ANTI-NEOPLASTIC STUDIES OF STANDARDIZED ROOT EXTRACT OF *EURYCOMAL LONGIFOLIA* (TAF273) TOWARDS LEUKAEMIA

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By

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DEDICATION

This thesis is dedicated to …

the soul of my parents

my brothers and sisters

and to

my beloved wife and kids

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ABL (ABL1)	Abelson murine leukemia viral oncogenes homolog 1
ABTS	[2,29-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)]
AC	Apoptotic cells
AI	Apoptotic index
ALL	Acute lymphoblastic leukaemia
ALT	Alanine aminotransferase
AML	Acute myeloid leukaemia
AP	Accelerated phase
APL	Acute promyelocytic leukaemia
ARG	Abelson-related gene
AST	Aspartate aminotransferase
ATRA	all trans retinoic acid
B	B lymphocyte
Baso	Basophil
Bax	Bcl-2-associated X
BC	Blast crisis
Bcl	B-cell lymphoma
BCR	Breakpoint cluster region
BFU-E	Erythroid burst forming unit
BIRC5	Baculoviral inhibitor of apoptosis repeat-containing 5
BM	Basement membrane
BV	Blood vessel
CAM	Chorioallantoic membrane

CBL	Casitas B-lineage lymphoma pro-oncogene protein
CCND1	Cyclin-D1
CCNE1	Cyclin E1
CCyR	Complete cytogenetic response
CD	Cluster of differentiation
CDKN1B	Cyclin-dependent kinase inhibitor 1B
cdks	Cyclin- dependent kinases
CEN	Checken erythrocyte nuclei
CFU-GM	Granulocyte–macrophage colony forming unit.
CHR	Chronic heart disease
CI	Combination index
c-Kit	Proto-oncogene with tyrosine kinase activity
CLL	Chronic lymphocytic leukaemia
CLP	Common lymphoid progenitor
CML	Chronic myelocytic leukaemia
CMP	Common myeloid progenitor
C-Myc	Cellular Proto-oncogene myc
CP	chronic phase
CRKL	CRK-like protein
C_T	Threshold cycle
DBL	Diffuse B-cell lymphoma oncogene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DRI	Dose reduction index

E. longifolia	*Eurycoma longifolia*
EC	Endothelial cell
ECGS	Endothelial cell growth supplements
ECM	extracellular matrix
ECM	Endothelial cell medium
ED	Effective dose
Eo	Eosinophil
F1	Fraction 1
F3	Fraction 3
F4	Fraction 4
FBS	Fetal bovine serum
FGF	Fibroblast growth factors
FITC	Fluorescein isothiocyanate
G1	G1 phase
G2	G2 phase
GADD45A	Growth arrest and DNA damage-45 alpha
GAP	Guanosine triphosphatase-activating function protein
GDP	Guanosine diphosphate
GEF	GDP-GTP exchange factor
GIR	Growth inhibition rate
GRB2	Growth factor receptor-bound protein 2
GTP	guanosine triphosphate
H&E	Hematoxylin and eosin
HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
HIFs	Hypoxia inducible factors

HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigens
HUVECs	Human umbilical vein endothelial cells
IC50	Half maximal inhibitory concentration
IHD	Ischemic heart diseases
IL	Interleukin
INF-α	Interferon-α
IP	Intraperitoneal
JAK	Janus kinase
JNK	Jun N-terminal Kinase
kb	Kilo base
LTA	Lymphotoxin-alpha
MAP	Mitogen-activated protein
MC	Mitotic cells
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MCyR	Major cytogenetic response
MDS	Myelodysplastic syndrome
mg	Milligram
mgQE/g	Quercetin equivalent per g
MMPs	Matrix metalloproteinases
MNCS	Mononuclear cells
Mono	Monocyte
MPDs	Myeloproliferative disorders
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-

	2H-tetrazolium]
NADH	Nicotinamide adenine dinucleotide
NC	Necrotic cells
NCEs	Newly chemical entities
N-CoR	Nuclear receptor corepressor
Neut	Neutrophil
P53	Tumor suppressor protein 53
PAs	Plasminogen activators
PBS	Phosphate buffer saline
PC	Pericytes
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PCV	Polycythemia vera
PD-ECGF	Platelet-derived endothelial cell growth factor
PDGF	Platelets derived growth factor
PDGFR	Platelet-derived growth factor receptor
PE	Plating efficiency
PEDF	Pigment epithelium-derived factor
Ph	Philadelphia
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PlGF	Placenta growth factor
PLT	Platelets
PS	Phosphatidylserine
QC	Quality control
qRT-PCR	Quantitative real time PCR

RAC	RAS-like GTPase
RAR	Retinoic acid receptor
RARα	Retinoic acid receptor alpha
RAS	Rat sarcoma
RBC	Red blood cells
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
S	Synthetic phase
SCD	Sickle cell disease
SCT	Stem cell transplant
SD	Standard deviation
SF	Survival fraction
SH1	SRC homology domain1
SH2	SRC homology domain2
SH3	SRC homology domain3
SHC	SRC homology 2-containing protein
SMART	Complex and silencing mediator for RAR and TR
SRC	Sarcoma
STAT	Signal transducers and activators of transcription
T	T lymphocyte
TAF273	Fraction 2
TGF	Transforming growth factor
TNF-a	Tumor necrosis factor alpha
TNFRSF10	Tumor necrosis factor receptor superfamily member 10

TNFRSF9	Tumor necrosis factor receptor superfamily member 9
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain
TRAF2	TNF receptor-associated factor 2
TRAF4	TNF receptor-associated factor 4
VC	Viable cells
VEGF	Vascular endothelial growth factor
VEGI	Vascular endothelial growth inhibitor
wt-HIF- 1α	Wild-type hypoxia-inducible factor 1 alpha
mg/kg	Milligram/kilogram
µg/ml	Microgram/millilitre

LIST OF SYMBOLS

α	Alpha
γ	Gamma
β	Beta
<	Less than
>	More than
μ	Micro
^{0}C	Celsius

KAJIAN ANTINEOPLASTIK MENGGUNAKAN EKSTRAK STANDARD AKAR *EURYCOMA LONGIFOLIA* (TAF273) TERHADAP LEUKEMIA

ABSTRAK

Aktiviti antineoplastik ekstrak akar Eurycoma *longifolia* telah dikaji ke atas penyakit leukemia menggunakan model *in vitro* dan *in vivo*. Empat subjenis spara tulen (F1, TAF273, F3 dan F4) ekstrak methanol akar *E. longifolia* asli yang diperolehi secara resin kromatografi disaring untuk aktiviti-aktiviti antileukemia, antiangiogenesis dan antioksidan. Aktiviti antileukemia telah diuji secara *in vitro* dengan lini sel leukemia K-562, HL-60 dan Kasumi-1 sementara kajian *in vivo* dengan K-562-xenograf menggunakan model tikus. Mekanisma tindakan, kombinasi kesan ubat dan toksik oleh TAF273T juga telah dikaji.

Kajian ini menunjukkan bahawa TAF273 mempunyai kesan sitotoksik yang paling efektif. Ia berpotensi untuk merencat pertumbuhan sel-sel K-562 dan HL-60 dengan nilai–nilai IC50 sebanyak 19 ± 3 dan 15 ± 2 μg/ml masing-masing. TAF273 merencat pertumbuhan sel-sel K-562 dan HL-60 (95%). Kesan sitotoksik TAF273 ke atas K-562 ditunjukkan juga dengan perencatan pertumbuhan koloni yang ketara dan kadar penurunan survival yang ekstrim (<0.5%).

Pemberian TAF273 secara intraperitoneal juga merencat pertumbuhan tumor di dalam K-562- xenograf tikus dengan kadar perencatan sebanyak 80.8% berbanding dengan control (P = 0.021). Tambahan pula, pewarnaan Hematoxylin/Eosin ke atas keratan tikus yang dirawat dengan TAF273 menunjukkan peningkatan yang ketara di dalam indeks apoptosis dan nekrosis (P < 0.001) serta penurunan yang jelas pada kehadiran saluran darah di dalam tumor berbanding dengan kontrol.

Kajian ini dengan jelas menunjukkan kesan sitotoksik adalah akibat daripada aruhan kepada apoptosis dan perencatan kitaran sel. TAF273 mengaruh kepada apoptosis dalam sel-sel K-562 dan HL-60 mengikut dos dan masa. Peningkatan apoptosis dibuktikan melalui 'externalization phosphatidylserine', kondensasi kromatin ($P < 0.01$) dan fragmentasi DNA. Analisa laluan apoptosis dalam sel 562 yang dirawat dengan TAF273 menunjukkan ekspresi pelbagai gen yang mengawal apoptosis juga turut terlibat. LTA, TNFRSF9, TNFSF10, CD70, TP53, TRADD, TRAF2 dan TRAF4 secara signifikan menunjukkan 'upregulated' sementara AKT1, BCL2, BID, BNIP2, BNIP3, NOD1, BAX, BCL2L11, BCL2L2, BFAR dan BRAF secara signifikan menunjukkan 'down-regulated' berbanding dengan kontrol ($P < 0.01$). Profil ekspresi gen mencadangkan bahawa mekanisma ekstrinsik and intrinsik terlibat di dalam aruhan apoptosis oleh TAF273.

Analisa aktiviti taburan kitaran sel dalam sel K-562 menunjukkan bahawa TAF273 menyekat pusingan sel pada fasa G1, S dan G2. Ini disokong oleh analisa laluan kitaran sel yang menunjukkan 'up-regulation' CDKN1B, GADD45A, GTF2H1, HERCS, HUS1 dan SERTAD1 serta, menunjukkan 'down-regulation' BCL2, CCNE1 dan PCNA berbanding dengan kontrol ($P < 0.01$).

Tambahan pula, TAF273 meningkatkan aktiviti sitotoksik imatinib ke atas sel-sel K-562. Kombinasi TAF273 dan imatinib menunjukkan kesan sinergistik ke atas sel K-562 pada nilai-nilai ED50, ED75 dan ED90. Kesan sinergistik ini menurunkan indeks dos sehingga 5 untuk imatinib dan kepada 2.5 untuk TAF273. Penurunan dos imatinib (sebanyak 5 kali) boleh mengurangkan kesan toksiknya.

Walaupun TAF273 menunjukkan kesan sitotoksik pada pada sel-sel leukemia, penting dinyatakan bahawa ia tidak menunjukan perubahan kepada parameter-

parameter hematologi, biokimia dan histologi apabila diambil secara oral sebanyak 200 mg/kg. Penemuan ini disokong oleh kesan sitotoksik TAF273 yang rendah ke atas sel-sel mononuklear periferi normal (IC50 180 µg/ml). Ini menunjukkan terdapat kesan sitotoksik selektif kepada sel-sel leukemia.

Di samping kesan sitotoksik, TAF273 juga menunjukkan aktiviti antiangiogenik yang kuat dan ini ditunjukkan dengan perencatan yang signifikan dalam penyebaran salurdarah mikro di dalam aorta tikus dengan IC_{50} sebanyak 11.5 µg/ml. TAF273 menunjukkan perencatan yang ketara (63.13%) pada pertumbuhan salur darah baru dalam membran 'chorioallantoic' embrio ayam. Kajian histologi ke atas tumor mendedahkan pengurangan yang jelas di dalam penyebaran salurdarah berbanding dengan kontrol ($P<0.01$). TAF273 dalam kajian *in vitro* merencat secara signifikan proses angiogenesis melalui langkah-langkah seperti proliferasi, migrasi dan pembezaan pada HUVECs.

Sebagai kesimpulan, penyeragaman komponen TAF273 daripada ekstrak methanolic akar *E. longifolia* berpotensi sebagai agen antitumor dan berkemungkinan dijadikan sebagai agen kemoterapi untuk rawatan leukaemia jenis kronik mieloid.

ANTI-NEOPLASTIC STUDIES OF STANDARDIZED ROOT EXTRACT OF *EURYCOMAL LONGIFOLIA* (TAF273) TOWARDS LEUKAEMIA

ABSTRACT

The anti-neoplastic activity of root extracts from Eurycoma *longifolia* was investigated towards leukaemia using *in vitro* and *in vivo* models. Four partially purified sub-fractions (F1, TAF273, F3 and F4) derived from resin chromatography of the crude methanolic extract of *E. longifolia* roots were screened for the anti-leukaemic, anti-angiogenic and anti-oxidant activities. The *in vitro* anti-leukaemic activity was tested using K-562, HL-60 and Kasumi-1 leukaemic cell lines whereas in vivo anti-leukaemic activity was tested using K-562-xenograft mice model. The toxicity, mechanism of action and drug combination effect of TAF273 were also investigated.

The results showed that TAF273 was the most effective cytotoxic fraction. It potentially inhibited the growth of K-562 and HL-60 cells with IC50 values of 19 ± 3 and 15 ± 2 μg/ml, respectively. The exposure of K-562 and HL-60 cells to TAF273 resulted in a significant growth inhibition (95%). The cytotoxicity of TAF273 on K-562 cells was also indicated by the remarkable inhibition of colony formation and the extremely low survival rates (< 0.5%).

Intraperitoneal administration of TAF273 also significantly inhibited tumor growth in the K-562-xenograft mice with growth inhibition rate of 80.8% compared with control (P = 0.021). In addition, Hematoxylin/Eosin stained sections from TAF273-treated mice revealed significant increase in the apoptotic and necrotic

indices ($P < 0.001$) and a remarkable reduction of intratumor blood vessels compared with control.

This study clearly indicates that the cytotoxic effect of TAF273 is most likely due to the induction of apoptosis and cell cycle arrest. TAF273 induced apoptosis in K-562 and HL-60 cells in a dose and time-dependent manner. The induction of apoptosis was evidenced by significant phosphatidylserine externalization, chromatin condensation ($P < 0.01$) and DNA fragmentation. Furthermore, analysis of the apoptotic pathway in K-562 cells treated with TAF273 demonstrated that the expression of various genes regulating apoptosis was also affected. LTA, TNFRSF9, TNFSF10, CD70, TP53, TRADD, TRAF2 and TRAF4 were significantly up-regulated, while AKT1, BCL2, BID, BNIP2, BNIP3, NOD1, BAX, BCL2L11, BCL2L2, BFAR and BRAF were significantly down-regulated compared with control ($P < 0.01$). The gene expression profile suggested that the extrinsic and intrinsic mechanisms were involved in TAF273-induced apoptosis.

Analysis of cell cycle distribution in K-562 cells showed that TAF273 induced cell cycle arrest at the G1, S and G2 phases. This was supported by an analysis of the cell cycle pathway which demonstrated up-regulation of CDKN1B, GADD45A, GTF2H1, HERCS, HUS1 and SERTAD1, and down-regulation of BCL2, CCNE1 and PCNA compared with control ($P < 0.01$).

Furthermore, TAF273 enhanced the cytotoxic activity of imatinib on K-562 cells. TAF273-imatinib combination showed a synergistic effect at ED50, ED75 and ED90 values. This synergistic effect resulted in a dose reduction index of up to five (5) times for imatinib and up to 2.5 for TAF273. The reduction in the dose of imatinib decreases its toxicity.

Despite the cytotoxicity of TAF273 on the leukaemic cells, it is important to note that TAF273 showed no remarkable alterations in the haematological, biochemical or histological parameters when taken orally at 200 mg/kg. These findings were also supported by the extremely low cytotoxicity of TAF273 on normal peripheral blood mononuclear cells (IC50 180 µg/ml). This indicates a selective cytotoxicity towards the leukaemic cells.

Beside the cytotoxicity, TAF273 also revealed a potent anti-angiogenic activity as indicated by the significant suppression in the sprouting of microvessels in rat aorta with IC50 value of 11.5 µg/ml. TAF273 showed remarkable inhibition (63.13%) of neovascularization in the chorioallantoic membrane of a chick embryo. Tumor histology also revealed a marked reduction in the extent of vascularization compare with control ($P < 0.01$). *In vitro,* TAF273 significantly inhibited the major angiogenesis steps, such as proliferation, migration and differentiation, of HUVECs.

In conclusion, the standardized TAF273 fraction of *E. longifolia* root methanolic extract showed promising anti-tumor activity and may be a potential chemotherapeutic agent for the treatment of chronic myeloid leukaemia.

CHAPTER 1 INTRODUCTION

1.1 Leukaemia

The term “leukaemia” comprises a diverse range of biologically-distinct diseases that affect the blood and bone marrow (Xie et al., 2003). The bone marrow cells are characterized by increased and uncontrolled proliferation of clone cells (Bock et al., 2007; Lichtman, 1995; Shah et al., 1998). The blockage of leukaemic cells at the disease’s early stages of development and failure of these cells to mature into functional cells are the main abnormality (Nowak et al., 2009).

Aetiologically, leukaemia results from a sequential series of mutational events, including: amplification, point mutations and most frequently, specific chromosome breakages and fusions that cause inappropriate associations of two different genes occurring in the bone marrow stem cells. These stem cells are normally capable of self-renewal and serve to sustain the population of cells by differentiating into the different blood cell lineages (Gilliland et al., 2004; Yu et al., 2011).

Leukaemia can also develop from other disorders, such as myelodysplastic syndrome (MDS), aplastic anemia, monoclonal B cell disorder and HIV infection (Aboulafia et al., 2002; Marti et al., 2005). Other diseases that can transform into leukaemia include idiopathic myelofibrosis, primary thrombocythaemia, polycythemia vera (PCV) and clonal sideroblastic anemia (Cervantes et al., 2005; Lichtman, 2005).

Environmental factors such as exposure to radiation or chemotherapy may be associated with leukaemia (Allan and Travis, 2005).

Leukaemia is an economical burden and a health care problem worldwide (Redaelli et al., 2004). It occurs among all ages and can appear as early as a few weeks of birth to a late age (Meadors, 1956).

The incidence of leukaemia in the world is about 1 per 100,000 per year (Parkin et al., 1999). In the United States of America (USA), 30,800 individuals were diagnosed with leukaemia and of that number, 21,700 individuals died of the disease (Jemal et al., 2002). Additionally in the USA, the incidence of acute leukaemia in the first year of life is 30 cases per million of live births. Also the annual incidence of acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML) is 20, and 10.6 cases per million, respectively (Felix and Lange, 1999).

In developed countries, (Stiller and Parkin, 1996) reported that acute leukaemias affect between 30–45 million children annually.

In Malaysia, leukaemia ranked fourth among cancers in males and fifth in females with an age standardized incidence rate (ASR) of 3.5 in males and 2.7 in females (Zainal Ariffin and Nor Saleha, 2011).

Leukaemia is clinically and pathologically subdivided into four major subtypes: acute myeloid leukaemia (AML), acute lymphoblastic leukaemia (ALL), chronic lymphocytic leukaemia (CLL) and chronic myeloid leukaemia (CML) (Xie et al., 2003).

1.2 Types of Leukaemias

1.2.1 Acute Myeloid Leukaemia (AML)

Acute myeloid leukaemia (AML) refers to a group of distinct subtypes that differ with respect to pathogenesis, genetic abnormalities, clinical features, response to therapy and prognosis (Löwenberg et al., 2003). AML has an incidence of about 5 cases/100,000 individuals/year and accounts for about 80% of acute leukaemia in adults, and 15–20 % in children (Deschler and Lübbert, 2006). In children, AML is characterized by a high white blood cell count and bulky extramedullary disease. The survival rate, however, is better than in older people (Felix and Lange, 1999).

The presence of more than 20% leukaemic blasts in a bone marrow aspirate is essential for a definitive diagnosis of acute leukaemia (Vardiman et al., 2009). The AML stem cell has been defined immunophenotypically as CD34+, CD38–, HLA–DR–, CD71-, CD90-, CD117–, and CD123+. The expression of CD90, CD117 and CD123 on the AML stem cell distinguishes it from the normal haematopoietic stem cell (HSC) (Gilliland et al., 2004; Reya et al., 2001). AML is the consequence of more than one mutation, such as point mutations, gene rearrangements and/or chromosomal translocations (Gilliland et al., 2004).

1.2.2 Acute Lymphoblastic Leukaemia (ALL)

Acute lymphoblastic leukaemia (ALL) is a clonal expansion of genetically altered lymphoblasts. It is the most frequent cancer in childhood, accounting for 23% of cancers diagnosed among children under 15 years of age. In the USA, it is estimated that the annual incidence rates per million are 77, and 17 at the age of 2 years and 14 years respectively (van den Berg and van der Lelie, 2000). ALL accounts for approximately 20% of adult leukaemia (Champlin and Gale, 1989).

Despite the high incidence rate of ALL in the childhood group, the cure rate, however, is high in this group (van den Berg and van der Lelie, 2000).

In contrast, the results of treatment in adults with ALL remain poor despite the application of strategies used successfully in children (Copelan and McGuire, 1995).

ALL, particularly T-cell ALL, is associated with the expression of one or more of the following specific oncogenes: LYL1, LMO2, HOX11, TAL1, LMO1, HOX11L2, and MLL-ENL (Pui et al., 2004).

1.2.3 Chronic Lymphocytic Leukaemia (CLL)

Chronic lymphocytic leukaemia (CLL) is characterized by the proliferation and accumulation, in the bone marrow and lymphoid organs, of immunologically non-competent B–lymphocytes arrested at the early stages of differentiation (Dameshek, 1967). CLL is rarely seen in people younger than 40 years and is manifested as immune disorders, such as hypogammaglobulinemia and auto immune phenomena (Montserrat and Rozman, 1995).

CLL occurs most frequently in Western countries, with an annual incidence rate of 2.5 cases/100,000 individual. CLL accounts for 30% of all leukaemias in Western populations, while it accounts for 3–5% in Asian populations (Finch and Linet, 1992). Its aetiology is not yet fully understood.

The involvement of bcl-1 and bcl-2 oncogenes in the development of leukaemia is controversial. The t(14;19) is rare and no relationship with viruses or genes has been demonstrated (O'Brien et al., 1995; Montserrat and Rozman, 1995).

1.2.4 Chronic Myeloid Leukaemia (CML)

Chronic myeloid leukaemia (CML), a malignant disease of the human haematopoietic stem cell results from the BCR/ABL gene rearrangement. It is characterized by a marked increase in granulocytes, marked bone marrow hyperplasia, and splenomegaly (Au et al., 2009; Lawler et al., 1974).

CML accounts for approximately 15% of all leukaemias. CML has a worldwide incidence of 1–2 per 100,000 (Epstein et al., 1999). In the USA, about 5,000 cases are diagnosed annually and 470 patients will die from the disease (O'Brien et al., 2009; Terasawa et al., 2010).

The Philadeplphia (Ph) chromosome is a cytogenetic hallmark of this disease. It results from reciprocal translocation t(9;22)(q34;q21) found specifically in the leukaemic cells of over 90% of CML patients (Naumann and Decker, 2003). The expression of the constitutively activated tyrosine kinase ($p210^{BCR/ABL}$) is necessary and sufficient to develop CML (Kurzrock et al., 2003; Calabretta and Perrotti, 2004).

1.3 Therapeutic Challenges

Chemotherapy is a very important approach in the treatment of leukaemias. Over the past three decades, treatment of leukaemia with chemotherapeutic agents has achieved highly successful survival rates, with complete haematological and cytogenetic responses.

Imatinib, an Abl kinase inhibitor, induces complete cytogenetic responses in more than 80% of newly diagnosed CML patients (Corbin et al., 2011). This improvement is basically attributed to an increase in the number and activity of therapeutic agents recently produced and using multi-agent chemotherapy rather than

single-agent therapeutic strategies. However, the emergence of resistance and relapse to frontline anti-leukaemic drugs brings difficulties in treatment of this disease.

A relapse of up to 40% has been reported in patients with AML who had responded successfully to initial therapy (Reddy and Perkins, 2004; Sievers et al., 2003).

In CML, 60% of patients treated with imatinib showed relapse (Martin et al., 2006). Resistance to imatinib, a frontline chemotherapeutic agent against CML, has been reported recently (Corbin et al., 2011; Quintas-Cardama et al., 2007). Cytogenetic resistance is reported in 15–25% of patients. Cytogenetic resistance is defined as failure to achieve any level of cytogenetic response, major cytogenetic response (MCyR), or complete cytogenetic response (CCyR) at 6, 12 and 18 months respectively (O'Brien et al., 2009).

Developing strategies to overcome the resistance to frontline chemotherapeutical agents of leukaemias pose a challenge in treating this disease. Thus the search for new novel compounds that can overcome or reverse this resistance become necessary. There exist several naturally occurring compounds in certain vegetables and herbs that exhibit chemopreventive properties (Reddy et al., 2003).

1.4 Natural Products are Main Sources for Anticancer Agents

Over the years, natural products have provided a rich source of compounds that have been used in medicine, pharmacy and biology (Gordaliza, 2007). Natural healing agents from microorganisms, plants and animals were eventually discovered, and for hundreds of years have been refined in the treatment and prevention of

complex human diseases. Doctors and researchers today continue to use these natural phenomena in modern medicine (Nobili et al., 2009; Pan et al., 2009).

Plants are an important source of new natural products; to the field of medicine, they continue to provide pools teeming with new molecules. In fact, medicinal herbs are still used in primary health care, despite the fact that the last few centuries have witnessed a revolution in synthetic variants (Pan et al., 2009). Natural products constitute around 60% of cancer chemotherapeutic drugs currently used today (Gordaliza, 2007).

Researchers are constantly searching for new recipes for anti-tumor drugs, and their searches usually begin with natural products.

1.4.1 *Eurycoma longifolia* Jack (*E. longifolia*)

Eurycoma longifolia Jack from the family Simaroubaceae is commonly found in South East Asia (SEA) including Thailand, Malaysia, Laos, Cambodia, Myanmar and Indo-China (Osman et al., 2003). It is a slender, evergreen flowering plant and it is known locally as 'Tongkat Ali' or 'Pasak Bumi' in Malaysia and Indonesia (Bhat and Karim, 2010; Wernsdorfer et al., 2009). This plant has gained a considerable reputation as a traditional medicine; its local use has been a sorted list of treatments, including aiding childbirth, as an aphrodisiac and as a treatment for dysentery.

E. longifolia has many compounds that are biologically active, with anti-plasmodial activity (Chan et al., 2004). Several classes of compounds have been isolated and identified, including quassinoids, β-carboline, triterpene tirucallane type, alkaloids, canthin-6-one alkaloids, biphenylneolignan and squalene derivatives (Mahfudh and Pihie, 2008).

1.5 Justifications of the Study

Cancer has risen to the top in terms of causes of death in both developed countries and developing countries (Jemal et al., 2011). In Malaysia, leukaemia accounts for 4.1% of all cancers (Zainal Ariffin and Nor Saleha, 2011).

The emergence of resistance to and high toxicity of currently used anti-leukaemic drugs such as imatinib creates difficulties for the treatment of leukaemia. Therefore, a search for new anti-leukaemic agents has become an urgent need.

There are several reports on remedial uses of *E. longifolia* to alleviate angiogenesis-related diseases (Kavitha et al., 2010) and there is even evidence that this plant is endowed with abundance of bioactive constituents, particularly the quassinoids, with significant anti-tumor and anti-angiogenic activities (Bhat and Karim, 2010; Kuo et al., 2004; Salamah et al., 2010). Several studies showed anti-proliferative activity on varying types of solid tumors, including lung cancer (Ueda et al., 2002; Wong et al., 2012), breast cancer (Tee et al., 2007), cervical carcinoma (Mahfudh and Pihie, 2008) and HepG2 (Zakaria et al., 2009). This has drawn attention toward hypothesizing that *E. longifolia* could contain potential anti-neoplastic activity against the blood cancer: leukaemia.

The anti-neoplastic activity of *Eurycoma longifolia* against leukaemia disease has not been addressed yet. Additionally, studying the mechanism by which *Eurycoma longifolia* exerts its effect on leukaemic cells may lead researchers to explore new therapeutic targets. Also, the evaluation of the

combination effect of extracts from *Eurycoma longifolia* with imatinib may help reverse the resistance to or enhance the effects of imatinib.

1.6 Hypothesis

Root extract of *E. longifolia* (TAF273) has anti-neoplastic activity on leukaemia *in vitro* and *in vivo*.

TAF273 has no toxicity in nude mice model.

TAF273 induces apoptosis and cell cycle arrest in leukaemic cells.

TAF273 enhances the activity of imatinib.

Root extracts of *E. longifolia* has anti-angiogenic and anti-oxidant activities.

1.7 Objectives of Study

1.7.1 Main Objective

This study aimed to investigate the anti-neoplastic activity of *E. longifolia* extracts on blood cancer: leukaemia.

1.7.2 Specific Objectives

- To investigate the anti-neoplastic activity of standardized root extract of *E. longifolia* (TAF273) against leukaemia models, *in vitro* and *in vivo*.
- To evaluate the toxicity of TAF273 extract in nude mice model.
- To determine the mechanism of action of TAF273 extract on K-562 leukaemia cells.
- To evaluate the effects of the combination of TAF273 extract with a standard anti-leukaemia drug (imatinib) on K-562 cells.
- To investigate the anti-angiogenic and anti-oxidant activities of *E. longifolia* root extracts.

CHAPTER 2 LITERATURE REVIEW

2.1 Origin of Blood Cells

Blood is one of the most highly regenerative tissues in the human body and is composed of an assortment of cells suspended in plasma. Most blood cells that circulate throughout the body have a relative short lifespan, dying off while newly formed cells take their place via haematopoiesis. Approximately 10^{12} cells are produced daily in adult human bone marrow (Doulatov et al., 2012; Peixoto et al., 2011) and this rate may be increased to 10 times or even more when the demand to blood production increases (Kaushansky, 2006; Ogawa, 1993).

Haematopoiesis (from ancient Greek: haima blood; poiesis to make) is the formation of blood cells in a living body. Haematopoiesis is a complex process involving the continuous generation of a spectrum of highly specialized, differentiated cell types (Orkin, 2000) as shown in Figure 2.1. The haematopoietic system consists of pluripotential stem cells (most primitive and self sustaining), progenitor cells (lineage restrictions and little or no self renewal capacity) and lineage-restricted, maturing cells (Lansdorp, 1995; McCulloch, 1983).

The final products of the haemopoietic stem cell differentiation are the progenitors of all blood cell lineages, such as erythrocytes, neutrophils, eosinophils, basophils, monocytes, macrophages, B and T lymphocytes, natural killer cells and platelets (Kaushansky, 2006; McCulloch, 1983).

The level of blood cells is controlled by a number of cellular and humoral factors and adjusts rapidly according to requirement (Kaushansky, 2006; Ogawa, 1989). For example, in response to microbial infection, an immediate release of mature neutrophils and an increased production of granulocytes and monocytes will take place until the infectious agents are cleared. Upon haemorrhage or acute

haemolysis, a rapid release of bone marrow reticulocytes along with an increase in red cell production will occur to adequately compensate for the loss (Takizawa et al., 2012).

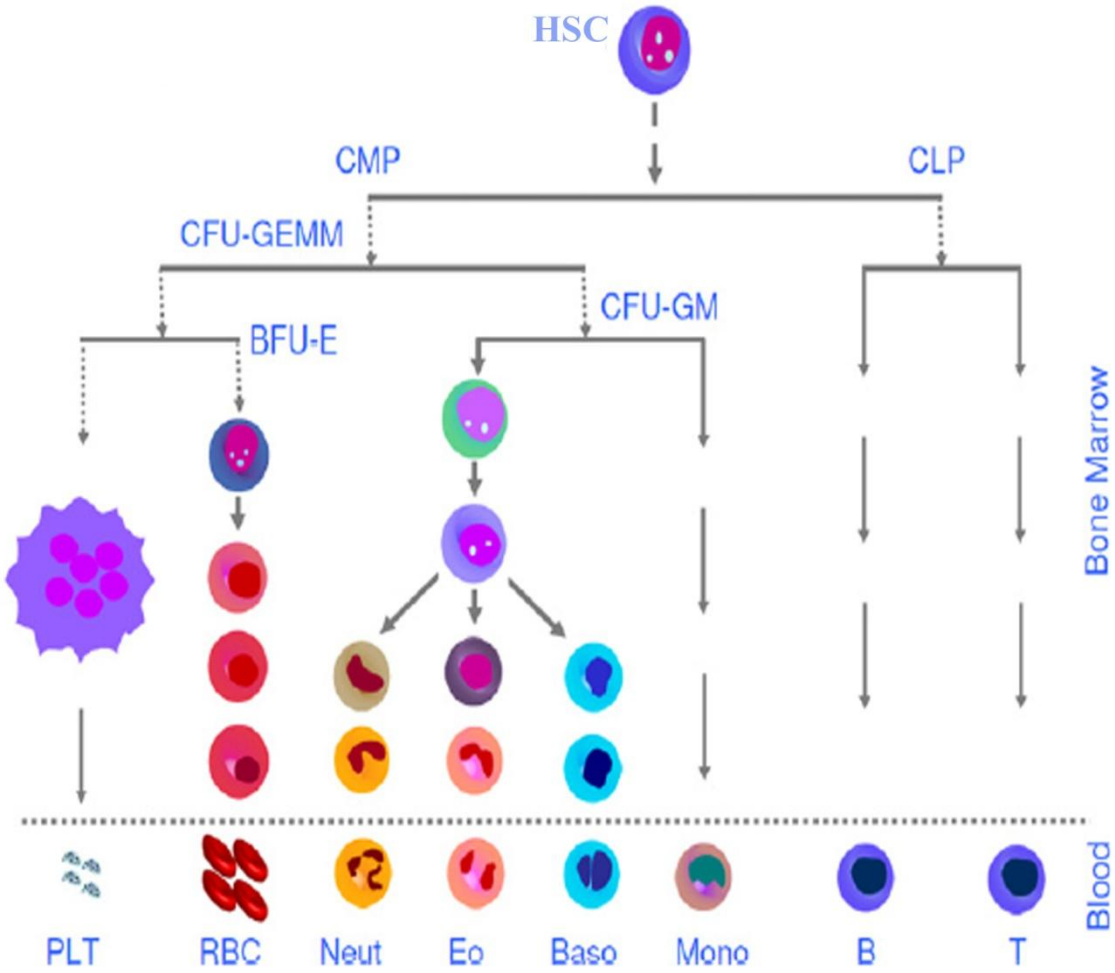

Figure 2.1 Schematic diagram of haematopoiesis.

Haematopoiesis has a tree-like structure with the haematopoietic stem cells at the root of the process. Each cell division gives rise to progeny cells that can retain the properties of their parent cell (self-renewal) or differentiate, ‘‘moving down’’ the haematopoietic tree. As the progeny move further away from the HSC, their pluripotent ability is increasingly restricted (CMP: common myeloid progenitor; CLP: common lymphoid progenitor; BFU-E: erythroid burst forming unit; CFU-GM: granulocyte–macrophage colony forming unit). PLT; platelets; RBC: red blood cells; Neut: neutrophil; Eo: eosinophil; Baso: Basophil; Mono: monocyte; B: B lymphocyte; T: T lymphocyte. Adapted from (Peixoto et al., 2011)

2.2 Haemopoietic Stem Cell Disorders

2.2.1 Classification

The haemopoietic stem cell disorders are classified into two principal pathogenetic disorders: aplasia (stem cell failure) and clonal haemopathies (injury to a single cell within the haemopoietic stem cell pool) (Lichtman, 1995a).

Aplasia comprises a heterogeneous group of diseases characterized by peripheral blood pancytopenia with a hypocellular bone marrow and remarkable reduction of blast cells (Lichtman, 1995a; Raghavachar et al., 1995).

Clonal haemopathies or clonal haematopoiesis is a group of haematopoietic disorders including the pre-leukaemias and myeloproliferative neoplasms (MPN). These disorders are characterized by an overwhelming production and excessive accumulation of granulocytes, erythrocytes, and platelets in the bone marrow, peripheral blood, and tissues (Wadleigh and Tefferi, 2010; Vardiman et al., 2009).

Chronic MPNs include chronic myelogenous leukaemia (CML), chronic monocytic leukaemia (CMML), chronic neutrophilic leukaemia, idiopathic myelofibrosis, polycythemia vera and primary thrombocythemia (Cervantes et al., 2005; Lichtman, 1995a; Lichtman, 2005; Vardiman et al., 2009).

The development of AML occurs when MPN reaches its terminal stage. AML develops in 75% of patients with CML and in 15% of patients with primary thrombocythaemia and idiopathic myelofibrosis (Lichtman, 1995a; Wadleigh and Tefferi, 2010).

2.2.2 Leukaemia

Leukaemia is characterized by an uncontrolled proliferation of one cell line or a stem cell common to several cell lines. Leukaemia is one of the most common forms of cancer, especially in children (Lichtman, 1995a; Reya et al., 2001).

Leukaemias are classified into two major groups: (i) chronic, in which the onset is insidious and is usually less aggressive (Geary et al., 1975); (ii) acute, which is characterized by a rapid onset, aggressiveness and poor differentiation with many blasts. AML is more common in adults, while ALL is more common in children (Hoelzer et al., 2002; Muntean and Hess, 2012; Xie et al., 2003).

Chronic leukaemias represent a group of diseases that include both CML and CLL malignancies (Cotta and Bueso-Ramos, 2007; Bergsagel, 1967).

2.2.2.1 Chronic Myeloid Leukaemia (CML)

Chronic myeloid leukaemia (CML) is a clonal disorder involving a pluripotent haematopoietic stem cell (Morrison, 1994). The disease was described in 1845 by three pathologists, Craigie, Bennett, and Virchow, working independently (Steinberg, 2007; Wong and Witte, 2004). CML is a disease commonly associated with the elderly with a slight male predominance. The average age of patients with CML at diagnosis is 45 to 55 years (Epstein et al., 1999; Morrison, 1994).

The characteristic presenting feature of CML is an elevated white blood cell count (50 000 -300 000 per ml) with a high percentage of mature neutrophils with metamyelocytes and myelocytes and few blasts (Radich, 2007; Wertheim et al., 2002). Basophils and eosinophils are also frequently present. Some patients exhibit

splenomegaly; however, nearly 40% are asymptomatic. Bone marrow biopsy reveals a marked myeloid hyperplasia (Radich, 2007; Wertheim et al., 2002).

Clinically, CML is classified into three phases: the chronic phase (CP), the accelerated phase (AP) and the blast crisis (BC) (Melo and Barnes, 2007; Rizzieri and Moore, 2012). Patients who suffer from CML may remain in CP for up to 5 years and are generally asymptomatic (Melo and Barnes, 2007; Rizzieri and Moore, 2012). The accelerated phase is characterized by genetic instability and constitute up to 10–15% blasts, 20–30% blasts with promyelocytes, over 20% basophils, and a platelet count of less than 100 x 10^9/L (Rizzieri and Moore, 2012). The AP is more aggressive than CP and generally lasts between 4–6 months. The blast crisis phase is by far the most tenacious of the phases of CML, and only has a median survival rate of 4–6 months (Melo and Barnes, 2007; Saglio et al., 2010).

Patients in the BC phase could exhibit several symptoms, including fever, cold sweats, fatigue, and anorexia. Furthermore, patients may experience splenomegaly, and may complain of bone pain (which could signify an infection) (Morrison, 1994; Steinberg, 2007; Wong and Witte, 2004). Splenomegaly is reported in 40–60%, of cases and hepatomegaly in 10–20% (Garcia-Manero et al., 2003).

2.2.2.1.1 Molecular basis of CML

2.2.2.1.1.1 Philadelphia Chromosome

The Ph chromosome is the hallmark of CML and was discovered in 1960 by Nowell and Hungerford (Steinberg, 2007). It is found in more than 95% of CML patients, in 5% of children, and in 10–20% of adults with ALL (Clark et al., 1989; Epstein et al., 1999; Laurent et al., 2001; Steinberg, 2007).

As shown in Figure 2.2, one can see that the Ph chromosome is the product of a reciprocal translocation, located between the long arms of chromosomes 9 and 22; t(9;22)(q34;q11). This translocation adds a 3' segment of the ABL gene found in chromosome 9q34 to the 5' section of the BCR gene in chromosome 22q11, thus producing a hybrid BCR/ABL gene—which in turn is transcribed into a chimeric BCR/ABL mRNA (De Braekeleer et al., 2011; Deininger et al., 2000; Epstein et al., 1999).

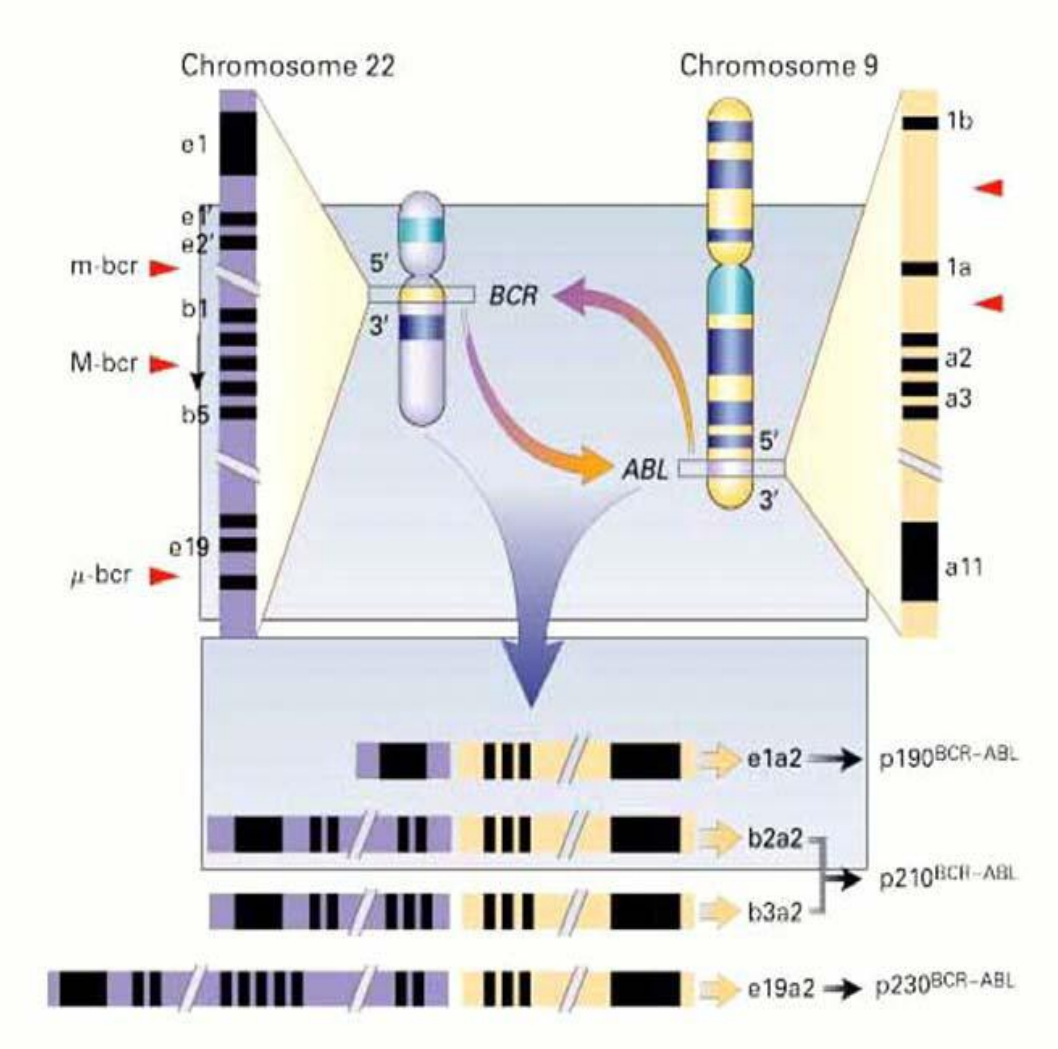

Figure 2.2 Schematic illustration of t(9;22)(q34;q11), break points and ABL/BCR chimeric proteins in CML

(Epstein et al., 1999)

The ABL or ABL1 (v-abl Abelson murine leukaemia viral oncogenes homolog 1) proto-oncogene encodes a non-receptor tyrosine kinase with a molecular mass of 145 kd (p145ABL). ABL protein is ubiquitously expressed and is involved in signal transduction via integrins, in cell cycle regulation and in response to DNA damage. The ABL gene spans 230 kb and is located at band 9q34. It includes the 5′ alternative first exons 1b and 1a and 10 common exons numbered from 2 to 11. ABL protein has 2 isoforms arising from an alternative splicing of the first exon (De Braekeleer et al., 2011; Deininger et al., 2000; Epstein et al., 1999).

The breakpoint cluster region (BCR) spans 130 kb and contains 23 exons (Laurent et al., 2001). The BCR gene encodes two major proteins, named Mr160 kd and Mr 130. Other BCR proteins, such as Mr 190kd, Mr 155 kd, Mr 135kd, Mr 125kd, and Mr 108kd are also described (Laurent et al., 2001). The BCR proteins are signalling proteins and are reported to be involved in two major signalling mechanisms, including phosphorylation and the GTP-binding protein (Laurent et al., 2001; Ren, 2005).

The major breakpoint cluster region (M-bcr) of the BCR gene on chromosome 22 extends over 5.8 kb and is located between exon 12 and 16 (originally referred to as exons b1-b5) (Deininger et al., 2000). Two fusion transcripts, e13a2 and e14a2 (b2a2 and b3a2, respectively) are generated and both translate into a chimeric protein of 210 kd, named $p210^{BCR/ABL}$ (Deininger et al., 2000; Laurent et al., 2001; Ren, 2005). In 95% of the BCR/ABL positive CML, the leukaemic cells contain either the b2a2 transcript or the b3a2 transcript, where both fusion products are present in about 5% of the cases (Melo, 1996). Those who exhibit b2a2 and b3a2 transcripts have a very similar set of symptoms, as well as response to treatment and prognosis.

But there is one difference: patients with b3a2 transcripts have a higher platelet count (Junia, 1996).

The minor breakpoint cluster region (m-bcr) results from the fusion of e1 exon of the BCR gene with the a2 exon of the ABL gene producing fusion transcript called e1a2, which encodes for a 190 kd protein, p190BCR/ABL (Deininger et al., 2000; Laurent et al., 2001) (Figure 2.2).

P190BCR/ABL is rare in CML and is mainly found in children and adults with Ph chromosome positive ALL (Deininger et al., 2000; Verma et al., 2009).

Another unique breakpoint cluster region (μ-bcr) was determined downstream of exon 19, producing a 230-kd fusion protein ($P230^{BCR-ABL}$). It is resulted from the fusion between exon 19 of the BCR gene and exon 2 of ABL (e19a2). This breakpoint is associated with the rare Ph chromosome positive chronic neutrophilic leukaemia and rarely occurs in CML (Deininger et al., 2000; Verma et al., 2009).

2.2.2.1.1.2 The BCR/ABL Oncoprotein

The leukaemogenic potential of p210BCR/ABL results from the constitutive activation of tyrosine kinase of the ABL protein (Epstein et al., 1999). ABL proteins are non-receptor tyrosine kinases, and play a significant part in both regulating cell growth and acting as a signal transduction (Wang, 1993). Isoform 1a and isoform 1b have been reported as two isoforms of ABL.

The high expression of isoform 1b in early haematopoietic progenitor cells is associated with myristoylation at the second glycine residue at the N-terminal (Jackson and Baltimore, 1989). A defect in myristoylation in ABL markedly enhances its tyrosine kinase activity (Hantschel et al., 2003).

2.2.2.1.1.3 Regulatory Function of SRC Homology Domains

There are three SRC homology domains downstream from the myristoylation site (located at the N-terminal portion of ABL); SRC homology domains 1 (SH1), SRC homology domains 2 (SH2) and SRC homology domains 3 (SH3). The SH2 and SH3 regulate the tyrosine kinase function of ABL in a negative way, while SH1 harbors its tyrosine kinase activity. By removing the SH3 or mutation in SH3 will increase ABL tyrosine kinase activity (Ben-Neriah et al., 1986; Franz et al., 1989). Any defects that may be located in SH2's structure would lessen phosphotyrosine binding and thus decrease the transforming abilities of ABL (Faderl et al., 1999).

The ungoverned BCR/ABL tyrosine kinase activity results from the combination of various BCR sequences. It was found that the coil-coiled N-terminal motif of BCR promotes dimerization and enhances BCR/ABL tyrosine kinase activity. This motif also enables the binding of F-actin to ABL (Goldman and Melo, 2003; McWhirter et al., 1993). The BCR activates via its serine-threonine kinase domain—the signalling pathways mediated by BCR/ABL tyrosine kinase (Muslin et al., 1996) (Figures 2.3 and 2.4).

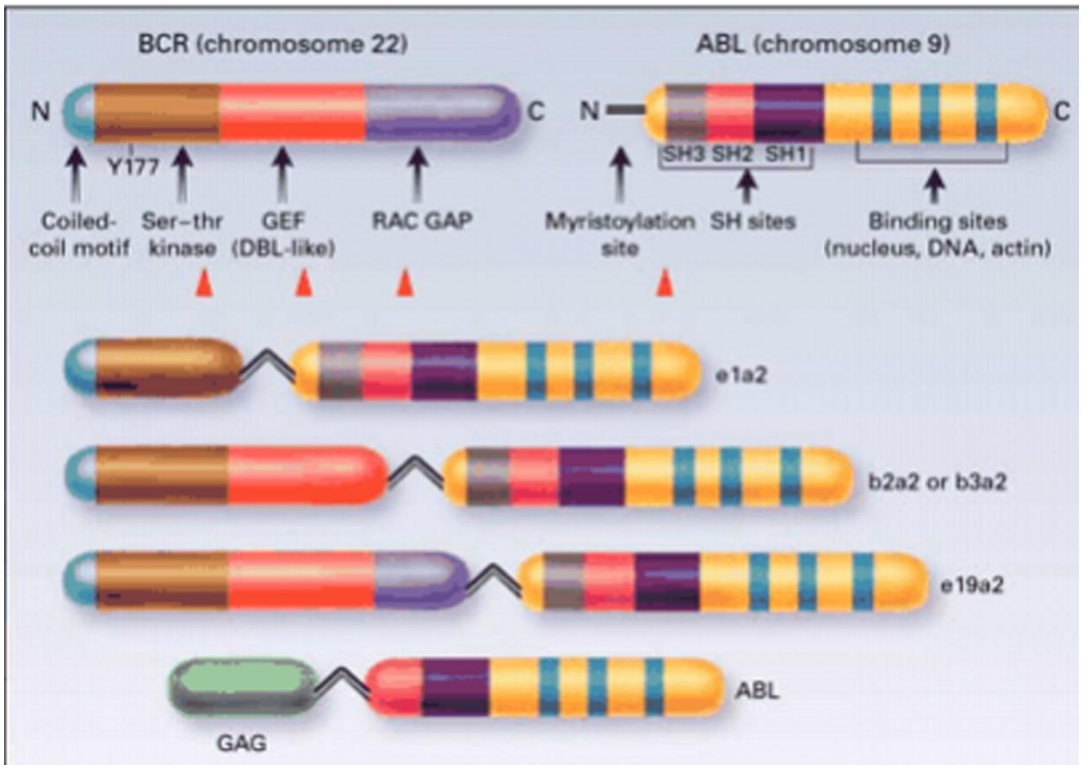

Figure 2.3 Functional domains in p160BCR, p145ABL and p210BCR-ABL.

Important functional domains of the BCR and ABL gene products as well as different fusion-protein products are shown ($p190^{BCR/ABL}$, $p210^{BCR/ABL}$ and $p230^{BCR/ABL}$). Breakpoints are indicated by arrowheads. N: N-terminal amino acid sequence, C: C-terminal amino acid sequence, Ser-thr: serine-threonine, GDP: guanosine diphosphate, GTP: guanosine triphosphate, GEF: GDP-GTP exchange factor, DBL: diffuse B-cell lymphoma oncogene, RAC: a RAS-like GTPase, GAP: guanosine triphosphatase-activating function protein, and SH: SRC homology domain (Epstein et al., 1999).

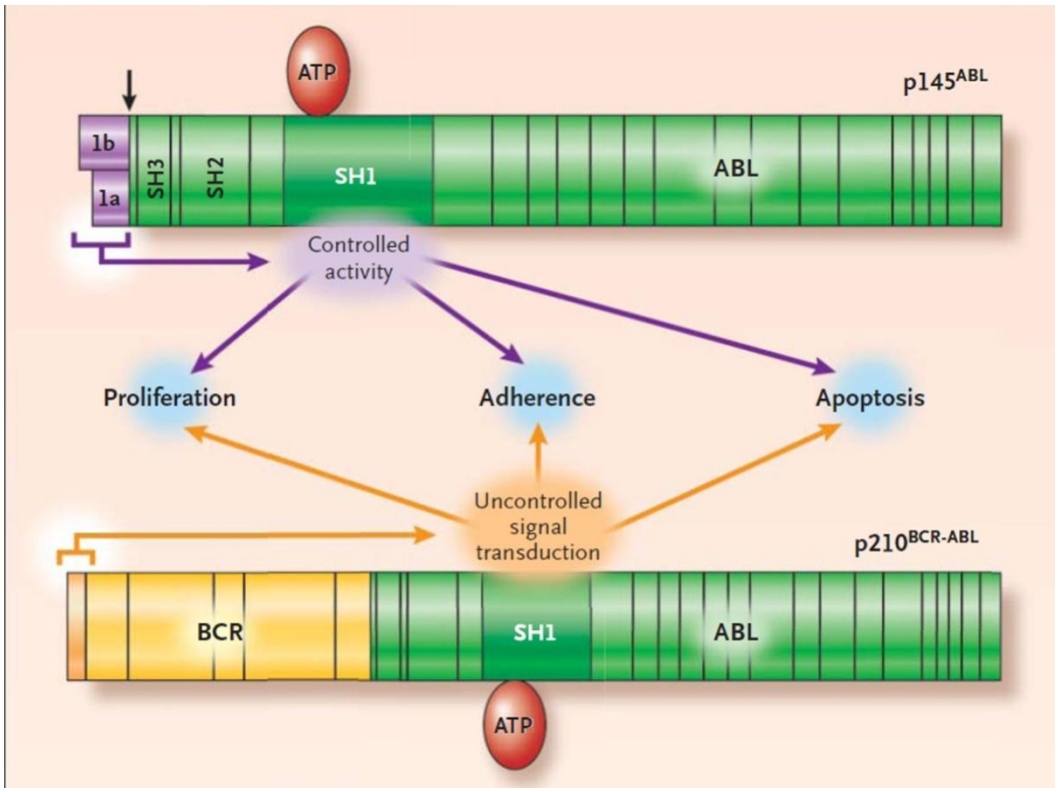

Figure 2.4 Physiologic regulation by the normal ABL protein and deregulation by BCR/ABL of key cellular processes.

(Goldman and Melo, 2003)

The BCR/ABL protein interacts with various proteins resulting in transduction of the oncogenic signals responsible for several cellular effects such as the repression or activation of gene transcription, the degradation of inhibitory proteins, cytoskeletal organization, and mitochondrial processing of apoptotic responses (Dai et al., 2001).

As of yet, the key pathways implicated include RAS, MYC, mitogen-activated protein (MAP) kinases, phosphatidylinositol 3-kinase (PI3K), signal transducers and activators of transcription (STAT). Numerous domains in BCR/ABL bind to various adapter proteins, such as CRK-like protein (CRKL), CRK, growth factor receptor-bound protein 2 (GRB2), DOK, casitas B-lineage lymphoma SRC homology 2-containing protein (SHC), and pro-oncogene proteins (CBL) (Goldman and Melo, 2003; Puil et al., 1994). See Figure 2.5 below.