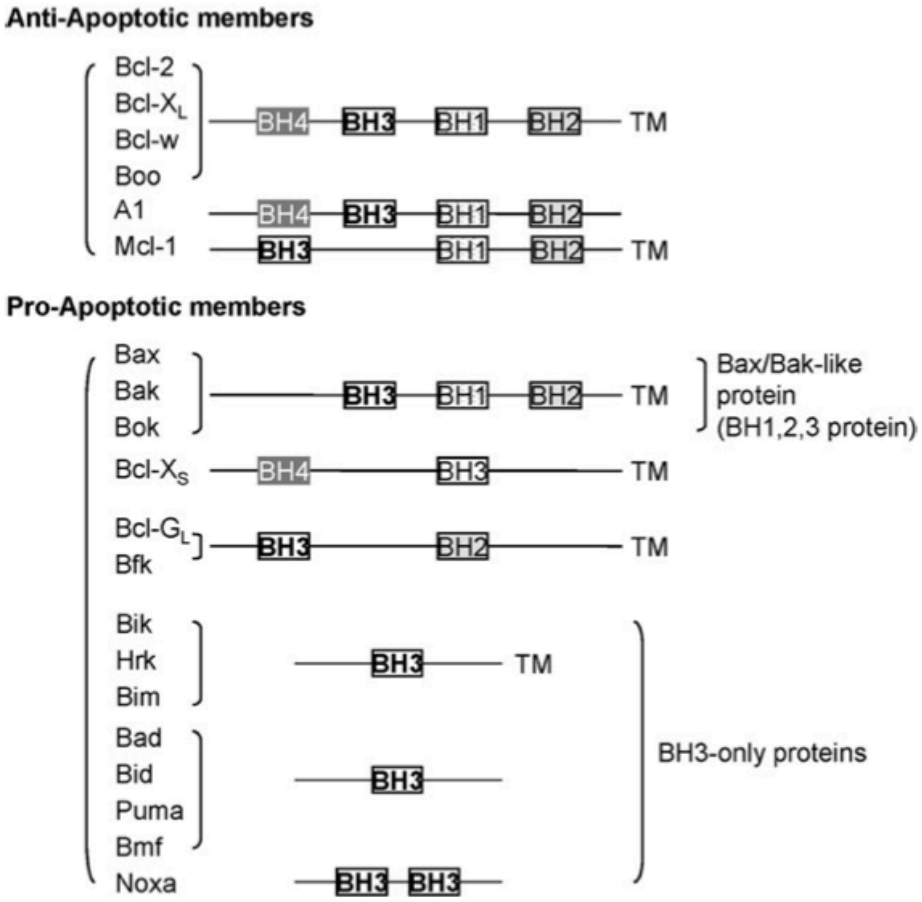

Figure 2.5. List of anti-apoptotic and pro-apoptotic members of Bcl-2 protein family. Taken from "Involvement of BH3-only Proteins in Hematologic Malignancies" by J. Kuroda and M. Taniwaki, 2009, *Critical Reviews in Oncology/Hematology*, 71, p. 90.

Following the death trigger, this protein will translocate to the mitochondria and interact with other pro-apoptotic proteins such as Bak or truncated Bid. Thus, mitochondrial pores are formed, eventually leading to the release of cytochrome c (Czabotar et al., 2007). Some studies have shown that Bax protein can also interactwith ANT and/or VDAC to induce the mitochondrial permeability and the formation of pores (Marzo et al., 1998). Another pro-apoptotic protein Bak localizes at the outer membrane of mitochondria and endoplasmic reticulum. The activity of these apoptotic proteins can be inhibited via their binding with anti-apoptotic Bcl-2

family members (Czabotar et al., 2007; Huang & Strasser, 2000; Puthalakath & Strasser, 2002).

However, a previous study suggested that the pathways can be linked and the molecules involved can affect the others in another pathway (Daniel & Korsmeyer, 2004). Moreover, many recent evidences have shown greater complexity and diversity in each pathway and enable to cross-activate to not only intrinsic or extrinsic pathway but also necrotic sub-pathways (Duprez et al., 2009; Whelan, Kaplinsky & Kitsis, 2010).

(b) Apoptosis and Cancer

In cancer, the malignant transformation of cells involves a complex multi-step process, allowing the cells to protect themselves from the mechanism of apoptosis (Coates et al., 2010). Consequently, the cells can survive against various adverse events such as metabolic stress, oncogene activation, nutrient and growth factor depletion, lack of blood supply, hypoxia and therapeutic intervention (Folkman, 2003; Mathew et al., 2007). Thus, many research have been done that target the mechanisms of cancer progression (Indran et al., 2011).

The most common factor causing the inhibition of apoptosis in human cancer cells is the loss or inactivation of tumour suppressor p53. This is followed by other factors such as, the inactivation of the apoptosome-mediated pathways and defect in the components of apoptotic machinery like APAF-1 and caspase-9 (Soengas et al., 1999). Loss of p53 function has been shown contribute to the instability of mitochondrial genome resulting to an increase level of cellular ROS and thus will

distract the mitochondrial-dependent apoptotic pathway (Brandon et al., 2006; Chen et al., 2010; Pani, Koch & Galeotti, 2009). Moreover, mutations or altered expression of p53 downstream effectors such as Bax, Bak and APAF-1 or upstream regulators such as ATM and Mdm 2 have also been reported in many types of cancer. Therefore, mutant p53 is always associated with resistance to standard chemotherapy as it blocks the mechanism of apoptosis induced by the chemotherapeutic agents (Ekert et al., 2004). Meanwhile, the inactivation of the apoptosome-mediated pathways were previously reported to occur in human cancer cells, including malignant melanoma, ovarian cancer, leukemia, and non-small cell lung carcinoma (Wolf et al., 2001).

Additionally, the roles of IAPs that are selectively overexpressed in various types of cancers will also lead to the defect in apoptotic pathways and have been shown in previous study (Nachmias, Ashhab & Ben-Yehuda, 2004). They proposed an effective therapeutic target by specifically inhibiting the IAPs through cell-permeable Smac peptides, leading to the inhibition of its binding to caspase. This may sensitize the cancer cells to chemotherapy both *in vitro* and *in vivo* (Fulda et al., 2002)

2.3.2 Type II Cell Death

This type of cell death refers to a process called autophagy. It is a fundamental cellular homeostatic mechanism, whereby cells autodigest parts of their cytoplasm for removal or turnover. The examples include cell injury and accumulation of aggregated proteins, damaged organelles or membranes and intracellular parasites (Mathew et al., 2007; Mizushima et al., 2008). This mechanism has the ability to recycle old components into new building blocks that