In vitro screening of natural/synthetic compounds for their potential to induce differentiation and/or apoptosis in myeloid leukemia cells: Understanding differentiation pathways in myeloid cell development

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Dedicated to my Family

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CERTIFICATE

This is to certify that the thesis entitled ***"In-vitro screening of natural/synthetic compounds for their potential to induce differentiation and/or apoptosis in myeloid leukemia cells: Understanding differentiation pathways in myeloid cell development"*** submitted to Jawaharlal Nehru University, New Delhi in the partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy comprises the work done by Ms. Pooja Pal under my guidance at the CSIR-Central Drug Research Institute, Lucknow. The work presented is original and has not been submitted in part or full for any other degree or diploma of any University/Institute.

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Abbreviations

A	Adenine
Abl	Abelson
ABI	Abl Interactor
AGM	Aorta-Gonad-Mesonephros
ALL	Acute Lymphoid Leukaemia
AML	Acute Myeloid Leukaemia
ETO	Eight Twenty One
AP	Accelerated Phase
APL	Acute Promyelocytic Leukemia
APC	Adenomatous Polyposis Coli
AR	Androgen Receptor
ARG	Abelson Related Gene
ARF	ADP Ribosylation Factor
ATP	Adenosine Triphosphate
ATRA	*All trans* Retinoic acid
AS	Angelman Syndrome
Bad	Bcl-2 Associated Death Promoter
BAG	Bcl-2 Associated Gene
BAALC	Brain and Acute Leukemia Cytoplasmic
Bak	Bcl-2 homologous Antagonist Killer
Bax	Bcl-2 Associated X Protein

BC	Blast Crisis
Bcl	B-Cell Lymphoma
Bcl-XL	B-Cell Lymphoma-Extra Large
Bcr-Abl	Break Point Cluster Region-Abelson kinase
Bid	BH3 Interacting Domain
Blk	B-Lymphocyte Kinase
BR	Basic Region
BrdU	5'-bromo-2'-deoxyuridine
BSA	Bovine Serum Albumin
bZIP	Basic Leucine Zipper
C	Cytosine
CAD	Caspase Activated DNase
CD	Cluster of Differentiation
CDRI	Central Drug Research Institute
CDK	Cyclin Dependent Kinase
CEBPA	CCAAT/enhancer binding protein alpha
CHX	Cycloheximide
CLP	Common Lymphoid Progenitor
CLL	Chronic Lymphocytic Leukemia
CML	Chronic Myeloid Leukemia
CMP	Common Myeloid Progenitor
CMV	Cytomegalovirus
CO_2	Carbon Dioxide

CP	Chronic Phase
CP	Cyclophosphamide
CP	Core Protein
CTLs	Cytotoxic T-Lymphoctes
CSIR	Council of Scientific and Industrial Research
CSMMU	Chatrapati Sahuji Maharaj Medical University
CXCR4	Chemokine Receptor Type-4
DAPI	4',6-diamidino-2-phenylindole
DCFDA	2'-7'-Dichlorofluorescin Diacetate
DISC	Death inducing Signaling Complex
DMBA	Dimethylbenz[*a*]anthracene
DMEM	Dulbecco`s Modified Eagle Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
DUB	Deubiquitinating Enzymes
dUTP	Deoxyuridine Triphosphate
E2	Estradiol
E2F	Elongation Factor
E6AP	E6-Associated Protein
ECL	Enhanced Chemi Luminescence
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid

EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
EMS	Ethylmethane Sulfonate
ER	Endoplasmic Reticulum
ER	Estrogen Receptor
ERK	Extracellular Signal-Regulated Kinase
FADD	Fas-Associated Protein with Death Domain
FACS	Fluorescence
FAB	French-American-British
FBS	Foetal Bovine Serum
Fbxw7	F-box/WD repeat containing protein 7
FISH	Fluorescence *In Situ* Hybridization
FITC	Fluorescein Isothiocyanate
G	Guanine
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
G-CSF	Granulocyte-Colony Stimulating Factor
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage-Colony Stimulating Factor
GMP	Granulocyte-Macrophage Progenitor
GPCR	G-Protein Coupled Receptor
GSK	Glycogen Synthase Kinase
GST	Glutathione S-Transferase
HCl	Hydrochloric Acid

HECT	Homologous to E6-AP Carboxyl Terminus
HLA	Human Leukocyte Antigen
HPV	Human Papillomavirus
HRP	Horseradish Peroxidise
HSC	Haemopoietic Stem Cell
HSP	Heat Shock Protein
IAP	Inhibitors of Apoptosis Protein
ICAD	Inhibitor of Caspase Activated DNase
IC_{50}	Inhibitory Concentration 50
ICMR	Indian Council of Medical Research
IgG	Immunoglobulin G
IL	Interleukins
IM	Imatinib Mesylate
INF	Interferons
IP	Immunoprecipitation
ITD	Internal Tandem Duplication
JC-1	5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolyl carbocyanine iodide
JNK	c-Jun N-Terminal Kinase
kDa	Kilodalton
KO	Knock Out
K-Ras	Kirsten-Rat Sarcoma
LAP	Leukemia-Associated Protein
LDL	Low Density Lipoprotein

LT-HSC	Long-Term HSC
LZ	Leucine Zipper
MAPK	Mitogen Activated Protein Kinase
Mdm2	Murine double minute 2
MEP	Megakaryocyte-Erythroid Progenitor
MHC	Major Histocompatibility Complex
MLL	Mixed Lineage leukemia
MMC	Mitomycin C
MPT	Mitochondrial Permeability Transition
mRNA	Messenger RNA
MTT	Thiazolyl Blue Tetrazolium Bromide
MYB	Myeloblastosis
NaCl	Sodium Chloride
NFκ B	Nuclear Factor *kappa*-light-chain-enhancer of activated *B* cells
NPM1	Nucleophosmin 1
NP-40	Nonidet P-40
NR Box	Nuclear Receptor Box
NuMA	Nuclear Mitotic Apparatus Protein
OD	Optical Density
ORM	Ormeloxifene
PARP	Poly (ADP-Ribose) Polymerase
PBMC	Peripheral Blood Mononuclear Cell

PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline-Tween 20
PCR	Polymerase Chain Reaction
PDGFR	Platelet-Derived Growth Factor Receptors
PE	Phycoerythrin
Ph	Philadelphia
PHD	Plant Homeodomain
PI	Propidium Iodide
PI3K	Phosphatidylositol 3-Kinase
PKC	Protein Kinase C
PMA	Phorbol Myristate Acetate
PML-RARα	Promyelocytic Locus- Retinoic Acid Receptor Alpha
Rb	Retinoblastoma
PS	Phosphotidyl Serine
PVDF	Polyvinylidene Fluoride
pY	Phospho Tyrosine
RD	Regulatory Domain
qRT-PCR	Quantitative Real Time Transcription
PCR	Polymerase Chain Reaction
Ras	Rat Sarcoma
RBCs	Red Blood Cells
RING	Really Interesting New Gene
RIPA	Radioimmunoprecipitation Assay

RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
RP	Regulatory Protein
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
SCF	Skp1-Cullin-F-box protein
SDF-1	Stromal cell Derived Factor-1
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SERM	Selective Estrogen Receptor Modulator
SEM	Standard Error of Mean
SFM	Serum Free Medium
SH	Src Homology
STAT	Signal Transducers and Activators of Transcription
ST-HSC	Short-Term HSC
STDEV	Standard Deviation
T	Thymine
TAD	Transactivation Domain
Taq	*Thermus aquaticus*
Tal-1	T-cell Acute Lymphocytic Leukemia Protein-1
TBP	TATA Box Binding Protein
TBS	Tris-Buffered Saline
T-Cells	Thymocyte Cells

TdT	Terminal deoxynucleotidyl Transferase
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
TF	Transcription Factors
TGF	Transforming Growth Factor
TK	Tyrosine Kinase
TNFR	Tumor Necrosis Factor Receptor
TPA	12-O-tetradecanoylphorbol-13-acetate
TRADD	Tumor Necrosis Factor Receptor Type 1-Associated Death Domain Protein
TUNEL	Terminal Deoxynucleotidyl Transferase Nick End Labeling
U	Uracil
UBC	Ubiquitin Conjugating
UBE3A	Ubiquitin-Protein Ligase E3A
UBP	Ubiquitin Binding Proteins
UV	Ultra Violet
WHO	World Health Organization

CONTENTS

Chapter 1

Introduction

1.1 Haematopoiesis

Haematopoietic system consists of various cell types with defined functions. Red blood cells (erythrocytes) carry oxygen to the tissues, platelets helps to prevent bleeding, granulocytes neutrophils, basophils and eosinophils) and macrophages (collectively known as myeloid cells) fight infections from bacteria, fungi and other parasites such as nematodes. B-lymphocytes produce antibodies while T-lymphocytes can directly kill or isolate them by inflammation that are recognized as foreign to the body which includes virus-infected cells and cancer cells. Many blood cells are short-lived and need to be replenished continuously; the average human requires approximately one hundred billion new haematopoietic cells each day. The continued production of these cells depends directly on the presence of Haematopoietic Stem Cells (HSCs). The process by which HSC commit and differentiate to all blood lineage cells is defined as haematopoiesis.

Haematopoiesis is the process of formation of cellular components of blood. Haematopoietic stem cells give rise to the cellular components which reside as rare cells in the bone marrow in adult mammals and sit atop a hierarchy of progenitors that become progressively restricted to several or single lineages (1). More than $2x10^{11}$ haematopoietic cells from at least 11 lineages are produced daily in humans from a small pool of self-renewing adult stem cells (2). Production of each cell type is highly regulated and responsive to environmental stimuli. Due to stress and mutations several haematological disorders can occur. A family tree of blood cell lineage with the transcription factors required for the haematopoiesis is depicted in Figure 1. Haematopoietic stem cells yield blood precursors devoted to uni-lineage differentiation and production of mature blood cells, including red blood cells, megakaryocytes, myeloid cells (monocytes/macrophages and neutrophils), and lymphocytes (3-6). HSCs are capable of self-renewal—the production of additional HSCs—and differentiation, specifically to all blood cell lineages. In mammals, the sequential sites of haematopoiesis include the yolk sac, aorta-gonad-mesonephros (AGM); a region of embryonic mesoderm, the fetal liver, and the bone marrow (7-12).

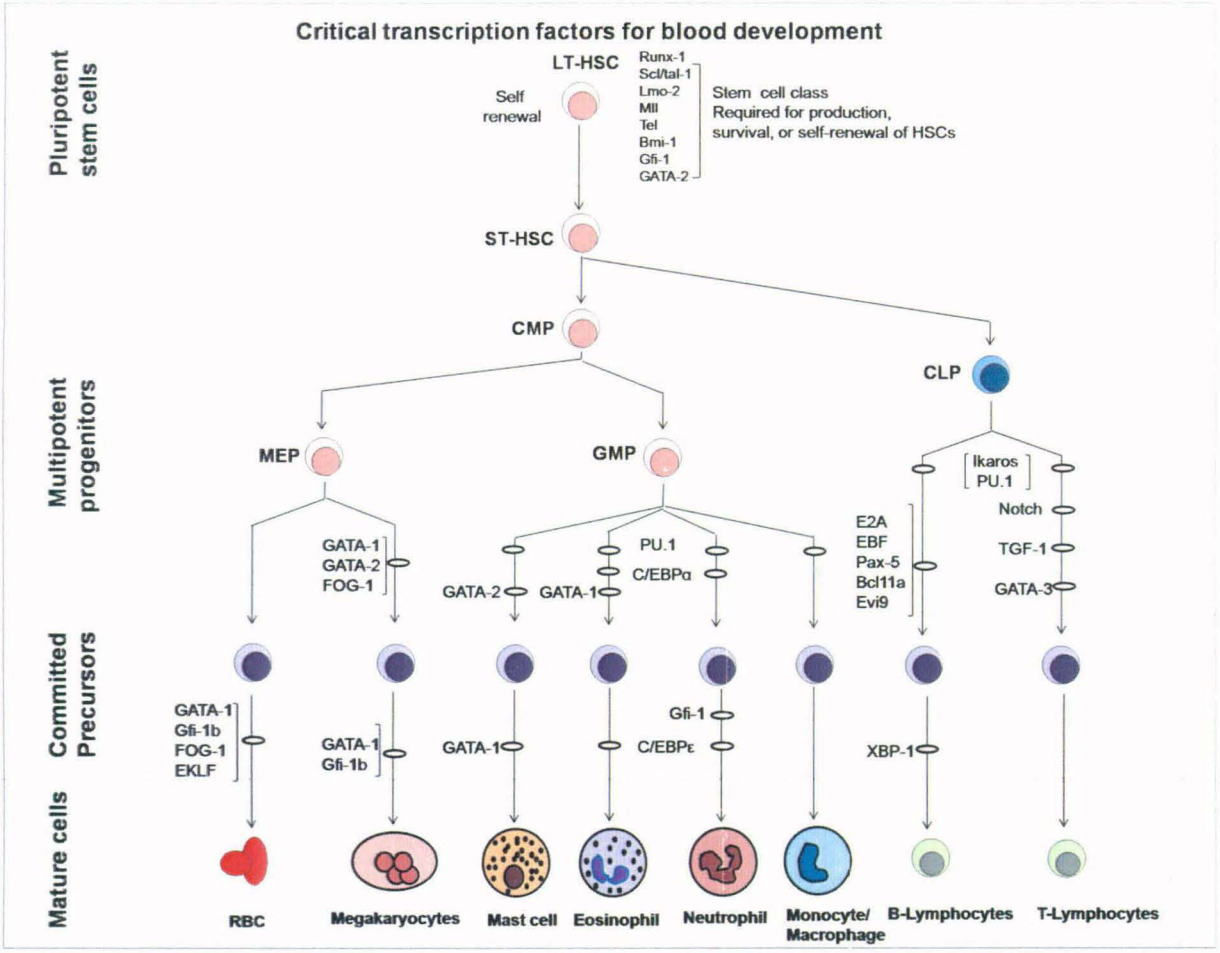

Figure1. Haematopoiesis and transcription factors required in the process.
The stages at which haematopoietic development is blocked in the absence of a given transcription factor, as determined through conventional gene knockouts, are indicated by red bars. The factors depicted in black have been associated with oncogenesis. Those factors in light font have not yet been found translocated or mutated in human/mouse haematologic malignancies. Abbreviations: LT-HSC, long-term haematopoietic stem cell; ST-HSC, short-term haematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocytes/erythroid progenitor; GMP, granulocyte/macrophage progenitor; RBCs, red blood cells (adapted from Orkin et.al. Cell, 2008(13)).

Recently placenta has also been recognized as an additional site (14). Stem cell development depends on the microenvironment, the niche for regulation of self renewal and differentiation (15).

1.1.1 Haematopoietic Lineage Differentiation

The haematopoietic lineage differentiation is a hierarchical fashion, haematopoietic stem cells first produce the progenitor cells and then the precursors with varying commitments to multiple or single pathways are formed. After a transient harboring in the yolk sac and AGM, HSCs migrate to the secondary sites of haematopoiesis: the liver, spleen, thymus and bone marrow, where the totipotent HSCs give rise to a population with restricted differentiation capacity (16, 17). These progenitor cells which can be identified by expression of specific lineage markers and bioassays of developmental potential in tissue culture or transplantation eventually differentiate into different haematopoietic lineages. The common lymphoid progenitors (CLP) give rise to B-cells and T-cells (18), whereas the common myeloid progenitors (CMP) give rise to monocytes, platelets, granulocytes and erythrocytes (19). The developmental potential of these cells is generally limited to only one or two of the haematopoietic lineages and these cells progressively display the antigenic, biochemical, and morphological characteristic of the mature cells of the appropriate lineages and lose their capacity for self-renewal.

1.1.2 Molecular Regulation of Haematopoiesis

Haematopoiesis involves a complex interplay between the intrinsic genetic processes of blood cells and the environment surrounding it. This interplay determines whether HSCs, progenitors and mature blood cells remain quiescent, proliferate, differentiate, self-renew, or undergo apoptosis. The best characterized environmental regulators of haematopoiesis are cytokines (20). Cytokines are a broad family of proteins that mediate positive and negative effects on cellular quiescence, apoptosis, proliferation, and differentiation. Cytokines function through a specific receptor and activate a variety of signaling pathways. This includes activation of tyrosine kinases such as focal adhesion kinase, pp60src, c-Abl, MAP kinases, Jun N-Terminal Kinase (JNK), and protein kinase C (PKC) (21). Cytokines including interleukin-3 and GM-CSF induce cell proliferation, while other cytokines including flt-3 ligand and kit ligand protect cells from apoptosis and sensitize them to the effects of growth promoting cytokines (22, 23) Cytokines may also facilitate the interactions between stem cells and elements in the microenvironment including extracellular matrix (ECM) components (24). Haematopoietic regulatory cytokines are

produced through both autocrine and paracrine mechanisms and in many cases are produced by non-haematopoietic cells including bone marrow stroma and endothelium.

Chemokines are another class of compounds that are important regulators of haematopoiesis (25). Chemokines are composed of a large family of proteins that mediate a variety of processes including inflammation, leukocyte migration and development, angiogenesis, tumor cell growth and metastasis (26). Chemokines bind to one or more of a large family of structurally related guanine protein-coupled transmembrane receptors (GPCRs). In haematopoiesis, chemokines can inhibit progenitor growth, regulate migration of haematopoietic progenitors, and mediate T-cell development in the thymus (27). Chemokine SDF-1 (which binds the receptor CXCR4) is essential for trafficking of haematopoietic cells in the developing embryo (26). A number of other chemokines likely play important roles in haematopoiesis. Other important environmental regulators of haematopoiesis include the extracellular matrix (ECM) components, other haematopoietic and nonhaematopoietic cells, nutrients and vitamins, and a variety of physiologic processes.

The haematopoiesis program is controlled by multiple factors and pathways. Gene silencing studies have shown effects of various genes on the haematopoietic program. T-lymphoid acute leukemia oncoprotein Tal-1/SCL (28-31) and LMO2 knockout mice (32) lack the precursor determination or maintenance both in yolk-sac and fetal liver which indicates that these two genes play important roles in early development of primitive and definitive haematopoietic progenitors. Although no specific primitive haematopoietic deficient mutant has been identified so far, studies from these knock-out mice indicate that the requirements for primitive and definitive haematopoiesis are distinct. Similarly, absence of the erythroid cell in GATA1 knock-out mice (33) and lymphoid cells in Ikaros knock-out mice (34, 35) demonstrated that GATA1 and Ikaros are key regulators in erythropoiesis and lymphopoiesis respectively.

1.1.3 Properties of Haematopoietic Stem Cells

The core property of haematopoietic stem cells is the ability to choose between self-renewal (remain a stem cell after cell division) and differentiation (start the path towards becoming a mature haematopoietic cell). In addition, HSCs migrate in regulated manner during development

process and are subject to regulation by programmed cell death (apoptosis). The balance between these activities determines the number of stem cells in the body.

1.1.3.1 Self-Renewal

One essential feature of HSCs is the ability to self renew i.e. to make copies with the same or very similar potential. This is an essential property because more differentiated cells, such as haematopoietic progenitors, cannot do this, even though they can expand significantly during a limited period of time after being generated. The key components or signals which allow self renewal are still unknown but one noteworthy key link is telomerase, the enzyme necessary for maintaining telomeres, the DNA regions at the end of chromosomes that protect them from accumulating damage due to DNA replication (36). Expression of telomerase is associated with self-renewal activity (37, 38). However, while absence of telomerase reduces the self renewal capacity of mouse HSCs, forced expression is not sufficient to enable HSCs to be transplanted indefinitely; other barriers must exist. It has proven surprisingly difficult to grow HSCs in culture despite their ability to self-renew.

1.1.3.2 Differentiation

The property of HSCs to differentiate into progenitors and mature cells that fulfill the functions performed by the haematopoietic system along with the self renewal property, defines the core function of HSCs. Differentiation is driven and guided by an intricate network of growth factors and cytokines. Once the HSCs commit to differentiate they cannot revert to a self-renewing state. Thus, specific signals, provided by specific factors seem to be required to maintain HSCs. This strict regulation reflects the proliferative potential present in HSCs, deregulation of which could easily result in malignant diseases such as leukemia or lymphoma.

1.1.3.3 Migration

Migration is another important property of HSCs which occurs at specific times during development (*i.e.*, seeding of fetal liver, spleen and eventually, bone marrow) and under certain conditions (*e.g.*, cytokine-induced mobilization). These migrating cells enter empty haematopoietic niches in the bone marrow and provide sustained haematopoietic stem cell self-

renewal and haematopoiesis (39, 40). It is assumed that this property of mobilization of HSCs is highly conserved along the evolution (mouse, dog and humans) and presumably results from contact with natural cell-killing agents in the environment after which regeneration of haematopoiesis requires restoring empty HSC niches. This means that functional, transplantable HSCs course through every tissue of the body in large numbers every day in normal individuals.

1.2 Leukemia

Cancer begins when cells in a part of the body start to grow out of control. There are many kinds of cancer, but they all start because of out-of-control growth of abnormal cells. Cancer cell growth is different from normal cells; instead of dying, cancer cells continue to grow and form new, abnormal cells. Cancer cells can also invade other tissues something that normal cells cannot.

Leukemias are proliferative diseases of the hematopoietic system that fail to obey normal regulatory signals. They are derived from stem cells or progenitors of the hematopoietic system and almost certainly include several stages of progression. During this progression, genetic and/or epigenetic changes occur; either in the DNA sequences itself (genetic) or other heritable modifications that affect the genome (epigenetic). These epigenetic changes alter cells from the normal hematopoietic system into cells capable of robust leukemic growth. There are varieties of leukemia, usually classified by the predominant pathologic cell types and/or the clinical course of the disease.

1.2.1 Types of leukemia

Based on cell type and phenotype of blast cells, leukemia can be divided into 4 main types:

- Acute myeloid (or myelogenous) leukemia (AML)
- Chronic myeloid (or myelogenous) leukemia (CML)
- Acute lymphocytic (or lymphoblastic) leukemia (ALL)
- Chronic lymphocytic leukemia (CLL)

Acute leukemia versus Chronic leukemia

The first factor in classifying a patient's leukemia is whether most of the abnormal cells are mature (look like normal white blood cells) or immature (look more like stem cells). In *acute* leukemia, the bone marrow cells do not mature properly and these immature leukemia cells continue to reproduce and build up. Without treatment, most patients with acute leukemia would live only few months. Some types of acute leukemia respond well to treatment and many patients can be cured while other types of acute leukemia have a less favorable outlook. In *chronic* leukemia, the cells can mature partly but not completely. These cells may look fairly normal but they are not. Unlike normal white blood cells, they do not fight infections; rather they survive longer, build up and crowd out normal cells. Chronic leukemias tend to progress over a longer period of time, and most patients can live for many years. But chronic leukemias are generally harder to cure than acute leukemias.

Myeloid leukemia versus lymphocytic leukemia

The second factor in classifying leukemia is the type of bone marrow cells that are affected. Leukemias that start in early forms of myeloid cells i.e. white blood cells (other than lymphocytes), red blood cells, or platelet-making cells (megakaryocytes) are *myeloid* leukemias (also known as myelocytic, myelogenous, or non-lymphocytic leukemias). If the cancer starts in early forms of lymphocytes, it is called *lymphocytic* leukemia (also known as lymphoid or lymphoblastic leukemia). Lymphomas are also cancers that start in lymphocytes. But whereas lymphocytic leukemias develop from cells in the bone marrow, lymphomas develop from cells in lymph nodes or other organs.

1.2.2 Acute myeloid leukemia

AML is a heterogeneous malignant hematopoietic disorder characterized by accumulation of undifferentiated cells due to mutations that prevent their normal differentiation and allow undifferentiated cells to survive. Common symptoms of untreated AML are fatigue, bleeding due to thrombocytopenia, organ infiltration and fatal infections due to neutropenia, all resulting from the suppression of normal bone marrow function. AML is diagnosed on the basis of the accumulation of myeloid blasts in the bone marrow. According to the French-American-British

(FAB) cooperative group the diagnosis of AML requires at least 30% myeloid blasts in the bone marrow (41, 42). The classification by WHO is based on morphology, histochemistry and cytogenetics. The WHO system defines four major categories of AML, namely; i) AML with recurrent genetic abnormalities, ii) AML with multilineage dysplasia, iii) therapy related AML and iv) AML not otherwise categorized.

French-American-British (FAB) Classification: In the 1970s, a group of French, American, and British leukemia experts divided acute myeloid leukemias into subtypes, M0 through M7, based on the type of cell from which the leukemia developed and how mature the cells are. This was based largely on how the leukemia cells looked under the microscope after routine staining. The French-American-British (FAB) classification divided AML into 8 subtypes designated as M0 to M7 (41) listed in table 1 below.

FAB Classification of acute myeloid leukemia	
Classification	**Cell type**
M0	Undifferentiated leukemia; Myeloperoxidase negative Myeloid markers positive
M1	Myeloblastic without differentiation; immature white blood cells
M2	Myeloblastic with differentiation; can be divided into those with t(8;21) AML1-ETO fusion
M3	Promyelocytic; APL; most cases have t(15;17)(q24;q21) PML-RARα or another translocation involving RARα
M4	Myelomonocytic; myelodysplastic syndrome
M4eo	Myelomonocytic with bone marrow eosinophilia; Characterized by inversion of chromosome 16 involving CBFb, which forms a heterodimer with AML1
M5	Monoblastic leukaemia; involves t(9;11) translocation
M5a	Monoblastic without differentiation
M5b	Monoblastic with differentiation
M6	Erythroleukemia (M6a), pure erythroid leukemia (M6b)
M7	Megakaryoblastic leukemia; associated with GATA1, t(1;22)(p13;q13) mutation. Increased risk in those associated with Down's syndrome.

The molecular changes that occur in AML patients with no detectable cytogenetic abnormalities make up the largest subgroup and represent a variety of cytogenetically silent molecular genetic abnormalities. The number of known mutations associated with AML continue to grow at an unprecedented pace with over 300 different chromosomal translocations and other mutational events having already been described. Therefore, it is obvious that there are many more leukemic genotypes than phenotypes. Gilliland (2001) proposed a 'two-hit' model (figure 2) for leukemogenesis in an attempt to provide a unified molecular theme to explain how different mutations can generate essentially similar phenotypes.

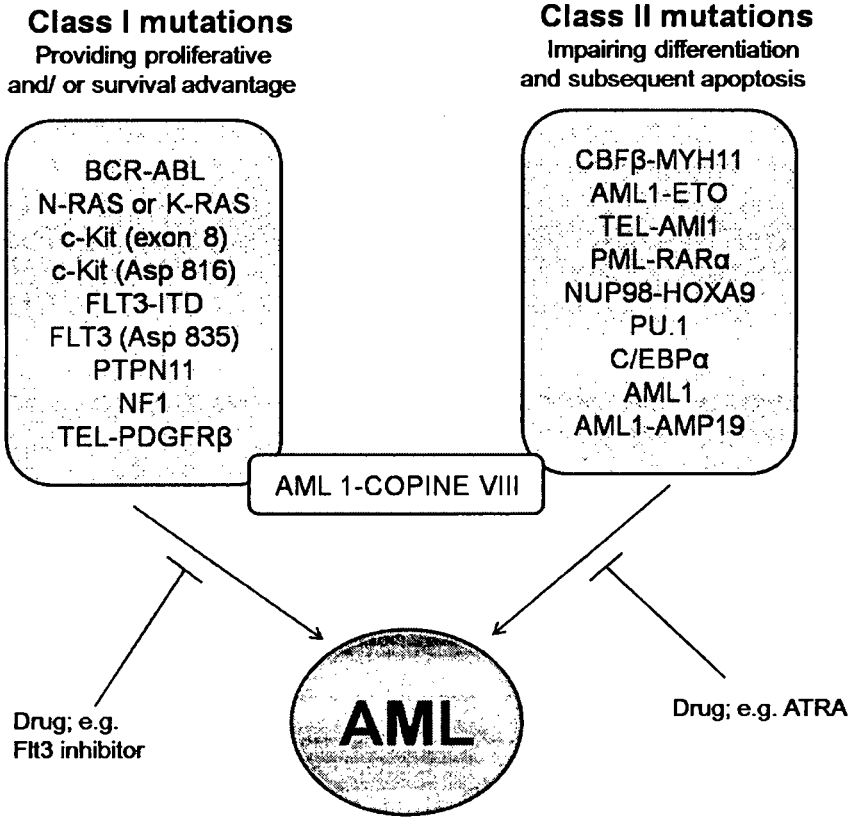

Figure 2: The "two-hit" model of AML development. Adapted from Gilliland et.al. Blood, 2002 (43-45). Class I mutations include mutations which confer a growth advantage whereas class II mutations impair the hematopoietic differentiation. AML1- COPINE VIII is a unique mutation with a resulting fusion protein with both class I and class II activities (46).

The basis of the hypothesis is that AML is the consequence of a collaboration between at least two broad classes of mutation; class I mutations that confer a proliferative and/or survival

advantage to cells (BCR-ABL, N-RAS, KRAS, c-Kit (exon 8), c-Kit (Asp816), FLT3-ITD, FLT3 (Asp835), PTPN11, NF1 and TEL-PDGFR.) and class II mutations that primarily impair haematopoietic differentiation and cellular apoptosis (CBFβ–MYH11, AML1–ETO, TEL–AML1, PML–RARα, NUP98–HOXA9, PU.1, C/EBPα, AML1 and AML1–AMP19) (45). The model predicts that AML results from the combined effects of only two mutations, one from each class. However, a limitation of the model has been the lack of identifiable class I and class II mutations in the majority of AML cases. The mutations of receptor tyrosine kinases (RTKs) class III and RAS frequently provide the 'missing' proliferative signal in AML. Mutated tyrosine kinases such as FLT3 or kit, activated alleles of N-RAS or K-RAS and constitutive expression of transcription factors, such as NF-kB have been found in a significant percentage of AML patients. The mutations are associated with a significantly greater risk of relapse and reduced survival (44).

1.2.2.1 Prognostic factors

The median survival for AML patients receiving supportive therapy alone is only 3-4 months and very few patients survive for more than one year. The prognostic factors include the cytogenetic test (showing chromosome or gene changes), the patient's age and the white blood cell count. Other important factors include pre-existing blood disorders (such as a myelodysplastic syndrome) and a history of treatment with chemotherapy and/or radiation therapy for an earlier cancer. There are numerous chromosomal aberrations and other genetic defects detected in AML; recurrent cytogenetic abnormalities are used in prognosis and guidance for therapeutic decisions on hematopoietic stem cell transplantation or intensive chemotherapy alone.

Chromosome abnormalities

Chromosome changes give one clue to prognosis; however, not all patients have these abnormalities. Based on the cytogenetics, patients can be classified into three major subgroups with different prognosis. The group with favorable cytogenetics includes about 25% of the patients. Examples of favorable cytogenetics are t(8; 21)(q22; q22) and t(15; 17)(q22; q21) which creates the fusion protein AML1-ETO (47) and PML-RARα (48) fusion proteins respectively. Patients with latter fusion protein receive targeted therapy with all-trans retinoic

acid (ATRA) in addition to conventional therapy. The adverse cytogenetic group includes about 10% of AML patients with multiple abnormalities as deletions of either chromosome.

Favorable abnormalities:

- Translocation between chromosomes 8 and 21; t(8; 21)
- Inversion of chromosome 16
- Translocation between chromosomes 15 and 17; t(15; 17)

Unfavorable abnormalities:

- Complex changes - involves several chromosomes.

Gene mutations

Several molecular genetics aberrations also have a prognostic impact in AML. The most important genetic aberration is in-frame internal tandem duplications (ITDs) of the receptor tyrosine kinase *FLT3* (44). *FLT3* mutations are associated with an adverse prognosis, and are the strongest separate marker for disease relapse in AML. Another frequent genetic aberration in AML is mutations in the nucleophosmin gene (*NPM1*) (49). These mutations are present in 40 – 50% of AML patients with normal karyotype and represent a favorable prognostic factor for patients without *FLT3* mutations. Several other molecular prognostic markers indicated are: Partial tandem duplications of the mixed lineage leukemia (*MLL*) gene associated with a short remission duration (50). High expression of the Brain and Acute Leukemia Cytoplasmic (*BAALC*) gene is an adverse prognostic factor (51). Mutations in CCAAT/enhancer binding protein alpha (*CEBPA*) give a favorable prognosis of disease outcome (52). Mutations in *TP53* are associated with secondary leukemia and chemo resistance (53). Gene expression profiling by DNA microarrays is a new method that is increasingly used for prognostic evaluation and identification of novel subclasses of AML. Gene expression profiling signatures have been correlated to clinical outcome in several studies and will probably become a valuable tool for future molecular diagnostics.

Other Factors

Age is also one of the many factors since older patients (over 60) generally do not fare as well as younger patients. Some of this may be because they are more likely to have unfavorable

chromosome abnormalities. Older patients may also have other medical conditions that can make it harder to treat them with more intense chemotherapy regimens. Having a prior blood disorder such as a myelodysplastic syndrome or having AML that develops after treatment for another cancer tends to lead to a worse prognosis because these types of AML are often harder to treat.

1.2.2.2 Treatment of AML

AML is not a single disease but a number of related diseases each distinguished by unique cytogenetic markers which in turn help determine the most appropriate treatment. These cytogenetic abnormalities, as well as other mutations, give rise to abnormal signal transduction and these abnormal pathways represent ideal targets for the development of new therapeutics.
The chemo drugs used most often to treat AML are cytarabine (cytosine arabinoside or ara- C) and the anthracycline drugs (such as daunorubicin/daunomycin, idarubicin, and mitoxantrone). These chemotherapeutic drugs have various side effects which include; hair loss, mouth sores, loss of appetite, nausea, vomiting and it also affects bone marrow, which often causes low blood cell counts in AML patients (54).

Acute Promyelocytic Leukemia with —t(15;17) translocation leading to formation of a fusion protein PML-RARα is the subtype of acute leukemia where the greatest progress has been made over the past decade (48). It is the most curable subtype of AML and the most important development leading to the dramatic improvement in survival has been the introduction of all-trans retinoic acid (ATRA) (55). ATRA is a form of vitamin A that is often part of the initial treatment of APL (56). It is often given along with chemo - an anthracycline drug with or without cytarabine. For patients who can't take an anthracycline (often because of heart problems), ATRA can also be given with arsenic trioxide for the initial treatment of APL, in which no regular chemo drugs are given (57). ATRA can have side effects which include headache, fever, dry skin and mouth, skin rash, swollen feet, sores in the mouth or throat, itching and irritated eyes. It can also cause blood lipid levels (like those of cholesterol and triglycerides) to go up. Often blood liver tests become abnormal.

While the incorporation of ATRA has led to these remarkable results, differentiation therapy with ATRA is associated with unique toxicities not previously observed with conventional cytotoxic therapy. AML is marked by the block in differentiation and it often

shows some morphological signs of differentiation induced by exposure to various soluble mediators like trans-retinoic acid (ATRA) and several cytokines (58). Several agents can induce leukemic cell differentiation in other AML subtypes, although these effects may differ between patients. Differentiation may then be associated with induction of apoptosis and differentiation-inducing therapy may therefore become useful in combination with intensive chemotherapy to increase the susceptibility of AML blasts to drug-induced apoptosis.

Bone marrow transplant is also effective in the treatment of AML (59). New therapeutics used in the treatment of AML includes antibody-based or cell-based immunotherapy, drug conjugates, radioimmunoconjugates, T cell adaptive immunotherapy and AML vaccines (60-63). Future studies in AML should therefore focus on: A) the identification of new agents with more predictable effects on differentiation and apoptosis; B) the use of clinical and laboratory parameters to define new subsets of AML patients in which differentiation/apoptosis induction has a predictable and beneficial effect, and C) further characterization of how AML blast sensitivity to drug-induced apoptosis is modulated by differentiation induction. In addition, differentiation therapy is also considered for other AML patients as a therapeutic approach with low treatment-related morbidity and mortality. Therefore, screening of compounds using *in-vitro* assays having potential to induce apoptosis and differentiation in myeloid blasts may have substantial impact in the development of better therapeutics for leukemia.

1.2.3 Chronic Myeloid Leukemia

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder of haematopoietic stem cells (64). This disease evolves in 3 distinct clinical stages: a chronic phase (CP) lasting three to six years is followed by transformation to an accelerated phase (AP) and then a terminal blast phase of short duration (65, 66). CML is one of the best understood diseases from the aspect of its cytogenetic abnormalities and the molecular mechanisms involved. CML is consistently associated with an acquired genetic abnormality, the Philadelphia chromosome (Ph1), a shortened chromosome 22 resulting from a reciprocal translocation of the long arms of chromosomes 9 and 22 (67). This translocation generates the BCR/ABL fusion gene, which is translated in p210BCR/ABL oncoprotein (figure 3) (68). Expression of p210BCR/ABL is necessary and sufficient for malignant transformation, as demonstrated with *in vitro assays* and

leukemogenesis in mice (69-71). BCR-ABL oncoprotein, a constitutively active tyrosine kinase recruits and activates several pathways transducing intracellular signals which ultimately lead to abnormal cellular adhesion, enhanced proliferation and inhibition of apoptosis. The BCR/ABL fusion protein helps in the proliferation and survival of myeloid progenitor cells by activating RAS and PI-3K/AKT pathway (72-74). Src family kinase interacts with BCR/ABL, gets activated which in turn phosphorylates STAT5B and thereby increases the expression of anti-apoptotic Bcl-XL protein (75, 76).

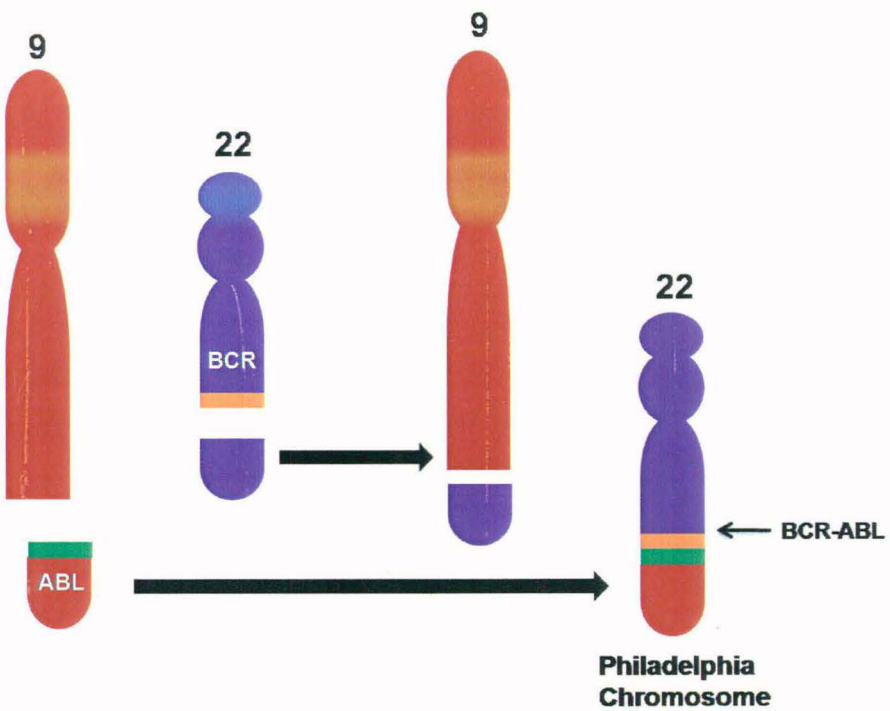

Figure 3 : Philadelphia chromosome (Ph) caused by reciprocal translocation between chromosomes 9 and 22 is generally believed to be the sole cause of CML (adapted from Druker, B. J. Adv. Cancer Res.2004, 91, 1-29).

Transition from chronic to blast crisis is unavoidable outcome of CML except in a cohort of patients receiving allogenic bone marrow transplants early in chronic phase. Two other BCR/ABL proteins, p190 and p230, generated by variant fusion genes, are only occasionally detected in CML (77-79).

The treatment of CML has long been in the frontier of cancer therapy. Imatinib mesylate (STI571, imatinib, Glivec® or Gleevec®, Novartis, Basel, Switzerland) is the treatment of choice for chronic-phase CML and due to its remarkable therapeutic effects; blast crisis

transition is postponed for several years in most patients with CML (80-82). However, Imatinib therapy alone cannot be the only solution to the treatment of CML due to growing evidences of drug resistance developed in CML patients. Therefore, screening of compounds which may kill Philadelphia chromosome harboring cells or may induce differentiation in arrested cells can be boon for such patients.

1.2.3.1 Pathology of CML

Philadelphia chromosome discovered by Peter Nowell in 1960 provided first evidence for genetic link of cancer (67). The leukemogenic potential of $p210^{BCR-ABL}$ resides in the fact that the normally regulated tyrosine kinase activity of the ABL protein is constitutively activated in the fusion oncoprotein (83). ABL proteins are non-receptor tyrosine kinases that have important roles in signal transduction and the regulation of cell growth. There are two isoforms of ABL, isoform 1a and isoform 1b. Isoform 1b, which is expressed at higher levels in early haematopoietic progenitor cells, is myristoylated on its second glycine residue at the N-terminal (84). Loss of myristoylation in ABL dramatically enhances its tyrosine kinase activity (85). Downstream to the myristoylation site, at the N-terminal segment of ABL, there are three SRC homology domains (SH3, SH2 and SH1). SH2 and SH3 regulate the tyrosine kinase function of ABL and SH1 harbors the tyrosine kinase activity of ABL. SH3 has a negative regulatory effect on the tyrosine kinase function. Deletion of SH3 or mutation in SH3 facilitates tyrosine kinase activity of ABL (84-86). Defects in the functional integrity of SH2 decrease phosphotyrosine binding and reduces the transforming capacities of ABL (87). The C-terminal part of ABL contains a DNA-binding domain, nuclear localization signals, and a binding site for actin (88). The disruption of ABL protein by genetic fusion is responsible for the up-regulated tyrosine kinase activity. The uncontrolled tyrosine kinase activity of BCR-ABL is also caused by the juxtaposition of alien BCR sequences. The N-terminal coiled-coil motif of BCR promotes dimerization and increases BCR-ABL tyrosine kinase activity and enables binding of F-actin to ABL (89). The serine-threonine kinase domain of BCR activates signaling pathways mediated by BCR-ABL tyrosine kinase (90). BCR which also contains SH2 binding sites, fusion to ABL adds a large amino acid sequence to the SH2 segment of ABL. BCR interferes with the adjacent SH3 and SH2 kinase regulatory domain, which in turn causes ABL to become constitutively active as

a tyrosine phosphokinase (91). Ectopic expression of BCR/ABL in growth factor-dependent cell lines activates numerous signal transduction pathways responsible for growth factor independence and reduced susceptibility to apoptosis of these cells (92).

1.2.3.2 Treatment of CML

Cytoreductive chemotherapies, INF-α and allogeneic stem cell transplantation were the standard treatment options for CML patients in chronic phase before the imatinib era. IFN-α is a member of glycoprotein family which has antiviral and antiproliferative properties. IFN-α was first shown to be an active agent in CML in the early 1980s and it became the non-transplant treatment of choice for chronic phase CML patients (93). IFN-α has been shown to increase survival while haematologic responses are seen in the majority (80%) of patients; cytogenetic responses are seen in only 30-50% of patients with complete cytogenetic responses in only 10-20% of IFN-treated patients (94-96). Unfortunately many patients tolerate IFN-α poorly, necessitating dose reduction or discontinuation of treatment. Currently, the only curative approach for CML is allogeneic stem cell transplantation. The outcome of this procedure depends on a series of risk factors, the most important of which are the patient's age and the phase of the disease (97). For young (age <40 years) patients undergoing transplants within 1 years from diagnosis, long- term survival rates are reported to be 70-80%. With advances in molecular HLA-typing, improvements in infection control and graft versus host disease prophylaxis, outcomes for related and unrelated donor transplants appear similar. Unfortunately, up to one third of CML patients are over the age of 60 for whom the allogeneic stem cell transplantation usually is not feasible due to high risk of treatment-related mortality (98). Therefore, for many patients with CML, stem cell transplantation is not an option.

1.2.3.3 Imatinib mesylate

Imatinib (STI571, Gleevec) is the first successful, rationally developed, receptor-targeted agent for chronic myelogenous leukemia (CML). Imatinib was initially developed by scientists at Ciba-Geigy (currently Novartis, Basel, Switzerland), as a specific platelet-derived growth factor receptor (PDGFR) inhibitor (82). As the BCR-ABL tyrosine kinase plays a key role in CML pathogenesis, attempts to target the BCR-ABL tyrosine kinase evolved as new therapeutic

strategies. It was also found to be a potent ABL tyrosine kinase inhibitor. Further optimization for v-ABL tyrosine kinase inhibition led to generation of imatinib mesylate which selectively inhibits ABL tyrosine kinase including BCR-ABL (figure 4) (81, 99).

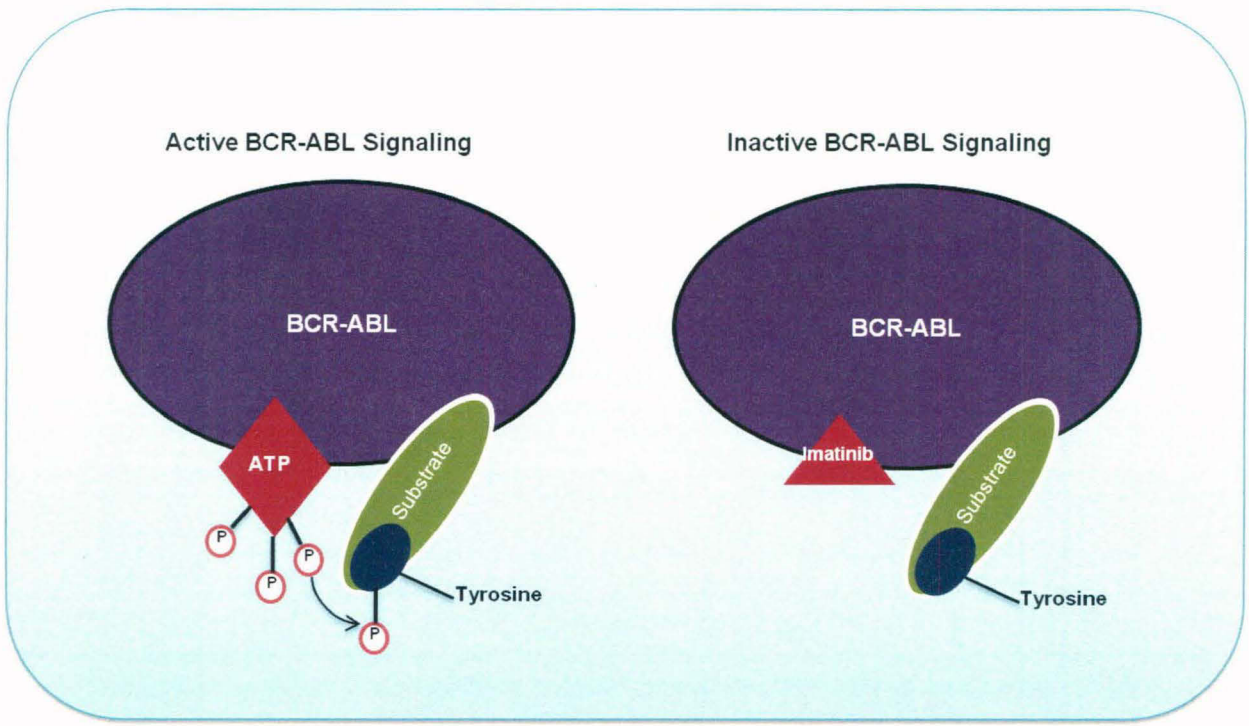

Figure 4: Imatinib a specific inhibitor of small family of tyrosine kinases, including BCR-ABL blocks the ATP binding site and prevents substrate phosphorylation, thereby interrupting BCR-ABL signal transduction pathways that lead to leukemic transformation (adapted from Schindler,T. et.al Science, 2000)

Further studies revealed that a limited number of other tyrosine kinases are also targeted by imatinib, including PDGFR (82), c-KIT (100) and ARG (101). Preclinical studies showed that imatinib selectively inhibits the proliferation of cell lines holding $p210^{BCR-ABL}$ and the clonal growth of myeloid cells from CML patients (102). It was also shown in mice models that imatinib had *in vivo* activity against *BCR-ABL* positive cells and that continuous exposure to imatinib was necessary to eradicate the tumors, suggesting this would be important for an optimal antileukemic effect (103). Though imatinib mesylate is widely used as a treatment of

choice for the CML, there are some side effects which act as a limiting factor in its therapeutic use.

(i) Molecular persistence:

Chronic phase patients treated with imatinib mesylate remain positive when tested by qRT-PCR for *BCR-ABL* transcripts though they have been expected to achieve complete cytogenetic remission i.e. they have a persisting minimal residual disease (104). Even those who have undetectable *BCR-ABL* transcripts may still harbor as many as $1x10^{7}$ leukemic cells in their bodies and there is a high likelihood for relapse if the drug is stopped (105-107). Bone marrow studies have shown that the residual Ph-positive cells are part of the leukemic stem cell compartment (108, 109). Studies performed *in vitro* suggest that many primitive Philadelphia-positive progenitor or stem cells are relatively insensitive to imatinib (110).

(ii) Resistance:

Primary and acquired resistance can be seen in all stages of CML patients treated with imatinib mesylate. Resistance to imatinib is multifaceted. Generally, there are two types of resistance, primary and acquired. Primary resistance may be defined at the haematologic, cytogenetic or molecular levels. Acquired resistance can be defined as: (i) progression into blast crisis; (ii) loss of a sustained CHR or cytogenetic response; and (iii) a 5- to 10-fold rise in *BCR-ABL* transcript number (111). However, the mechanism of primary resistance is still mainly unsolved. In general, there are two possible categories of the molecular mechanisms of imatinib resistance, i.e. BCR-ABL independent and BCR-ABL dependent (112). In the first category, secondary oncogenic changes can occur in the leukemic cells and render the cell proliferation independent of BCR-ABL. In this scenario, BCR-ABL is no longer a relevant target and even the most ideal BCR-ABL inhibitor would be ineffective in this setting. However, BCR-ABL-independent mechanisms are rare events. In the second, BCR-ABL-dependent category, some change is predicted in either the patient (host-mediated) or the leukemic clone (cell-intrinsic) that prevents the drug from effectively shutting down the target BCR-ABL protein. Host-mediated resistance can occur through enzymatic modification of imatinib by a P450 enzyme in the liver or by production of a protein that neutralizes drug activity, such as alpha-1 acid glycoprotein (113-

115). Cell-intrinsic resistance could occur by modification of the target BCR-ABL tyrosine kinase through gene amplification or *BCR-ABL* kinase domain mutations or by a reduction of intracellular drug concentration through over expression of multidrug resistance genes (116). Among these mechanisms, the *BCR-ABL* kinase domain mutations are the most studied. These different lines of evidence suggest that imatinib, although being highly active against the differentiated mass of CML cells, probably fails to eradicate leukemic stem cells (117-119).

In CML, the leukemic stem cells can be considered quiescent, spending most of their time in G_0. Under certain circumstances, leukemic stem cells can enter cell cycle and give rise to progenitors, which produce differentiated leukemic cells (120, 121). Expansion of Ph-positive progenitors is inhibited by imatinib and life-long imatinib therapy is likely required to continuously suppress the remaining leukemic cells in CML patients, even in the best responders. Development of treatment targeting these quiescent stem cells, e.g. immunotherapy, is a challenge in CML.

1.3 Ormeloxifene

Ormeloxifene ($C_{30}H_{35}O_3N.HCl$; trans-1-[2-{4-(7-methoxy-2,2-dimethyl-3-phenyl-3,4-dihydro-2H- 1-benzopyran-4-yl)-phenoxy}-ethyl]-pyrrolidine hydrochloride; also known as centchroman, is a potent non-steroidal selective estrogen receptor modulator (SERM) used as oral contraceptive for birth control (122). It was synthesized at CSIR-Central Drug Research Institute, Lucknow (123). This agent is a unique need-oriented contraceptive agent which is included in the National Family Welfare Programme of India. Since 1991, ormeloxifene has been used as a need-oriented contraceptive and is being given for treating dysfunctional bleeding of the uterus. This agent's contraceptive activity is well established in rodents and primates (124). Ormeloxifene inhibits implantation via inhibition of endometrial receptivity to blastocyst signals by antagonism of the action of estrogen, without altering the concentration or secretion pattern of estrogen and progesterone, hypothalamo-pituitary-ovarian axis, follicle maturation, ovulation, mating behavior, gamete transport or fertilization, and pre-implantation development of embryos (125-127). Clinically, ormeloxifene has been reported to provide good pregnancy protection in women in postcoital as well as weekly regimens and is marketed in India as a contraceptive pill.

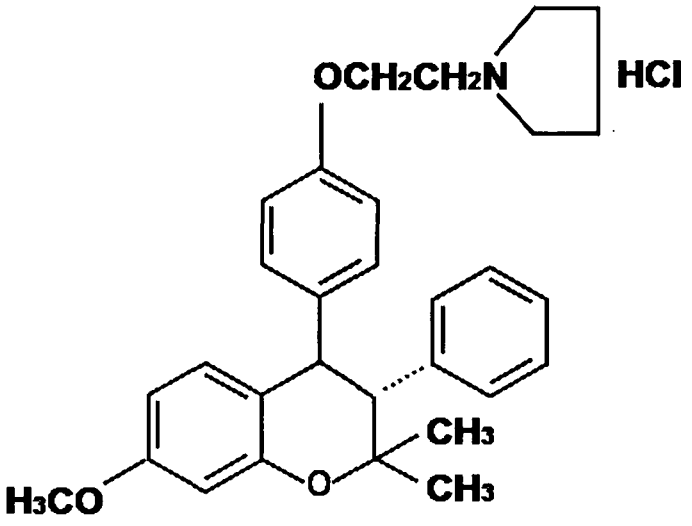

Figure 5: Structure of ormeloxifene (128)

Ormeloxifene is efficient in the management of hormone related clinical disorders (129). It is effective in the treatment of polycystic ovarian syndrome by induction of ovulation (130). Due to its potent anti-estrogenic and weak estrogenic activities (127, 131, 132), it is also effective against advanced breast cancer (133) and may be therapeutically effective for other clinical conditions such as dermatitis, osteoporosis, restenosis, endometriosis and uterine fibroids. It prevents bone loss by directly inhibiting the bone reabsorbing activity of osteoclast. Ormeloxifene as a racemate has been found to be a potent cholesterol-lowering pharmaceutical resulting in a significant decrease in serum concentrations. Ormeloxifene has also been reported to be efficacious in the management of dysfunctional uterine bleeding or menorrhagia. In nearly 78% percent of the patients the menorrhagia was cured without any side effect (134). Thus, it acts as an effective and safe therapeutic option for the medical management of menorrhagia. Ormeloxifene suppresses the receptors in the reproductive organs like the ovaries, uterus and breasts while it stimulates the estrogen receptors of other organs like the bones (129, 135, 136). Substantial evidence has been amassed to support the premise that ormeloxifene has potential anticancer activity (128, 133). In a published study, Mishra et. al, evaluated the role of ormeloxifene in breast cancer patients. Apparently, treatment with ORM was evaluated in 4 male and 75 female patients with advanced breast cancer. The overall response rate, including both male and female cases, was 40.5%. Among the female patients, the overall response rate was

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38.7%. One of the 4 male patients showed a complete response and 2 showed partial responses. The responses were more marked for bone, pulmonary, soft tissue, skin and lymph-node metastases than for liver metastases (133). In addition, ORM can reduce the mutagenic effects of known genotoxic compounds dimethylbenz[*a*]anthracene (DMBA), cyclophosphamide (CP), mitomycin C (MMC) and ethyl methanesulfonate (EMS) in *Salmonella*. These protective activities of ORM against the known positive mutagens in the *Salmonella* may be due to induction of apoptosis by ORM, which leads to the elimination of cells damaged by these mutagens from the cell population (128).

Ormeloxifene possesses excellent therapeutic index and has been well-tolerated, without any haematological, biochemical or histopathological evidence of toxicity during phase III trial in pregnant females. Ormeloxifene has no side effects after prolonged use. It is devoid of side effects such as nausea, headache, vomiting, dizziness, breakthrough bleeding, depression, mood change, nostalgia, and poor libido; androgenic ill effects such as acne and hirsutism, metabolic complications such as weight gain, hypertension, coagulation disorders, hyperglycemiâ, and abnormal lipid profile or pigmentation or ophthalmological or audiological effects.

Chapter 2

Materials and Methods

Materials

Cell culture media, culture flasks, chemicals, reagents, kits, antibodies, membranes and transfection reagents were obtained from the following companies:

Applied Biosystems- Real time PCR reaction buffer, cDNA synthesis kit

BD Biosciences- PE-conjugated antibodies CD11b, CD114, IgG isotype control, FACS tubes

Bio-Rad- Bradford Reagent

Dharmacon- On-Target plus E6AP siRNA, scrambled siRNA, Dharmafect transfection reagent

Fermentas- Molecular weight markers, DNA ladder

GE Healthcare- Hyperfilm ECL, ECL Advance western blotting detection kit, pH strips for 1-D gel electrophoresis and GST-Sepharose beads

Gibco (Invitrogen)- Transfection reagent Lipofectamine-2000, Lipofectamine-LTX, DMEM high glucose, RPMI-1640 liquid and powder medium, Fetal Bovine Serum

Himedia- Agar powder, Luria Bertani broth

Eurofins MWG Operon- Primers for semiquantitative and quantitative PCR

Merck- Acetone, Isopropanol, Methanol, Ethanol

Millipore- Sterile filters, Protein Agarose A and G beads, Enhanced Chemi Luminescence, PVDF membrane

Roche- Real Time PCR kit and plates

Sigma-Aldrich- RPMI-1640 media, DMEM powder, Sodium bicarbonate, Sodium chloride, L-glutamine, Giemsa stain, Sodium acetate, Ethidium bromide, Bromophenol blue, Ammonium bicarbonate, PCR primers, Tris-saturated phenol, Gene elute plasmid miniprep kit, Coomassie brilliant blue R, Coomassie brilliant blue G, Ponceau stain, Acrylamide, Sodium dodecyl sulphate, Ammonium persulfate, TEMED, Trizma HCl, Trizma base, Glycine, Triton X-100, Imidazole, Calcium chloride, Chloroform, Glycerol, Sodium deoxycholate, BSA, DAPI, Ammonium chloride, Nonidet P-40, Paraformaldehyde, Formaldehyde, DTT, EDTA, Potassium chloride, Sodium di-hydrogen phosphate, Di-sodium hydrogen phosphate, DMSO, Protease

inhibitor cocktail, Phosphatase inhibitor cocktail I and II, Silver nitrate, Ampicillin, Chloramphenicol, Developer, Fixer, Rabbit, Mouse and Goat secondary HRP conjugated antibodies, Lysozyme and Agarose

SRL- Sodium dodecyl sulphate, Tris-base, Sodium acetate, Sodium chloride, Methanol, Sodium hydroxide, Glycine, Sodium bicarbonate, Boric acid

Tarson- 90 mm culture dishes, disposable sterile pipettes, 50 ml and 15 ml falcon tubes

Nunc- 25cm^2 culture flasks, 75cm^2 culture flasks, 96, 6, 12 and 24 well plates

USB- Sodium hydrogen phosphate, Urea, Disodium hydrogen phosphate, N,N-methylene- bis-acrylamide

Methods

2.1 Cell Culture

2.1.1 Culture Media

To prepare 1L of culture medium, 16.4g of RPMI-1640 was dissolved in 1L of distilled water. This incomplete culture medium was then filter sterilized with 0.22μ of sterile filter. To prepare complete culture medium 10% heat inactivated fetal bovine serum was added along with 1X antibiotic solution. Cells were cultured in 5% CO_2 humified incubator. HEK293T cells were cultured in Dulbecco's modified eagle medium (DMEM). DMEM was prepared by dissolving 16.4g of DMEM powder to 1L of distilled water and was further filter sterilized with 0.22 μ of filter unit. Complete culture medium was prepared by the addition of 10% heat inactivated FBS, 1X antibiotic solution and 2mM sodium pyruvate solution.

2.1.2 Cell Culture

HL60, K562 and U937 cells were cultured in the RPMI-1640 medium supplemented with 10% FBS and 1X antibiotic solution. Cells were grown at 37°C in 5% CO_2 humfied incubator. Cell were sub cultured after every fourth day by centrifuging at 1200rpm for 5min at RT. Cell pellet was washed once with sterile PBS and then again centrifuged to remove debris and old medium. Further, cells were dissolved in fresh medium and replated in 75cm^2 cell culture flask. HEK293T is an adherent cell line and was sub cultured after every third day. For sub culturing, old medium was decanted, cells were washed once with sterile PBS and trypsinized with 1X trypsin-EDTA solution. Cells were flushed up and down with sterile pipette and PBS until homogenous single cell suspension was achieved, this was followed by centrifugation at 1200rpm for 5min at RT. Cell pellet was dissolved in fresh medium and was plated in culture flask.

2.1.3 PBMCs (Peripheral blood mononuclear cell) isolation from blood samples

PBMCs were isolated from CML patient blood samples using histopaque-1077 (Sigma) (137). 10ml blood sample was mixed in equal volumes of MACS buffer (PBS containing 2% FBS and 2mM EDTA) and centrifuged at 400g for 10min. Supernatant was completely removed and dark red pellet was again washed with 25ml of MACS buffer. The dark pellet was diluted in MACS buffer in 1:1 ratio and was fractionated through Histopaque-1077 by centrifugation at 400g for

45min. Mononuclear cells were collected from the interface, washed twice with RPMI and were resuspended in RPMI-1640.

2.1.4 Cryopreservation of cells

Cells were cryopreserved in liquid nitrogen at -196°C so that they can be revived later for future use. Cryopreservation medium included 90% FBS and 10% DMSO. Cells were washed once with PBS and pelleted down. Cryopreservation medium was added to the cell pellet and this cell suspension was aliquoted in cryo vials and was placed in slow cooling isopropanol boxes which were first kept at -20°C for 12hrs, placed at -80°C for next 12hrs and then shifted to -196°C in liquid nitrogen for the long term storage.

2.1.5 Stable cell line preparation

β-estradiol inducible K562 stable cell line was prepared using pBABE puro p42 C/EBP alpha-ER plasmid construct. $1x10^5$cells/ml were plated in 6 well plates one day before transfection in phenol red free RPMI supplemented with 10% charcoal stripped FBS and 1X antibiotics. Next day 4h prior to transfection, medium of cells was changed with phenol red free RPMI supplemented with 1X antibiotics without FBS. Lipofectamine LTX reagent was used for the transfection. After transfection selection of cells was performed in 2.0μg/ml puromycin supplemented medium for two weeks. Serial dilution of cells was carried out in 96 well plates to obtain single cell dilution. Cells were grown for 3 days and then were transferred to 6 well plates and later to $25cm^2$ cell culture flasks. 6 clones were selected and after cell growth of nearly two weeks, western blotting was performed to verify the clones. Giemsa staining was performed for the detection of functional clones as K562-p42 C/EBPα-ER stable clones differentiate into neutrophils after estradiol induction.

2.1.6 Plasmids

Expression plasmids for pcDNA3.1-C/EBPα-HA (138), pcDNA3.1-E6AP (139) and pCAG-HA-E6AP-C843A (140), pGEX4T-GST-E6AP (141) were kind gifts from G. J. Darlington, Nihar Jana, Ikuo Shoji and Zafar Nawaz respectively; while pCDNA6- C/EBPα-His, pMT123HA-Ubi and p(C/EBP)2TK-luc are previously described (142). pBabe Puro-p42 C/EBPα-ER was kind gift from Alan D. Friedman (143).

2.2 MTT Assay

MTT method of cell determination is useful in the measurement of cell growth in response to any compound or cytotoxic agent in cytotoxicity studies and in the derivation of cell growth curves (144). Yellow MTT (3-(4, 5-Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 500 and 600nm) by spectrophotometer. The absorption maximum is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the effectiveness of the agent in causing death of cells can be deduced through the production of a dose-response curve. Solutions of MTT solubilized in tissue culture media or balanced salt solutions, without phenol red, are yellowish in colour. Mitochondrial dehydrogenases of viable cells cleave the tetrazolium ring, yielding purple MTT formazan crystals which are insoluble in aqueous solutions. The crystals can be dissolved in DMSO. The resulting purple solution is spectrophotometrically measured. An increase in cell number results in an increase in the amount of MTT formazan formed and an increase in absorbance.

$1x10^4$ cells per well in a 96 well plates were plated one day before start of experiment. Next day, the cells were induced with different concentrations of ormeloxifene (ORM) i.e. 1μM, 2.5μM, 5μM, 7.5μM, 10μM, 15μM, 20μM. DMSO was taken as a vehicle control since ORM was dissolved in it. HEK293 was used as a non-myeloid control cell. After 48 hrs of induction, MTT solution was added to the $1/10^{th}$ of the culture medium. Cells were incubated for 3h at 37°C in CO_2 incubator after addition of MTT solution (MTT solution was prepared in PBS to the working concentration of 5mg/ml). After 3h cells were centrifuged and culture medium was removed and the formazon crystals were dissolved in DMSO. The absorbance of the samples was measured with a specific enzyme-linked immunosorbent assay (ELISA) reader at 560nm. The percentage cytotoxicity was measured by the graph plotted with these readings from three independent experiments.

2.3 Apoptosis Assays

2.3.1 Annexin V-PI

Changes in the cellular morphology including cell shrinkage, nuclear condensation along with biochemical events leading to loss of mitochondrial membrane potential, loss of plasma membrane asymmetry and DNA cleavage between nucleosomes characterize apoptosis (145). The appearance of phosphatidylserine (PS) residues (normally hidden within the plasma membrane) on the surface of the cell is another parameter which can be used to detect and measure apoptosis (146). The presence of PS on the cell surface creates one of the specific signals for recognition and removal of apoptotic cells by macrophages. These PS changes can be detected with the anticoagulant, Annexin V, which has a high affinity for binding to PS (147). As the apoptosis progresses, cell membrane integrity is lost. Propidium iodide is an impermeable dye for live cells; however, in case of dead cells integrity of cell membrane is lost which allows PI to enter inside cells and nucleus where it can intercalate in between DNA bases. The amount of intercalated dye is directly proportional to no. of dead cells. Therefore, using DNA specific viability dyes like Propidium Iodide (PI), it is possible to distinguish between early apoptotic, late apoptotic, and dead cells.

K562, HL60 and U937 cells were induced with different ORM concentrations for the indicated time points. Annexin V-PI staining Kit (Sigma) as per manufacturer protocol was used for flow cytometry analysis of apoptotic cells. Briefly, $1x10^6$ cells were washed with PBS. 5µl Annexin-V and 10µl PI solution was added in 1X binding buffer to prepare the buffer solution as per manufacturer's protocol for each tube. Cells were incubated in the AnnexinV-PI solution for 15min in dark at room temperature (RT) followed by addition of 400µl of binding buffer and subsequent analysis in Flow Cytometer (FACS Calibur, Becton Dickinson, USA).

2.3.2 TUNEL Assay

TdT-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) apoptosis detection system (Invitrogen) was used for the detection of apoptosis (148). During apoptosis, there is activation of nucleases that eventually degrade nuclear DNA into fragments of approximately 200 base pairs in length (145). DNA fragmentation exposes a large number of 3'-hydroxyl ends. These hydroxyl groups can then serve as starting points for terminal

deoxynucleotidyl transferase (TdT), which adds deoxyribonucleotides in a template-independent fashion. Addition of the deoxythymidine analog 5-bromo-2′-deoxyuridine 5′-triphosphate (BrdUTP) to the TdT reaction serves to label the break sites. Once incorporated into the DNA, BrdU can be detected by an anti-BrdU antibody using standard immunohistochemical techniques. This method of labeling DNA breaks is referred to as Terminal Deoxynucleotide **T**ransferase d**U**TP **N**ick **E**nd **L**abeling, or TUNEL.

Treated cells ($1\text{-}2x10^6$) were washed with 1X PBS followed by fixing with 4% formaldehyde on ice for 15min. Further, cells were washed with 1XPBS and incubated in 50µl DNA labeling solution for 1h at 37°C. DNA labeling solution contains TdT enzyme, Brd UTP and reaction buffer as provided in kit. After DNA labeling, cells were washed twice with rinse buffer and incubated with 100µl dye labeled anti-BrdU antibody for 30min at RT in dark. Antibody staining solution is prepared by mixing 5.0µL of the Alexa FluorR 488 dye labeled anti-BrdU antibody with 95µL of rinse buffer. 0.5ml of iodide/RNase staining buffer was added to each sample and incubated in dark for 30min at RT. Subsequently TUNEL positive cells were analyzed in FACS flow cytometer. In addition, for the microscopic analysis, after incubation with antibody cells were cytocentrifuged and were visualized under fluorescent microscope.

2.3.3 JC-1 mitochondrial membrane potential assay

Mitochondria plays an important role during apoptosis (149). Mitochondrial membrane potential ($\Delta\psi$) is generated by mitochondrial electron transport chain which drives a proton flow from matrix through inner mitochondrial membrane to cytoplasm, thus creating an electrochemical gradient. This gradient in turn is responsible for the formation of ATP molecules by F_0-F_1 ATP synthase. For this reason $\Delta\psi$ is an important parameter for mitochondrial functionality and an indirect evidence of energy status of the cell. Changes in the mitochondrial membrane potential are measured by the flow cytometry using JC-1 dye (5, 5, 6, 6-tetrachloro 1, 1, tetraethyl benzimidazolocarbo-cyanine iodide). It is a cationic dye which accumulates in the mitochondria as J aggregate. Cells were treated as per indicated conditions and 30min prior to cytometric analysis, JC-1 was added to the cells ($1x10^6$cells/ml) to a final concentration of 10µg/ ml and incubated at 37°C in 5% humified CO_2 incubator for 15min. 10,000 cells were examined for each samples FL-1 (530 nm) *versus* FL-2 (585 nm) dot plot on a Becton Dickinson Flow

Cytometer. JC-1 has dual emission depending on the state of the mitochondrial membrane potential.

2.3.4 Caspase glo Assay

The Caspase-Glo® 3/7 and 9 is homogeneous luminescent assay that measures caspases activity. This member of the cysteine aspartic acid-specific protease (caspase) family plays a key initiator role in the intrinsic apoptotic pathway of mammalian cells (150). Addition of this reagent results in the cell lysis followed by caspase cleavage of the substrate and generation of luminescent signal. The signal generated is proportional to the amount of caspase activity. Briefly, 10,000 cells per well were seeded in 96 well plates. After treatment with ormeloxifene, 1:1 ratio of caspase glow reagent (caspase 3/7 and caspase 9, Promega) to sample volume was added as per manufacturer's protocol and incubated for 1h at RT. Reading of luminescence was taken in luminometer. The graph was plotted for the relative luminescence produced in the treated sample as compared to the control.

2.4 Cell Cycle Analysis

2.4.1 DNA content analysis by FACS flow cytometry

Cell cycle analysis was done using propidium iodide (PI). Propidium iodide (PI) binds to DNA by intercalating between the bases with little or no sequence preference and with a stoichiometry of one dye per 4.5 base pairs of DNA. PI also binds to RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining. When bound to nucleic acids, the absorption maximum for PI is 535nm and the fluorescence emission maximum is 617nm. PI fluorescence is detected in the FL2 channel of flow cytometers.

For cell cycle analysis, K562 cells were treated with 7.5μM ORM for indicated time points. After treatment cells were washed once with PBS and then resuspended in 1ml PBS. Cell suspension was fixed with absolute ethanol to the final concentration of approximately 70%. Cells were incubated on ice for 15min and then washed once with PBS and resuspended in 500μl PI solution for 30min at RT in dark. After washing, cells were analyzed in Flow Cytometer (FACS Calibur, Becton Dickinson, USA) for the distribution of cells in different phases of cell cycle.

2.5 PCR Analysis

2.5.1 RNA isolation

Trizol reagent was used for the RNA isolation. Briefly, cells were centrifuged at 1200rpm for 5min at RT. Cell pellet was dissolved in 100µl PBS and then 1ml of trizol was added to it, mixed by vigorous shaking for 1min followed by incubation at RT for 5min. Further, 200µl of chloroform was added to each tube, mixed vigorously for 15sec and incubated at RT for 3min. After incubation, tubes were centrifuged at 16000rpm for 15min at 4°C. A colourless phase was obtained which was collected in a new tube, 1ml of isopropanol was added to it and incubated for 10min at RT. Tubes were centrifuged at 16,000rpm for 10min at 4°C. Pellet obtained was washed once with 1ml of 75% ethanol and centrifuged at 16,000rpm for 10min at 4°C. Supernatant was completely removed and pellet was air dried for 5min at RT. Pellet was redissolved in 20-25µl of nuclease free water and UV absorbance was taken at 260/280nm (151) to assess the purity of mRNA.

2.5.2 cDNA synthesis and Reverse Transcriptase PCR

cDNA synthesis was carried out from total RNA using cDNA synthesis kit (Applied Biosystems). PCR amplifications were carried out using *Taq* DNA polymerase for routine applications (Sigma). All the PCR reactions were carried out by standard procedures (152) using cDNA as template in a C1000^{TM} Thermal Cycler (BioRad).

2.5.3 Quantitative Real time PCR

cDNA was used for the real time quantitative PCR analysis on Roche Light Cycler 480 using SYBR green master mix from Applied Biosystems. Statistical analysis was performed using ΔΔ CT method.

Primer Sequences used in the Quantitative and Qualitative PCR analysis are given in the table 2.

Table 2: Primer Sequences

Primer		Sequences
c-myc	Left Primer	5'-TTCGGGTAGAAAACCAG-3'
	Right Primer	5'-CAGCAGCTCGAATTTCTTCC-3'
p21	Left Primer	5'-GACACCACTGGAGGGTGACT-3'
	Right Primer	5'-CAGGTCCACATGGTCTTCCT-3'
GAPDH	Left Primer	5'-CTTCAACACCCCAGCCAT-3'
	Right Primer	5'-TAATGTCACGCACGATTTCC-3'

2.6 Biochemical Assays for cell staining

2.6.1 Hoechst Staining

Hoechst is a blue fluorescent dye used to stain DNA. This dye binds to all the nucleic acid but preferably to AT rich regions. Hoechst staining is done to stain the nucleus. $5x10^4$ cells/2ml per well were plated a day before ORM treatment. Post ORM treatment, cells were washed and resuspended in 500µl PBS. Subsequently cells were cytospun on slides at 800rpm for 5min. Cells were stained with Hoechst stain (H6024, Sigma) (2µg/ml), washed with PBS to remove excess stain, air dried and analyzed under fluorescent microscope.

2.6.2 Giemsa staining

Giemsa stain is specific for phosphate group of DNA and it binds to the region of DNA rich in adenine and thymine. Gimesa is a mixture of methylene blue, eosin and azure B. K562p42-C/EBPα-ER cells were induced with 5µM estradiol for 5days. After induction, cells were cytospun on slides and air dried. Next day, cells were stained using May-Grunwald and Giemsa solution by placing in Coplin jar filled with May-Grunwald solution for 5min followed by washing with 1XPBS for 2min. Meanwhile, Giemsa solution was diluted 1:20 ratio in PBS and slides were stained in this diluted solution for 15-20 min. Cells were then washed in running tap water to remove the excess stain, air dried and subjected to microscopic examinations under light microscope and were photographed.

2.6.3 Immunofluorescence microscopy

HEK293T cells were plated in chamber slide one day before transfection. Next day cells were transfected with C/EBPα and E6AP expression plasmids. 24h post transfection, cells were washed with PBS, fixed in 4% paraformaldehyde for 10min, permeabilized with 0.5% Triton X-100 in PBS for 5min, washed with PBS, and then blocked with 1% BSA in PBS for 1h. Cells were then incubated with primary antibodies C/EBPα and E6AP (1:200) overnight at 4°C. Next day, cells were washed thrice with PBS, incubated with Alexa Flour 594 and 488 secondary antibodies (1:250 dilutions) for 1h; Again washed thrice with PBS followed by 4', 6-diamidino-2-phenylindole (DAPI) staining (Sigma). Cells were then mounted with vectasheild (vector) and visualized under confocal microscope (Leica) for analysis.

2.7 Western blot analysis for detection of protein expression

2.7.1 Protein lysate preparation and estimation

Protein lysates were prepared in the RIPA lysis buffer which contained 1% (w/w) NP40, 0.5% (w/v) Sodium Deoxycholate, 0.1% (w/v) SDS, 0.15M NaCl, 5mM EDTA and 1mM DTT. To this working solution, phosphatase inhibitors and protease inhibitors were added. For cell lysate preparation, cells were centrifuged at 1500rpm for 5min at 4°C and supernatant was removed. Cells were washed once with chilled PBS to remove the remaining medium and cell debris. Cells were again centrifuged and RIPA buffer was added to the cell pellets by repeated pipetting. Lysates were then placed on ice for 30min with repeated tapping after every 10min. Lysates were then centrifuged at 25,000rpm for 20min at 4°C. Supernatant was collected in a prechilled tube for further use. 5X Bradford assay reagent (BioRad) was used to quantify the concentration of proteins. Absorbance was taken at 595nm in spectrophotometer. A standard plot using BSA was prepared to calculate the protein concentration using absorbance reading.

2.7.2 SDS-PAGE and Western blotting

For western blotting, protein samples were prepared in 2X SDS sample loading buffer (125mM Tris pH 6.8, 4% SDS, 20% Glycerol, 10% 2-Mercapto-ethanol and pinch of bromophenol blue) and resolved on 10% SDS-polyacrylamide gel (142). Further, proteins were electroblotted on PVDF membrane (Millipore) in Tris-glycine buffer (48 mM Tris, 39 mM glycine, 0.04% SDS,

20% methanol). Blots were then blocked in 5% skimmed milk in PBS-T (PBS containing 0.5%v/v Tween-20) for 1h at RT followed by washing with PBS-T and incubation with required dilution of primary antibodies for overnight at 4°C. After three washes of 10min each with PBS-T, blots were incubated in HRP-conjugated secondary antibody for 1h at room temperature followed by three washing with PBS-T. Blots were developed using chemiluminescence substrate (ECL,Millipore) in LAS 4000. Primary antibodies used were Bax (Alexis), Cytochrome-c (Alexis), Caspase-3 (sc-7148), c-Abl (sc-23), CDK2 (sc-163), Bcl-2 (sc-492), p21 (sc-397), β tubulin (sc-9104), β actin (sc-47778), C/EBPα (sc-9314), ubiquitin (sc-8017), GST (sc-459), GAPDH (sc-13179) from Santacruz Biotechnology; PARP (9542), ERK1/2 (4370), c-myc (9402) from Cell signaling Technology and E6AP (E8655) from Sigma.

2.8 Protein-protein interaction assays

2.8.1 GST fusion protein purification and GST-pull down

For GST-pull down assay, GST-E6AP fusion proteins were expressed in *E. coli* after 0.2mM IPTG induction for 12h. Induced bacterial pellet was lysed in NETN buffer (150mM NaCl, 20mM Tris pH 8.0, 1mM EDTA pH 8.0, 0.1% NP40 and protease inhibitors), sonicated and centrifuged at 15000rpm. Supernatant was collected followed by subsequent protein purification using immobilised Glutathione Sepharose beads (Amersham Biosciences, USA). Sepharose beads bound with GST proteins were washed twice with NETN buffer on a rotating shaker at 4°C for 10min each and were then lysed in 2X SDS sample loading buffer (125mM Tris pH 6.8, 4% SDS, 20% Glycerol, 10% 2-Mercapto-ethanol and a pinch of bromophenol blue). Subsequently, it was resolved on 12% SDS PAGE and visualised by commassie blue staining for resolved proteins.

Cell lysates of C/EBPα over expressed HEK293T cells were prepared in RIPA buffer. For pull down experiments GST purified proteins were incubated with whole cell lysates in NETN buffer for 3h at 4°C on a rotating shaker. After pull down, protein bound GST sepharose beads were washed three times with NETN buffer. SDS loading dye was added to the beads and was resolved on 10%SDS-Polyacrylamide gel, immunoblotted with GST and C/EBPα antibody to confirm the interaction.

2.8.2 Co-Immunoprecipitation (CoIP) Assay

Co-immunoprecipitation is an *in-vitro* biochemical assay used to detect *in-vivo* interaction of two proteins. For this, HEK293T cells were cotransfected with C/EBPα and E6AP. 24h post transfection, cells were treated with MG132 (25μM) for 3h. Protein lysates after preclearing with IgG were incubated with C/EBPα antibody and Protein Agarose G beads (10μl) (Millipore) for 3h at 4°C in coimmunoprecipitation buffer (1% TBS, 0.5% NP40, Protease inhibitors). After incubation, beads were washed with IP buffer 3 times and bound proteins were eluted in laemmli buffer. Immunoprecipitated proteins were heated at 56°C for 90min in 2X SDS loading buffer and then boiled at 95°C for 5min. Samples were separated on 10% SDS-PAGE and were subsequently immunoblotted with C/EBPα and E6AP antibody.

2.8.3 *In-vivo* Ubiquitination Assay

HEK293T cells were transfected with C/EBPα, E6AP and ubiquitin constructs. 24h post transfection cells were harvested and RIPA lysates were prepared. Subsequently, co-immunoprecipitation was performed with 2μg of C/EBPα antibody using protein G Agarose beads (Millipore). After preclearing, protein lyastes were incubated with antibody and beads for 3h in IP buffer. The co-immunoprecipitated proteins were then separated by 10% SDS-PAGE and probed with ubiquitin antibody. The same blot was stripped and reprobed with C/EBPα antibody to confirm the immunoprecipitation.

2.9 Luciferase Reporter Assay

$1x10^5$ HEK293T cells/well were plated one day before transfections in 24 well plates. Next day cells were transfected with p(C/EBP)TK-luc promoter and expression plasmids for C/EBPα, E6AP and E6AP mutant (E6AP-C843A). 24h post transfection cell extracts were assayed for luciferase activity, using luciferase assay reagent (Gold Biotechnology). GFP expression was measured by multiplate fluorimeter. Luciferase activity values were normalized with GFP values and fold activity was calculated over untreated control. Data are presented as means of triplicate values obtained from representative experiments.

2.10 Fluorescence Activated Cell Sorting (FACS) Analysis

For the FACS analysis of differentiation surface markers on the K562-p42C/EBPα-ER stable cells were assayed. Cells were transfected with siE6AP and 48h post transfection, cells were induced with 5uM β-estradiol. It was followed by washing the cells once with PBS and subsequent resuspension in PE-conjugated cd11b antibody and the isotype IgG as control. Cells were analyzed under FACS flow cytometer (Becton Dickinson) for the expression of cd11b a myeloid differentiation marker.

Reagents and Buffers:

PBS: 137mM NaCl, 2.7mM KCl, 4.3mM Na_2HPO_4, 1.47mM KH_2PO_4. Adjust to a final pH of 7.4

Coomassie blue staining solution: 0.2% Coomassie blue, 7.5% Acetic acid, 50% Ethanol

Destaining solution: 50% (v/v) Methanol in water with 10% (v/v) Acetic acid

Laemmli Buffer (SDS loading buffer 2X): 125mM Tris pH 6.8, 4% SDS, 20% Glycerol, 10% 2-Mercapto β-ethanol and a pinch of Bromophenol blue

MACS Buffer: 2%FBS and 2mM EDTA in 1X PBS

Nuclear Extract Buffer A: 20mM HEPES, 10mM NaCl, 3mM $MgCl_2$, 1%NP-40, 10% Glycerol, 0.2mM EDTA, 1mM DTT and protease inhibitors

Nuclear extract buffer C: 20mM HEPES, 400mM NaCl, 20% Glycerol, 0.2mM EDTA, 1mM DTT, and Protease inhibitors

RIPA Lysis buffer: 1%NP40, 0.5%Sodium deoxycholate, 0.1%SDS, 0.15M NaCl, 5mM EDTA and 50mM Tris pH8.0

Urea Lysis buffer: 7M Urea, 2M Thiourea, 1% DTE, 4% CHAPS and 2.5mM EDTA

Running buffer (1X, 1L): 12g Tris base, 47.5g Glycine and 16g SDS in dH_2O

Transfer buffer (1X, 1L): 3g Tris base, 14.1g Glycine, 200ml Methanol and dH_2O

Blocking buffer: 5.0% milk in PBST

1X TBS: 20mM Tris Base, 150mM NaCl, pH 7.6

Co immunoprecipitation Buffer: 1% TBS, 0.5% NP40, Protease inhibitors

NETN Buffer: 150mM NaCl, 20mM Tris pH 8.0, 1mM EDTA pH 8.0, 0.1% NP40 and protease inhibitors

Stripping Buffer: 15g glycine, 1g SDS, and 10ml Tween 20; Adjust pH to 2.2. Volume maintained up to 1L with distilled water

Chapter 3

Ormeloxifene, a non-steroidal selective estrogen receptor modulator (SERM) induces apoptosis in myeloid leukemia cells

3.1 Introduction

Apoptosis

The term apoptosis (a-po-toe-sis) was first used by Kerr, Wyllie, and Currie in 1972 to describe a morphologically distinct form of cell death (153). Understanding of mechanisms involved in the process of apoptosis in mammalian cells transpired from the investigation of programmed cell death that occurs during the development of the nematode *Caenorhabditis elegans* (154). In this organism 1090 somatic cells are generated in the formation of the adult worm, of which 131 cells undergo apoptosis or "programmed cell death". These 131cells die at particular points during the developmental process which is essentially invariant between worms demonstrating the remarkable accuracy and control in this system. Apoptosis has since been recognized and accepted as a distinctive and important mode of "programmed" cell death, which involves genetically determined elimination of the cells.

The process of programmed cell death or apoptosis is generally characterized by distinct morphological characteristics and energy-dependent biochemical mechanisms. Apoptosis is considered a vital component of various vital processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death. Inappropriate apoptosis (either too little or too much) is a factor in many human disease conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. The ability to modulate the life or death of a cell is recognized for its immense therapeutic potential. Therefore, research continues to focus on the elucidation and analysis of the cell cycle machinery and signaling pathways that control the cell cycle arrest and apoptosis. To that end, the field of apoptosis research has been moving forward at an alarmingly rapid rate. Although many of the key apoptotic proteins have been identified, the molecular mechanisms of action or inaction of these proteins remains to be elucidated.

Morphology of Apoptosis

Light and electron microscopy have identified various morphological changes that occur during apoptosis (155). During the early process of apoptosis, cell shrinkage and pyknosis are visible by light microscopy (153) . With cell shrinkage, the cells are smaller in size, the cytoplasm is dense

and the organelles are more tightly packed. Pyknosis, result of chromatin condensation is the most characteristic feature of apoptosis. Extensive plasma membrane blebbing occurs followed by karyorrhexis and separation of cell fragments into apoptotic bodies during a process called "budding" (156). Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without a nuclear fragment. The organelle integrity is still maintained being enclosed within an intact plasma membrane. These bodies are subsequently phagocytosed by macrophages, parenchymal cells, or neoplastic cells and degraded within phagolysosomes. Macrophages that engulf and digest apoptotic cells are called "tingible body macrophages" and are frequently found within the reactive germinal centers of lymphoid follicles or occasionally within the thymic cortex (157). The tingible bodies are the bits of nuclear debris from the apoptotic cells. There is essentially no inflammatory reaction associated with the process of apoptosis or with the removal of apoptotic cells because: (1) apoptotic cells do not release their cellular constituents into the surrounding interstitial tissue; (2) they are quickly phagocytosed by surrounding cells thus likely preventing secondary necrosis; and, (3) the engulfing cells do not produce anti-inflammatory cytokines.

Biochemical Features

Apoptotic cells exhibit several biochemical modifications such as protein cleavage, protein cross-linking, DNA breakdown, and phagocytic recognition (158). Caspases are widely expressed in an inactive proenzyme form in most cells and once activated can often activate other procaspases, allowing initiation of a protease cascade. Some procaspases can also aggregate and autoactivate. This is proteolytic cascade in which one caspase can activate other caspases, amplifies the apoptotic signaling pathway and thus leads to rapid cell death. Caspases have proteolytic activity and are able to cleave proteins at aspartic acid residues, although different caspases have different specificities involving recognition of neighboring amino acids (150). Once caspases are activated, there seems to be an irreversible commitment towards cell death. Ten major caspases have been identified and broadly categorized so far. They are initiators (caspase-2,-8,-9,-10), effectors or executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (159). The other caspases that have been identified include caspase-11 which is reported to regulate apoptosis and cytokine maturation during septic shock; caspase-12

mediates endoplasmic-specific apoptosis and cytotoxicity by amyloid-β; caspase-13 and caspase-14 (160-163). Extensive protein cross-linking is another characteristic of apoptotic cells and is achieved through the expression and activation of tissue transglutaminase (164). DNA breakdown by Ca^{2+}-and Mg^{2+}-dependent endonucleases also occur resulting in DNA fragments of 180 to 200 base pairs (145). A characteristic "DNA ladder" is visualized by agarose gel electrophoresis with an ethidium bromide stain and ultraviolet illumination.

Another biochemical feature is the expression of cell surface markers that result in the early phagocytic recognition of apoptotic cells by adjacent cells, permitting quick phagocytosis with minimal compromise to the surrounding tissue. This is achieved by the movement of the normal inward-facing phosphatidylserine of the cell's lipid bilayer to the outer layers of the plasma membrane (146). Although externalization of phosphatidylserine is a well-known recognition ligand for phagocytes on the surface of the apoptotic cell, recent studies have shown that other proteins are also be exposed on the cell surface during apoptotic cell clearance. These include Annexin I and calreticulin. Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used for the detection of apoptosis (147, 165). Calreticulin is a protein that binds to an LDL receptor related protein on the engulfing cell and is suggested to cooperate with phosphatidylserine as a recognition signal (166).

Mechanisms of Apoptosis

The mechanisms of apoptosis are highly complex and sophisticated that involves an energy dependent cascade of molecular events. There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway. These two pathways are linked and the molecules in one pathway can influence the other (167). There is an additional pathway that involves T-cell mediated cytotoxicity and perforin-granzyme dependent killing of the cells. The perforin/granzyme pathway can induce apoptosis via either granzyme B or granzyme A. The extrinsic, intrinsic, and granzyme B pathways converge on the same terminal, or execution pathway.

The apoptotic pathway is initiated by the cleavage of caspase-3 and results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins,

formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells.

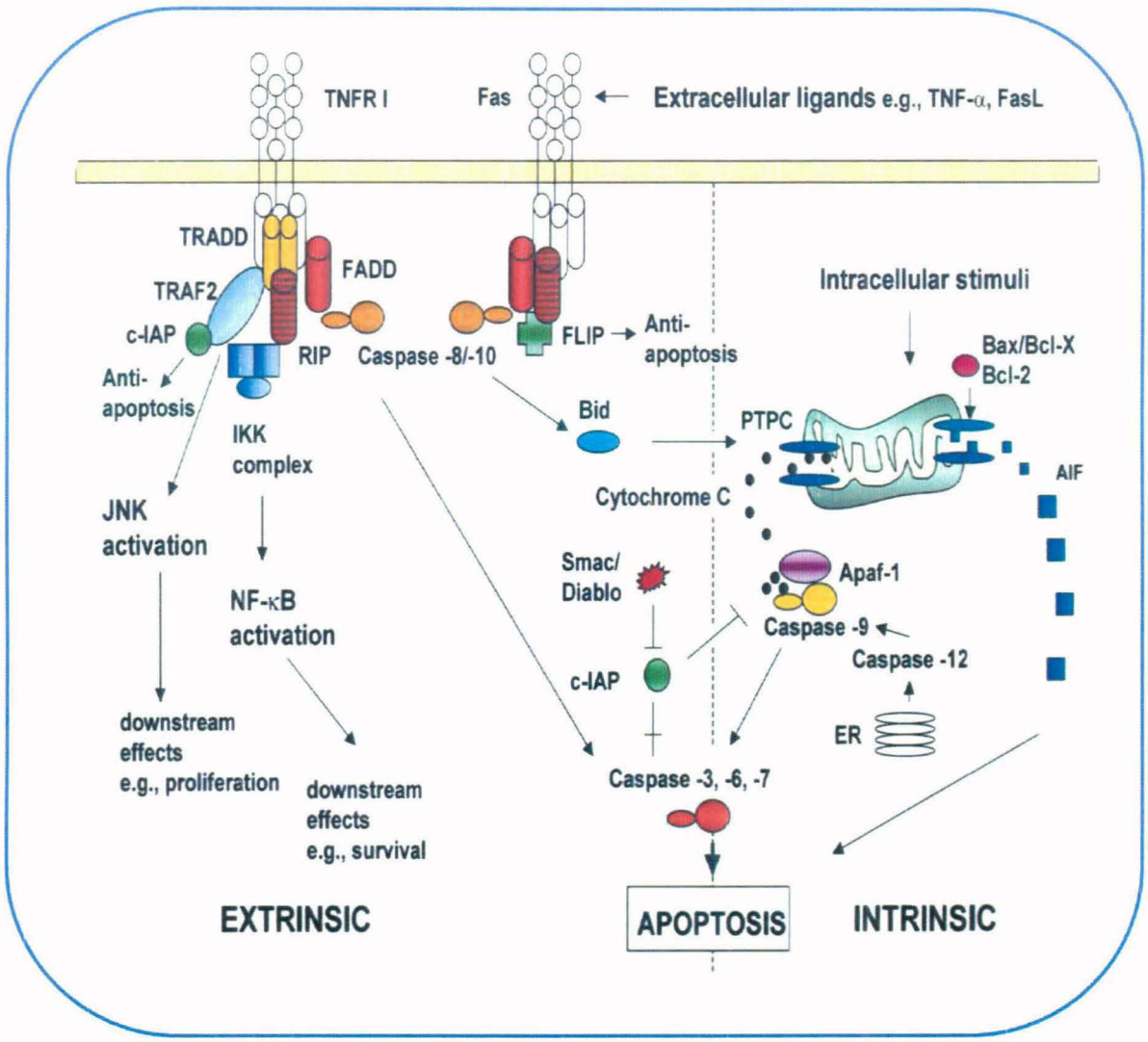

Figure 1: Major components of the core apoptotic cascade. The two major apoptotic pathways—the cell surface death receptor pathway (extrinsic) and the mitochondria-initiated (intrinsic) pathway are depicted. The extrinsic pathway emanates from extracellular stimuli, which are transduced through membrane-associated receptors of the TNFR superfamily. Intracellular stimuli induce apoptosis primarily through the mitochondria and, to a lesser extent, through the ER (adapted from C. J. Zeiss, Vet Pathol 40:5, 2003).

Extrinsic Pathway

The extrinsic signaling pathways that initiate apoptosis involve transmembrane receptor-mediated interactions. These involve death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily (168). Members of the TNF receptor family share similar cysteine-rich extracellular domains and have a cytoplasmic domain of about 80 amino acids called the "death domain" (169). This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathways. Ligands and corresponding death receptors include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (169-172). Upon ligand binding, cytoplasmic adapter proteins are recruited which exhibit corresponding death domains that bind with the receptors. The binding of Fas ligand to Fas receptor results in the binding of the adapter protein FADD and the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD and RIP (173-175). FADD then associates with procaspase-8 via dimerization of the death effector domain. At this point, a death-inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8 (176). Once caspase-8 is activated, the execution phase of apoptosis is triggered. Death receptor mediated apoptosis can be inhibited by a protein called c-FLIP which binds to FADD and caspase-8, rendering them ineffective (177). Another point of potential apoptosis regulation involves a protein called Toso, which has been shown to block Fas-induced apoptosis in T cells via inhibition of caspase-8 processing (178).

Intrinsic Pathway

Apoptosis can be initiated by intrinsic signals that are produced following cellular stress. Cellular stress may occur from exposure to radiation or chemicals or by viral infection. It might also be a consequence of growth factor deprivation or oxidative stress caused by free radicals (179). In general, intrinsic signals initiate apoptosis through the involvement of the mitochondria. The relative ratios of the various bcl-2 proteins can often determine how much cellular stress is necessary to induce apoptosis. The intrinsic signaling pathways that initiate apoptosis involve a diverse array of non-receptor-mediated stimuli that produce intracellular signals which act directly on targets within the cell and are mitochondrial-initiated events (180). The stimuli that initiate the intrinsic pathway produce intracellular signals that may act either in a positive or

negative fashion. Negative signals involve the absence of certain growth factors, hormones and cytokines that can lead to failure of suppression of death programs, thereby triggering apoptosis. Other stimuli that act in a positive fashion include radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals. All of these stimuli cause changes in the inner mitochondrial membrane that result in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release of two main groups of normally sequestered pro-apoptotic proteins from the intermembrane space into the cytosol (180). The first group consists of cytochrome *c*, Smac/DIABLO, and the serine protease HtrA2/Omi (149, 181, 182). These proteins activate the caspase dependent mitochondrial pathway. Cytochrome *c* binds and activates Apaf-1 as well as procaspase-9 forming an "apoptosome" (183). The clustering of procaspase-9 in this manner leads to caspase-9 activation. Smac/DIABLO and HtrA2/Omi are reported to promote apoptosis by inhibiting IAPs (inhibitors of apoptosis proteins) activity (184).

The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins (185). The tumor suppressor protein *p53* has a critical role in the regulation of Bcl-2 family of proteins (186). The Bcl-2 family of proteins governs mitochondrial membrane permeability and can either be pro-apoptotic or antiapoptotic. To date, a total of 25 genes have been identified in the Bcl-2 family. Some of the anti-apoptotic proteins include Bcl-2, Bcl-x, Bcl-XL, Bcl-XS, Bcl-w, BAG while Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk are proapoptotic. These proteins have special significance since they can determine if the cell commits to apoptosis or aborts the process. It is thought that the main mechanism of action of the Bcl-2 family of proteins is the regulation of cytochrome *c* release from the mitochondria via alteration of mitochondrial membrane permeability.

Perforin/granzyme Pathway

T-cell mediated cytotoxicity is a variant of type IV hypersensitivity where sensitized CD8+ cells kill antigen-bearing cells. These cytotoxic T lymphocytes (CTLs) are able to kill target cells via the extrinsic pathway; notably FasL/FasR interaction is the predominant method of CTL-induced apoptosis (187). However, they are also able to exert their cytotoxic effects on tumor cells and virus-infected cells via a novel pathway that involves secretion of the transmembrane pore-forming molecule perforin with a subsequent exophytic release of cytoplasmic granules through

the pore and into the target cell (188). The serine proteases granzyme A and granzyme B are the most important component within the granules (189).

Granzyme B cleaves proteins at aspartate residues and therefore activates procaspase-10 and can cleave factors like ICAD (Inhibitor of Caspase Activated DNAse) (190). Granzyme B can utilize the mitochondrial pathway for amplification of the death signal by specific cleavage of Bid and induction of cytochrome *c* release (191). However, granzyme B can also directly activate caspase-3. In this way, the upstream signaling pathways are bypassed and there is direct induction of the execution phase of apoptosis. Both the mitochondrial pathway and direct activation of caspase-3 are critical for granzyme B-induced killing (192).

Granzyme A is also important in cytotoxic T cell induced apoptosis and activates caspase independent pathways. Granzyme A activates DNA nicking via DNAse NM23-H1, a tumor suppressor gene product (193). This DNAse has an important role in immune surveillance to prevent cancer through the induction of tumor cell apoptosis. The nucleosome assembly protein SET normally inhibits the NM23-H1 gene. Granzyme A protease cleaves the SET complex thus releasing inhibition of NM23-H1 resulting in apoptotic DNA degradation. In addition to inhibiting NM23-H1, the SET complex has important functions in chromatin structure and DNA repair.

Execution Pathway

The extrinsic and intrinsic pathways both end at the point of the execution phase, considered the final pathway of apoptosis. Execution caspases activate cytoplasmic endonuclease which degrades nuclear material and proteases that degrade the nuclear and cytoskeletal proteins. Caspase-3, caspase-6, and caspase-7 function as effector or "executioner" caspases, cleaving various substrates including cytokeratins, PARP, the plasma membrane cytoskeletal protein alpha fodrin, the nuclear protein NuMA and others, that ultimately cause the morphological and biochemical changes seen in apoptotic cells (194).

Caspase-3 is considered to be the most important of the executioner caspases and is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 specifically activates the endonuclease CAD (Caspase Activated DNase). In proliferating cells CAD is complexed with its inhibitor, ICAD. In apoptotic cells, activated caspase-3 cleaves

ICAD to release CAD (190) which then degrades chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3 also induces cytoskeletal reorganization and disintegration of the cell into apoptotic bodies. Gelsolin, an actin binding protein, has been identified as one of the key substrates of activated caspase-3. Caspase-3 cleaves gelsolin and the cleaved fragments of gelsolin, in turn, cleave actin filaments in a calcium independent manner. It results in disruption of the cytoskeleton, intracellular transport, cell division, and signal transduction (195). Phagocytic uptake of apoptotic cells is the last component of apoptosis. Phospholipid asymmetry and externalization of phosphatidylserine on the surface of apoptotic cells and their fragments is the hallmark of this phase. Although the mechanism of phosphatidylserine translocation to the outer leaflet of the cell during apoptosis is not well understood, it has been associated with loss of aminophospholipid translocase activity and nonspecific flip-flop of phospholipids of various classes (146).

Perturbations in regulation of apoptosis have been implicated in a number of diseases. Cancer is a disease that is often characterized by too little apoptosis. Cancer cells typically possess a number of mutations that have allowed them to ignore normal cellular signals regulating their enhanced growth and become more proliferative than normal. Under normal circumstances, damaged cells will undergo apoptosis, but in the case of cancer cells mutations may have occurred that prevent cells from undergoing apoptosis. In these cases there is no check on the cellular proliferation and consequently the disease can progress to the formation of tumors. In many cases these tumors can be difficult to kill since many cancer treatments rely on damaging the cells with radiation or chemicals and mutations in the apoptotic pathway often produce cells that are resistant to this type of attack. Understanding how apoptosis is regulated in cancer is therefore of major interest in the development of treatments for this disease. In fact, suppression of apoptosis during carcinogenesis is thought to play a central role in the development and progression of some cancers (196). There are a variety of molecular mechanisms that tumor cells use to suppress apoptosis. However, effective cytoprotective therapies for these diseases remain a major unmet medical need. To a significant extent, the limited success of cytoprotective drug development can be traced to the simplified view that cell death is either intrinsically regulated by apoptosis or that it is unregulated, caused by stress (197).

Myeloid leukemia is a hematologic malignancy characterized by block in differentiation and abnormal proliferation of clonal myeloid cells. Despite extensive clinical research with numerous combinations of cytotoxic agents the overall prognosis of myeloid leukemia patients remains poor, thus the hunt for better effective agents is ongoing (198). Ormeloxifene (3,4-trans-2,2-dimethyl-3-phenyl-4-p-(beta-pyrrolidinoethoxy) phenyl-7-methoxychroman) also known as centchroman, is a potent non-steroidal selective estrogen receptor modulator (SERM) used as oral contraceptive for birth control. Ormeloxifene (ORM) suppresses the receptors in the reproductive organs like the ovaries, uterus and breasts while it stimulates the estrogen receptors of other organs like bones (129, 135, 136).

In the present study we have explored anticancer activity of Ormeloxifene (ORM) and few other synthetic and natural compounds in myeloid leukemia cells. We show that ORM at an IC_{50} of 7.5μM induces apoptosis in HL60, U937 and K562 cells. However, this induction of apoptosis was more prominent in K562 cells, therefore, we further chose K562 as a model cell line to understand mechanism of apoptosis induction by ORM. We show that ORM induces apoptosis in these cells through activation of Extracellular Signal-Regulated Kinase (ERK) leading to cytochrome-c release and subsequent mitochondria mediated caspase-3 activation. Further, we show that prior treatment of K562 cells with ERK inhibitor PD98059 drastically inhibits ERK phosphorylation, cytochrome-c relase and reduces ORM induced apoptosis. Chronic myeloid leukemia is associated with Bcr-Abl fusion protein which is an oncoprotein necessary for malignant transformation. We show that ormeloxifene treatment in K562 cells inhibits both phosphorylation as well as expression of Bcr-Abl fusion protein which actually is a constitutively active tyrosine kinase and helps in the survival and proliferation of myeloid cells. Thus, in summary, our data shows that ormeloxifene induced apoptosis in K562 cells involves inhibition of Bcr-Abl phosphorylation, phosphorylation of ERK and mitochondria mediated caspase activation.

3.2 Results

In house compounds of CSIR-CDRI were screened for the cytotoxicity in the myeloid leukemia cell lines K562, HL60 and U937. MTT assay was performed for the calculation of IC_{50} in these cells after 48h of treatment with the compounds. Human embryonic kidney fibroblast HEK293 cells as a control were treated with compounds in a dose dependent manner. The compounds selected for screening were those already known for their efficacy in other diseases and disorders.

CDRI-99/373 is an antiosteoporosis synthetic compound developed by CSIR-CDRI. It is antiresorbing agent. It decreases the expression of calcitonin receptors, the functional marker of activated osteoclasts, and also disrupts F-actin ring *in vitro*.

Ormeloxifene (ORM) (3,4-trans-2,2-dimethyl-3-phenyl-4-p-(beta-pyrrolidinoethoxy) phenyl-7-methoxychroman) also known as centchroman, is a potent non-steroidal selective estrogen receptor modulator (SERM) used as oral contraceptive for birth control. It is marketed under trade name Saheli by Hindustan Latex Limited. It is also known to have potential anticancer activity in breast cancer (133, 199).

Guggulsterone is a standardized fraction of the plant *Commiphora mukul*. It possesses hypolipidemic efficacy and is a cholesterol lowering drug. It is manufactured and marketed by Cipla Limited under trade name Gugulip. It is also known to induce apoptosis and differentiation in leukemic cell lines HL60 and U937 (200).

K058 is a natural compound synthesized by CSIR-CDRI. It is currently studied for its role in the treatment of metabolic disorder like diabetes.

Compounds	K562	HL60	U937	HEK293
K058	>30μM	>30μM	>30μM	>30μM
99/373	>30μM	>30μM	>30μM	>30μM
ORM	≥7μM	≥8μM	≥8μM	>25μM
Guggulsterone	>15μM	>10μM	>10μM	>30μM

Table 1: IC_{50} values of in house compounds of CSIR-CDRI. Data represents the mean ± SEM of three independent experiments.

IC_{50} values were calculated and compound having IC_{50}> 30μM were not considered for further study due to their low cytotoxicity on the leukemic cell lines. Based on MTT assay, IC_{50} of ORM was calculated to be ~7.0μM in leukemic cells compared to >30μM for HEK293 a non leukemic human kidney fibroblast cell line (Table 1). Thus, ormeloxifene was used for further studies in leukemic cell lines.

3.2.1 ORM induces growth inhibition in myeloid leukemia cells

In order to determine growth inhibitory concentration (IC_{50}) of ORM, we performed MTT assay in various cell lines. Activity of ormeloxifene in different myeloid leukemia cell lines K562, HL60 and U937 was assessed.

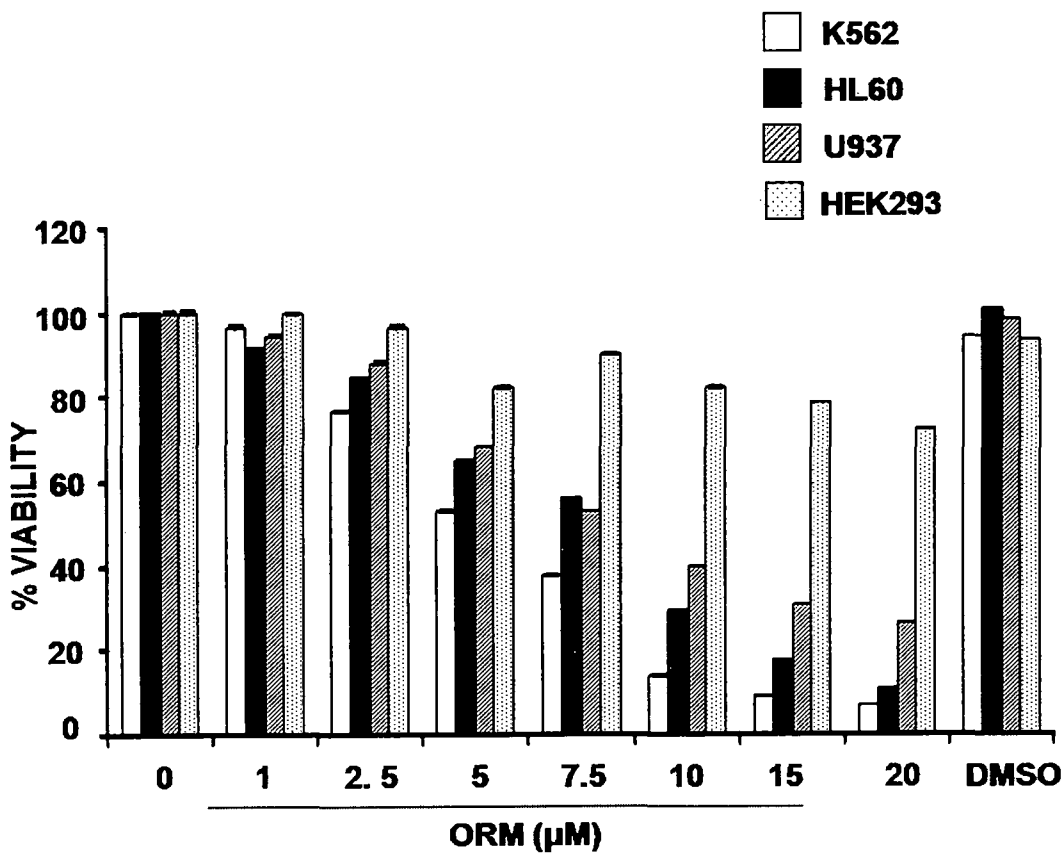

Figure 2: Cell viability as determined by MTT colorimetric assay, shows % viable cells after myeloid leukemia cells were treated with increasing doses of ORM. Assay was performed on 3 replicates for each treatment and repeated twice. 50% growth inhibition was observed at around 7.5μM. Data represents mean ± SEM.

K562, HL60 and U937 and Human embryonic kidney fibroblast HEK293 cells as control were treated with ormeloxifene for 48h in a dose dependent manner. Percentage viability of cells was measured with MTT assay. Based on MTT assay, IC_{50} of ORM was calculated to be ~7.0μM in leukemic cells compared to >30μM for HEK293, a non leukemic human kidney fibroblast cell line (Figure 2). Thus, showing selectivity index of more than 4 for leukemic cells compared to HEK293.

In previously published reports, ORM has been shown to induce apoptosis in breast cancer cells (199) suggesting it to have anticancer property. We therefore hypothesized that ORM might have anti cancer property in the myeloid leukemia cells as well and thus performed a series of experiments to assess anti cancer activities of ormeloxifene in these cells.

3.2.2 ORM induces apoptosis in myeloid leukemia cells

3.2.2.1 ORM induces apoptosis in a dose dependent manner

After calculating the IC_{50} of ormeloxifene in myeloid leukemia cells we further sought to assess if this cytotoxicity is via induction of apoptosis in these cells. To assess this, we performed Annexin V–PI assay which detects both early and late apoptosis. Notably, Annexin V is a recombinant phosphatidylserine-binding protein that interacts strongly and specifically with phosphatidylserine residues and can be used for the detection of apoptosis. K562, HL60 and U937 were treated with 1, 5 and 7.5μM of ORM for 48h and Post 48h treatment; cells were washed, stained with Annexin V-PI and analyzed in FACS Flow Cytometer. As shown in figure 3 the FACS flow analysis showed that ORM induces apoptosis in these leukemia cells in a dose dependent manner. The number of apoptotic cells (FITC-Annexin-V and PI double positive cells) in all the ORM treated cells drastically increased from 5-6% in control to 35-55% in cells treated with 5 and 7.5μM ORM. Thus, FACS flow analysis revealed that ORM induces apoptosis in myeloid cells in a dose dependent manner. Notably, maximum numbers of cells undergoing apoptosis were observed in K562 cells.

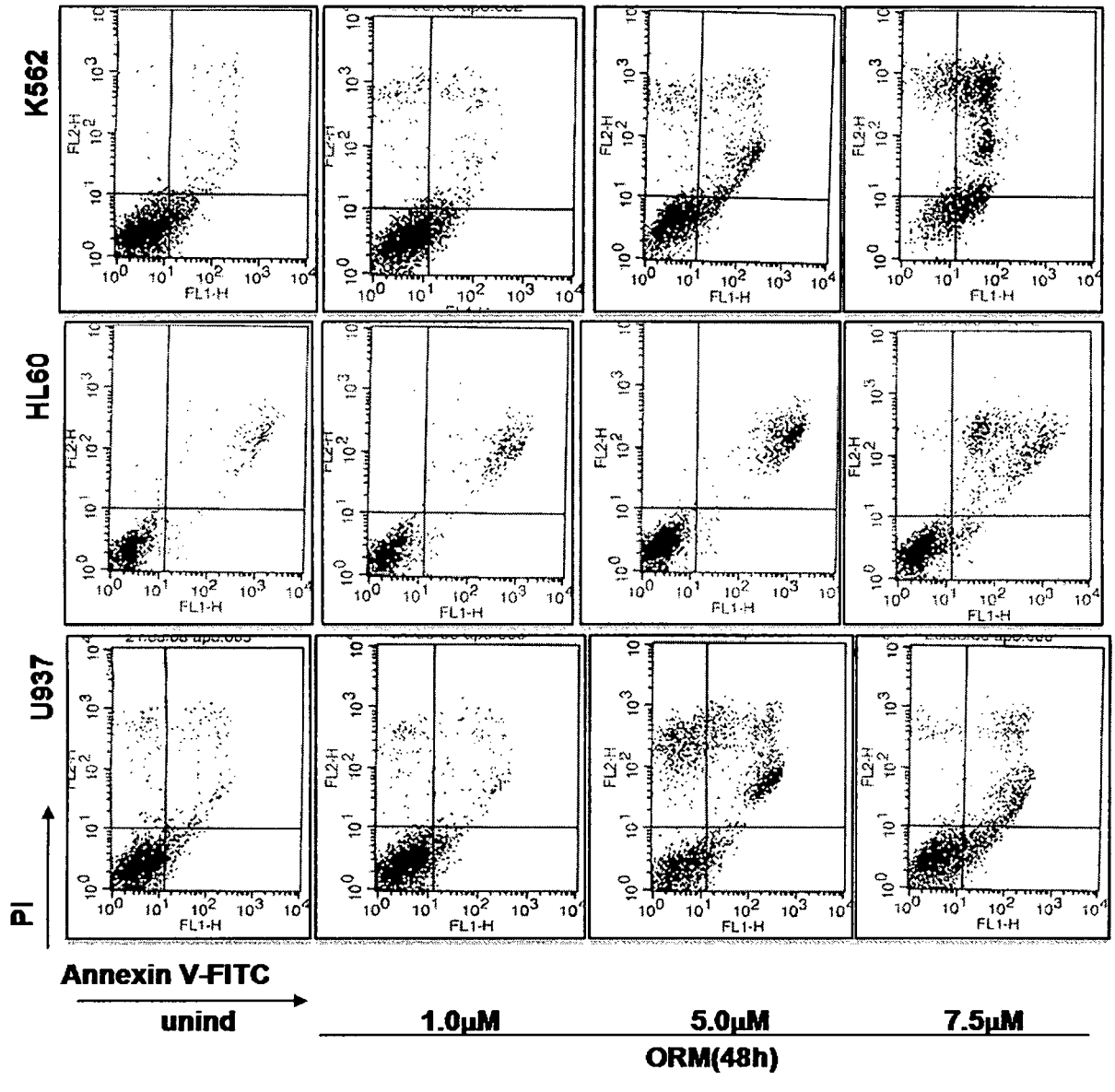

Figure 3: Myeloid leukemia cells were treated with increasing doses of ORM for 48h. After induction, Annexin V-PI double positive staining was performed to detect the apoptotic cells by flow cytometer. (unind-uninduced)

The percentage of apoptotic cell population was calculated and a graph was plotted comparing the percentage of apoptotic cells in each myeloid leukemia cell line (Fig 4).

This clearly indicates that the highest percentage of cells that show apoptosis after 48h of induction with 7.5μM of ORM belong to K562 cell line which is in accordance with observed cytotoxicity in Fig 1. This prompted us to use K562 as the representative cell line for all our further experiments.

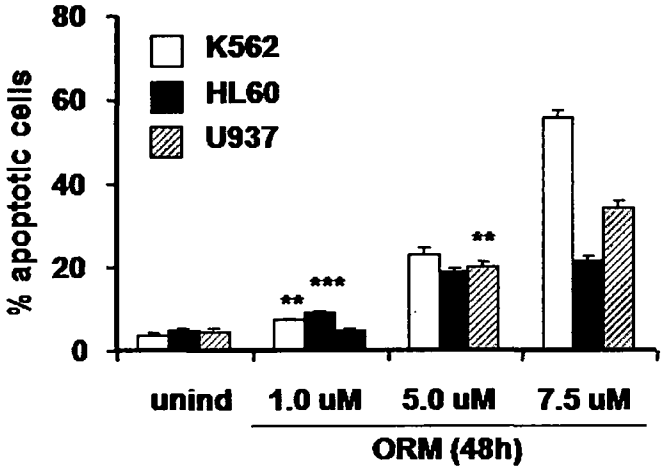

Figure 4: Percentage apoptotic cells by FACs flow cytometry were calculated and a graph was plotted for the comparative analysis of apoptosis in each myeloid leukemia cell line. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

3.2.2.2 DNA strand breaks are detected during ORM mediated apoptosis

Further, for validation of apoptosis in K562 cells, we performed TUNEL assay which detects the presence of a multitude of DNA strand breaks and is considered to be the gold standard for identification of apoptotic cells (201). K562 cells at the peak of their growth were treated with 5 and 7.5μM ORM, washed and stained with dUTP as per manufacturer's protocol. Further, dUTP labeled cells were sorted by FACS flow cytometer which showed that almost 19% of cells have nicked DNA as compared to 2.5% in untreated cells (Figure 5).

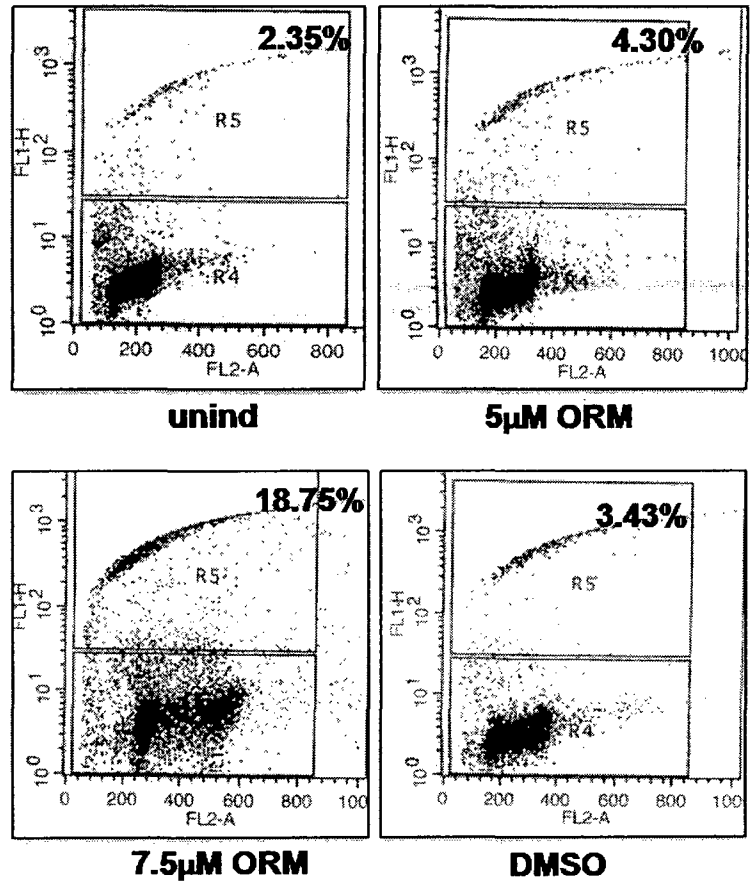

Figure 5: K562 cells were treated with 7.5μM ORM for indicated time points, TUNEL assay was performed using the APO-BrdU kit (Invitrogen), a terminal deoxynucleotidyl transferase (TdT) based end labeling assay for DNA strand breaks. TUNEL positive cells were analysed in Flow cytometry.

In addition, we also assessed the TUNNEL positive cells by fluorescence microscopy. Cells were cytospun, fixed and stained with dUTP for microscopic detection of labeled apoptotic cells. ORM induced apoptotic cell death as identified by Flow cytometer is consistent with the microscopic analysis confirming that ORM indeed induces apoptosis in leukemia cells.

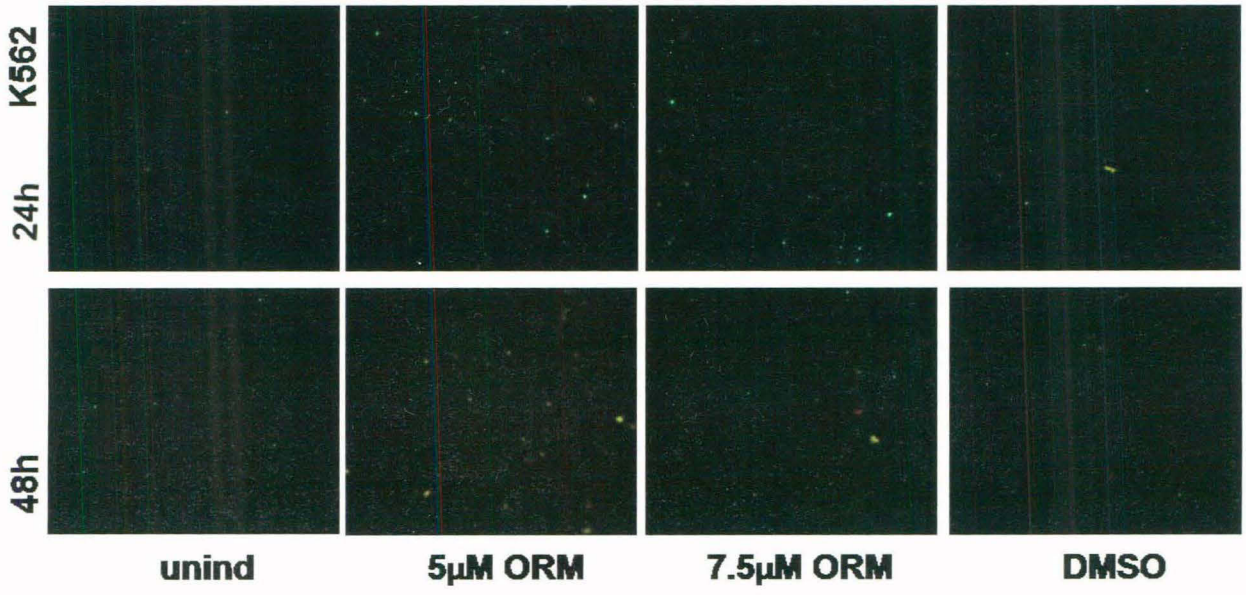

Figure 6: K562 cells were treated with 7.5μM ORM for indicated time points, TUNEL assay was performed using the APO-BrdU kit (Invitrogen), a terminal deoxynucleotidyl transferase based end labeling assay for DNA strand breaks. Cells were cytospun and Image analysis shows increased no. of apoptotic cells in 7.5μM ORM treated condition after both 24 and 48hrs.

3.2.2.3 ORM mediated apoptosis involves mitochondrial membrane potential loss

In a previous study, ormeloxifene has been reported to induce apoptosis through mitochondria and caspase-mediated pathway in breast cancer cells (133). Therefore, we asked if ORM induced apoptosis in these leukemia cells is also mitochondria mediated.

In order to investigate this, we measured the mitochondrial membrane potential. Note that membrane permeabilization leading to loss of membrane potential promotes apoptosis. For this, K562, HL60 and U937 cells were treated with ORM in a dose dependent manner for 0, 24 and 48hrs.

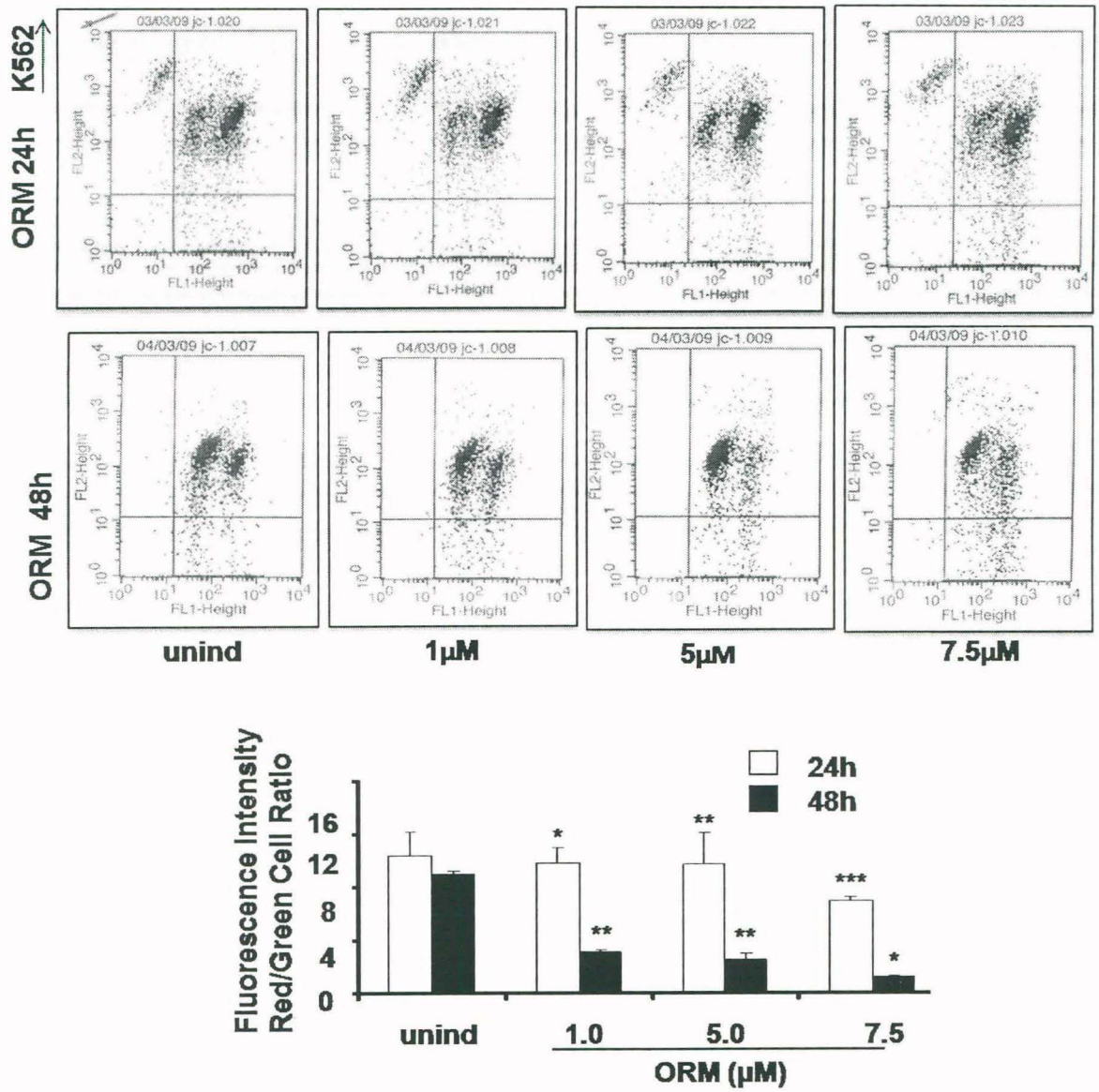

Figure 7: ORM induces mitochondrial membrane potential. After induction with ORM for indicated time points K562 cells were stained with JC-1 dye and membrane potential loss was analyzed in FACS Flow cytometer. Lower panel graphically shows the changes in mitochondrial potential. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

Cells were washed and stained with cationic dye JC-1. JC-1 dye forms aggregates in healthy cells with a high FL-2 fluorescence indicating a normal mitochondrial membrane potential. Upon onset of apoptosis, mitochondrial membrane potential collapses and results in reduction of FL-2 fluorescence with a concurrent gain in FL-1 fluorescence as the dye shifts from an aggregate to monomeric state (202).

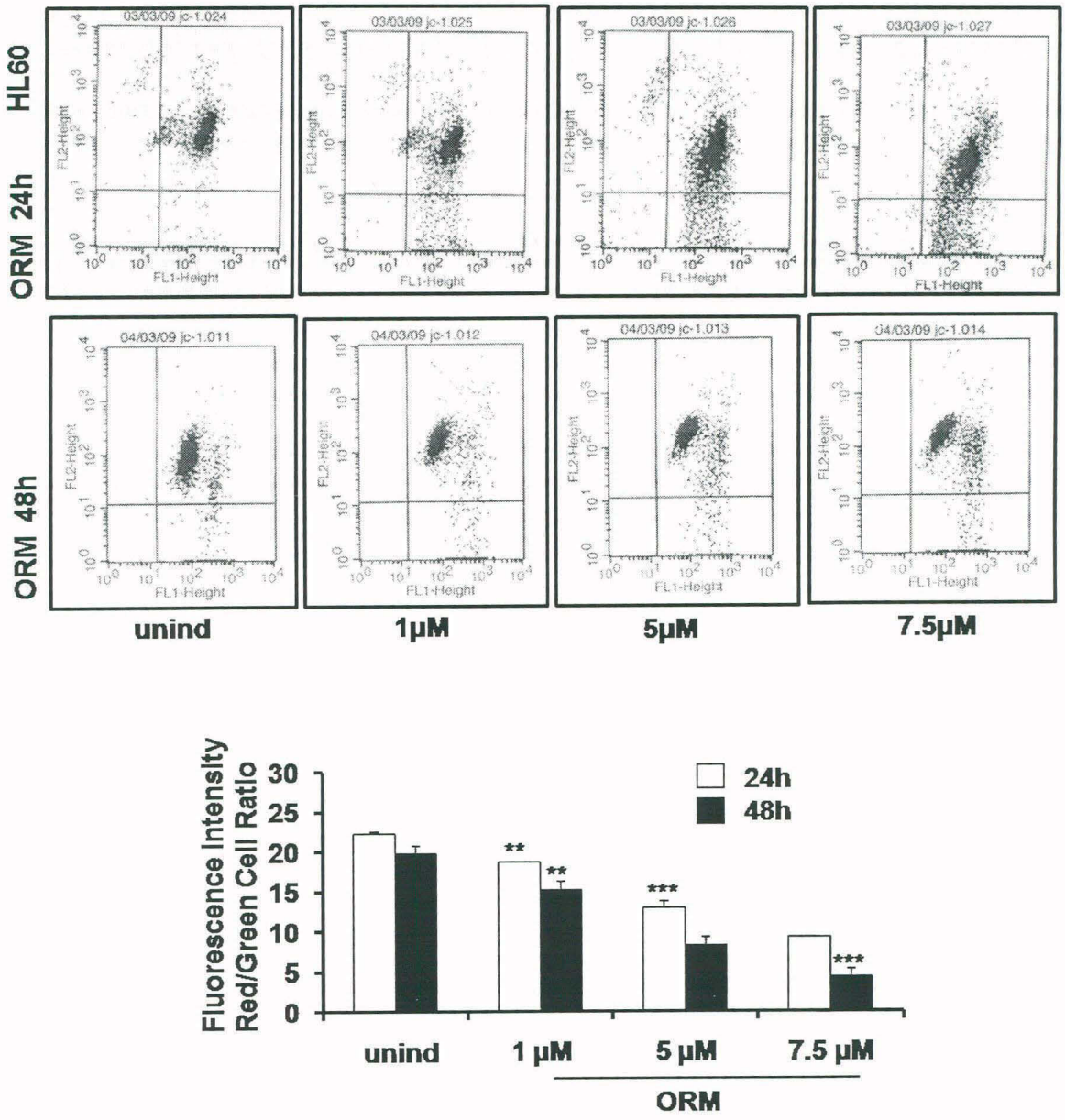

Figure 8: ORM induces mitochondrial membrane potential. After induction with ORM for indicated time points, HL60 cells were stained with JC-1 dye and membrane potential loss was analyzed in FACS Flow cytometer. Lower panel graphically shows the changes in mitochondrial potential. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

The, retention of the dye in the cell can be monitored through the increase in FL-1 fluorescence in FACS cytometer. As seen in figure 7, 8 and 9 for K562, HL60 and U973 cells respectively, the ratio (FL2/FL1) of cells depicted against ORM concentration in the representative graph after 24 and 48hrs shows dramatic change in the mitochondrial membrane potential.

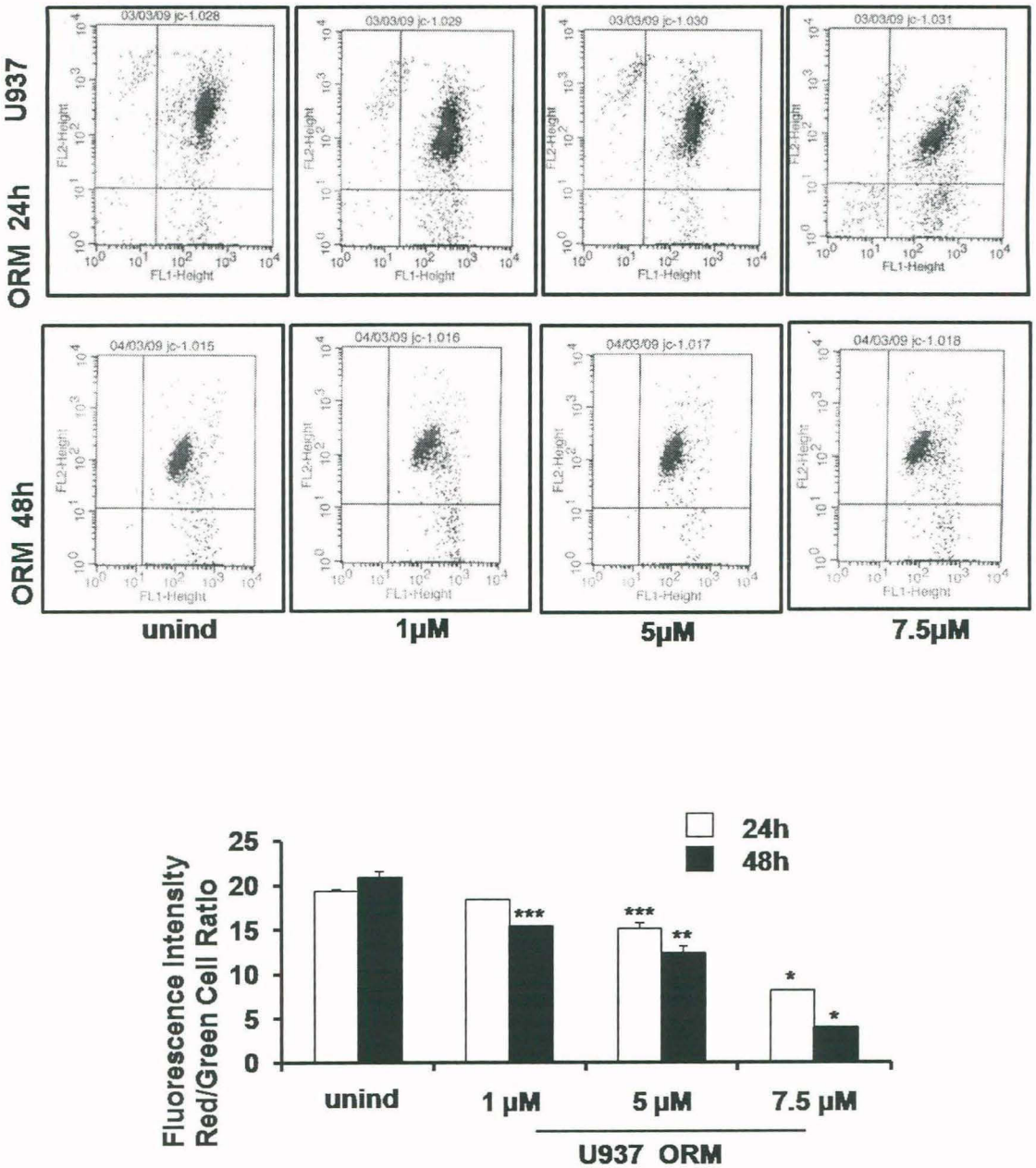

Figure 9: ORM induces mitochondrial membrane potential. After induction with ORM for indicated time points U937 cells were stained with JC-1 dye and membrane potential loss was analyzed in FACS Flow cytometer. Lower panel graphically shows the changes in mitochondrial potential. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

Thus, the mitochondrial membrane potential loss is visible in all the three myeloid cells lines K562, HL60 and U937 which suggests that ORM induced apoptosis in these cells is associated with the loss in integrity of the mitochondrial membrane.

3.2.2.4 ORM promotes mitochondria mediated caspase activation

ORM induced mitochondrial membrane potential loss in K562 cells prompted us to hypothesize that ORM induced apoptosis might involve mitochondria mediated apoptosis. Therefore to address the involvement of mitochondria mediated apoptosis we measured caspase 9 and 3 activity using caspase glo assay kit (Promega).

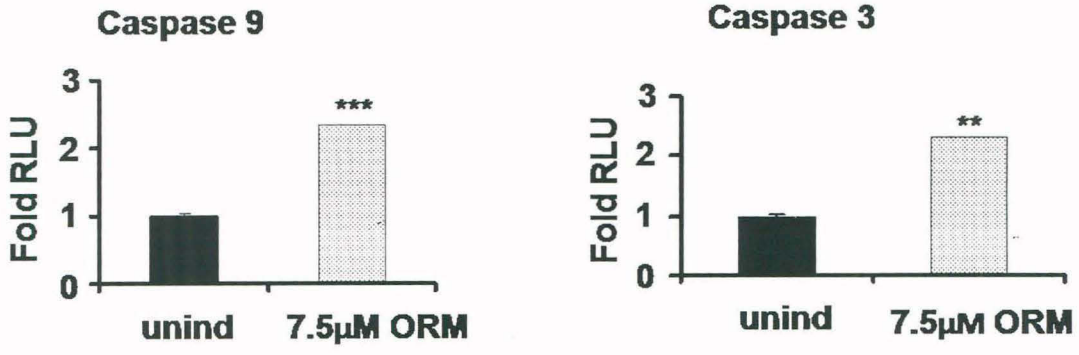

Figure 10: ORM mediated apoptosis involves caspase 9 and 3 activation as shown by relative luminescence units (RLU) measured using caspase glo assay. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

In this assay, the signal generated is proportional to the amount of caspase activity present. ORM treatment for indicated time points substantially enhanced caspase 9 and caspase 3 activity indicating ORM induced apoptosis in K562 to be apparently via mitochondria mediated caspase activation (figure 10).

To dissect and validate further, we assessed the changes in the expression of molecular signature proteins related to the mitochondria mediated apoptosis.

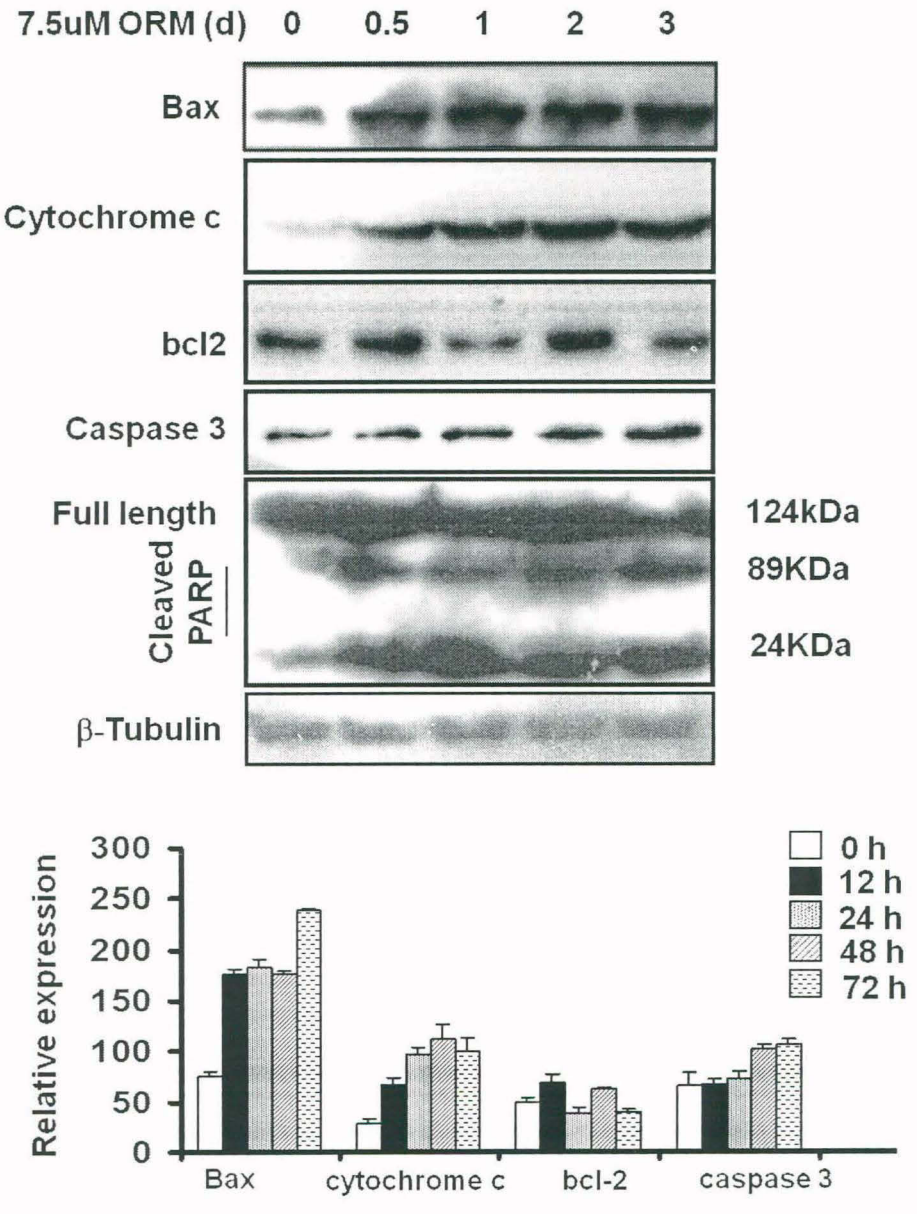

Figure 11: K562 cells were treated with 7.5μM ORM for indicated days; RIPA lysates were prepared and equal amounts of proteins were resolved in 10% SDS PAGE and immunoblotted with indicated proteins after stripping and reprobing the same membrane, lower panel graphically depicts relative expression of indicated proteins upon ORM treatment at different time points (densitometric analysis).

Mitochondria mediated apoptosis involves increased activation and expression of bax proteins leading to multiple pores in mitochondrial membrane which is followed by release of cytochrome-c from mitochondria intermembrane space into the cytosol (203).

In agreement with this we observed significant increase in the expression of bax and cytochrome-c levels (figure 11). In addition, we also observed differential expression of anti-apoptotic protein Bcl-2. Furthermore, since either intrinsic or extrinsic, both modes of apoptosis lead to PARP cleavage by activation of caspase-3 for execution of efficient apoptosis, we assessed the activation of caspase 3 and subsequent PARP cleavage by ORM. Immunoblot against caspase 3 and cleaved PARP clearly showed increase in caspase 3 and cleaved PARP products suggesting ORM indeed promotes efficient apoptosis.

Together with the loss of mitochondrial membrane potential, increase in bax expression, release of cytochrome-c followed by activation of caspase and PARP cleavage, these data demonstrate that ORM induced apoptosis is through mitochondrial mediated caspase activation.

3.2.2.5 Apoptosis related morphological changes are detected upon ORM treatment

Morphological changes pertaining to apoptosis upon ORM induction in K562 cells were assessed by hoechst staining. This dye stains minor groove of DNA with AT region selectivity. This dye binds to all the nucleic acid but AT rich dsDNA strands enhance fluorescence greater than GC rich strands of the DNA.

After induction with 7.5μM of ORM for 24h and 48h, cells were cytospun and stained with Hoechst dye. Microscopic analysis showed apoptosis related morphological changes such as formation of apoptotic bodies, membrane blebbing and chromatin condensation in ORM treated cells which further confirmed that ORM induces apoptosis in myeloid leukemia cells K562 (figure 12). White arrows indicate the vacuole and apoptotic bodies formed upon ORM treatment in K562 cells.

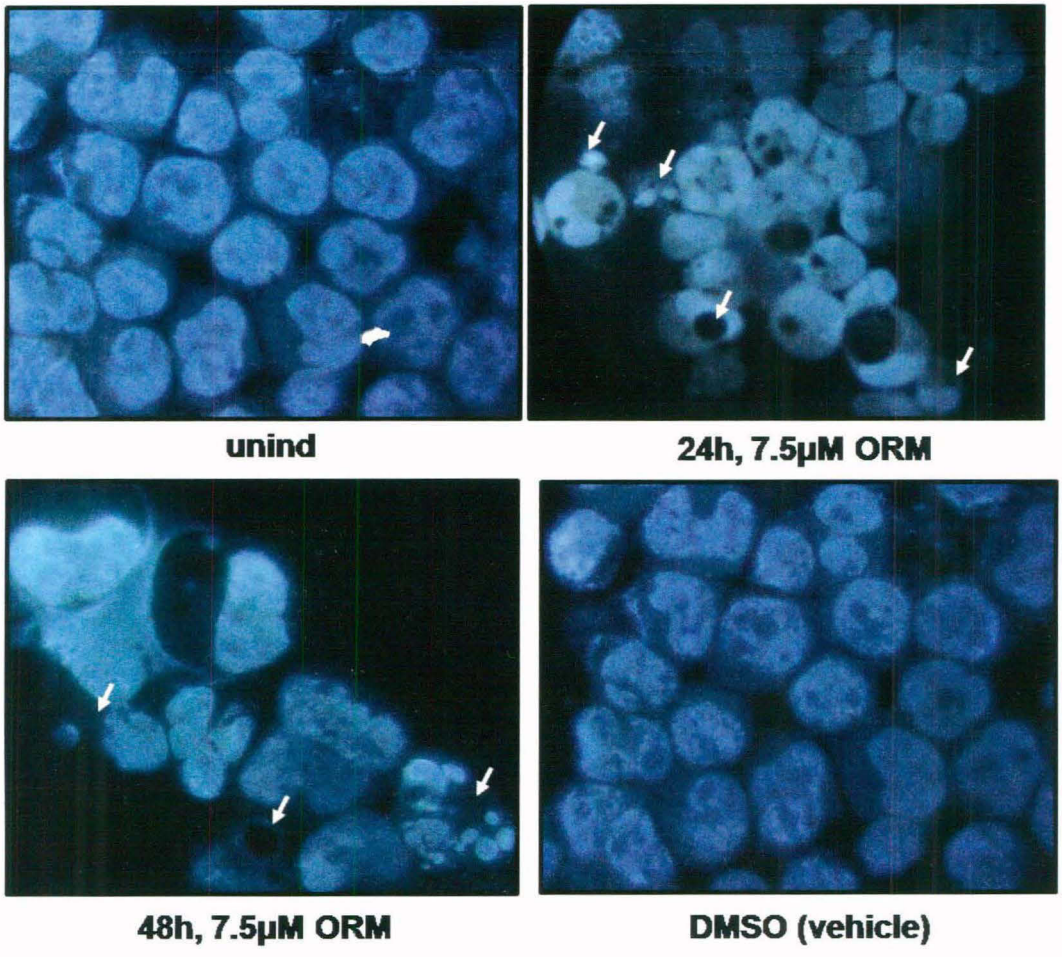

Figure 12: K562 cells were cytospun after treatment with 7.5μM ORM for 24 and 48h. Slides were stained with Hoechst stain and visualized under fluorescent microscope.

3.2.2.6 ORM induced apoptosis involves activation of Extracellular Signal Regulated Kinase (ERK)

In order to investigate further the signaling involved in ORM mediated apoptosis, we assessed the phosphorylation status of ERK; because in many cases where an anticancer drug/molecule induces apoptosis in K562 cells, ERK activation has apparently been involved (204).

To examine this, K562 cells were treated with 7.5μM ORM for indicated time points. Post ORM treatment, whole cell extracts were prepared, separated on 10% SDS PAGE and immunoblotted with pERK antibody which showed dramatic increase in ERK phosphorylation as shown in figure 13. Further, the same membrane was stripped and reprobed with cytochrome-c which showed a direct correlation between multitude of ERK activation and induction of

apoptosis (as measured by cyctochrome-c release). Histogram in the lower panel depicts densitometry analysis of the relative expression changes in the immunoblot.

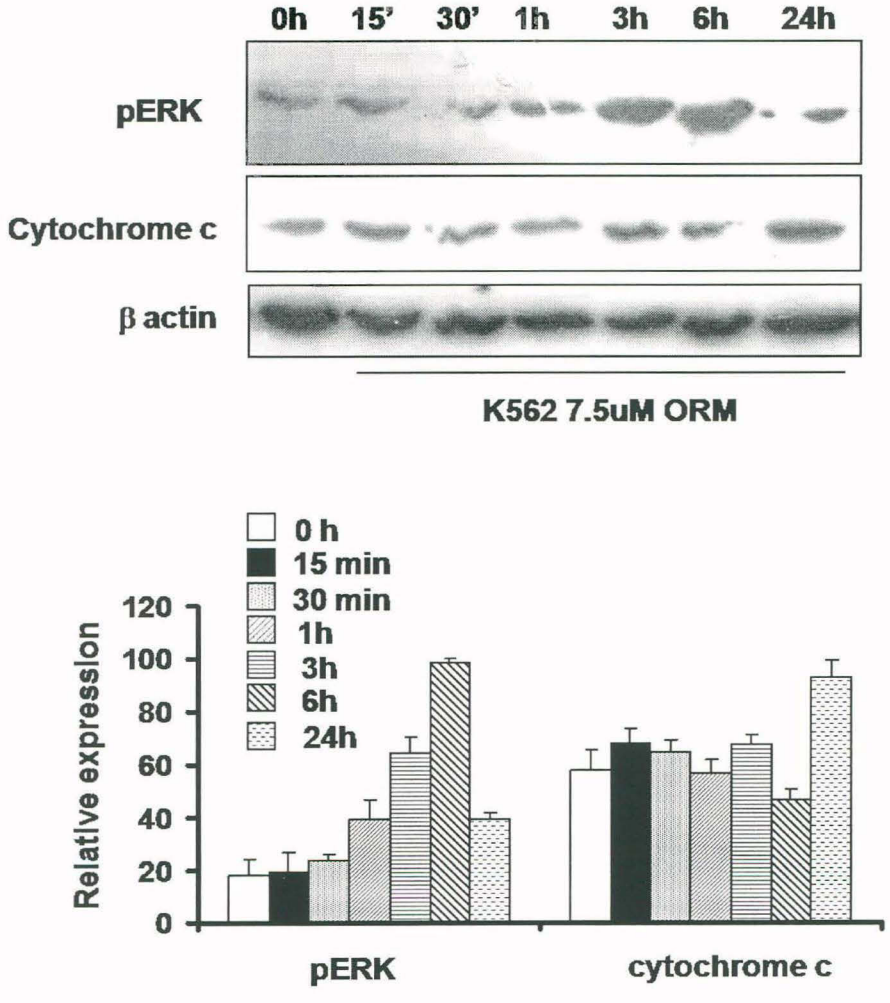

Figure 13: Whole cell extracts of ORM treated K562 cells after indicated time points were separated on 10% SDS PAGE and probed with phospho ERK, cytochrome-c and loading control (β-actin). Lower panel graphically depicts relative expression (densitometry) in immunoblots.

Next, K562 cells were treated with ERK inhibitor PD98059 for 1h prior to ORM treatment. This resulted in inhibition of ORM mediated ERK phosphorylation and dramatic block in cytochrome-c release with concomitant ERK inhibition by PD98059 suggesting that ORM mediated apoptosis involves ERK activation.

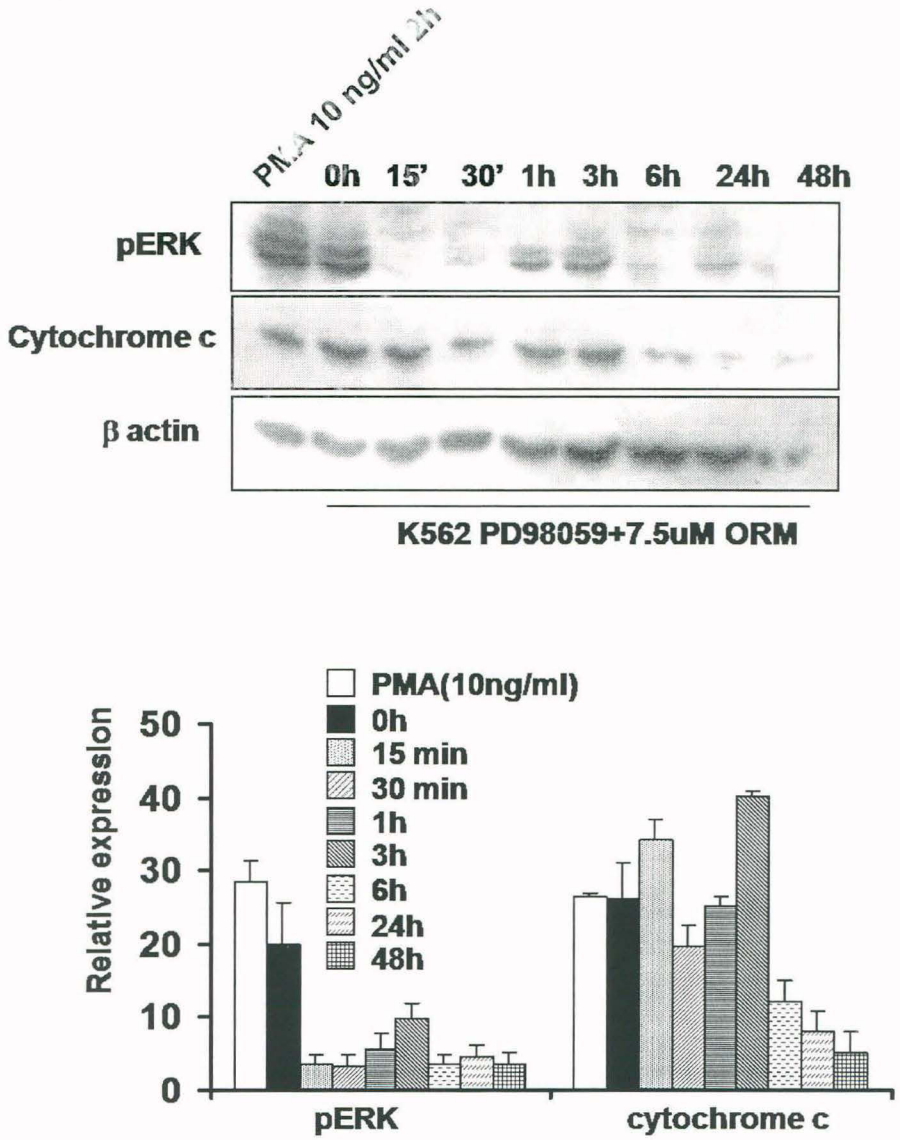

Figure 14: K562 cells pretreated for 1h with 10μM PD98059 were treated with ORM for indicated time points, whole cell extracts were prepared and separated on 10% SDS-PAGE, immunoblotted and probed with pERK, cytochrome-c and β-actin; PMA 10ng/ml for 2h was used as positive inducer of ERK phosphorylation. Lower panel graphically depicts relative expression (densitometry) in immunoblots.

Histogram in the lower panel depicts densitometry analysis of the relative expression changes in the immunoblot.

This finding was further manifested by reversal of ORM mediated apoptosis in FACS Flow cytometer analysis where these cells were treated with 10μM ERK inhibitor (PD98059) 1h prior to the ORM treatment. Flow cytometry analysis performed in K562 cells after treatment with

ORM in the absence or presence of ERK inhibitor PD98059 showed that ORM mediated apoptosis is substantially inhibited in the presence of PD98059 (figure 15).

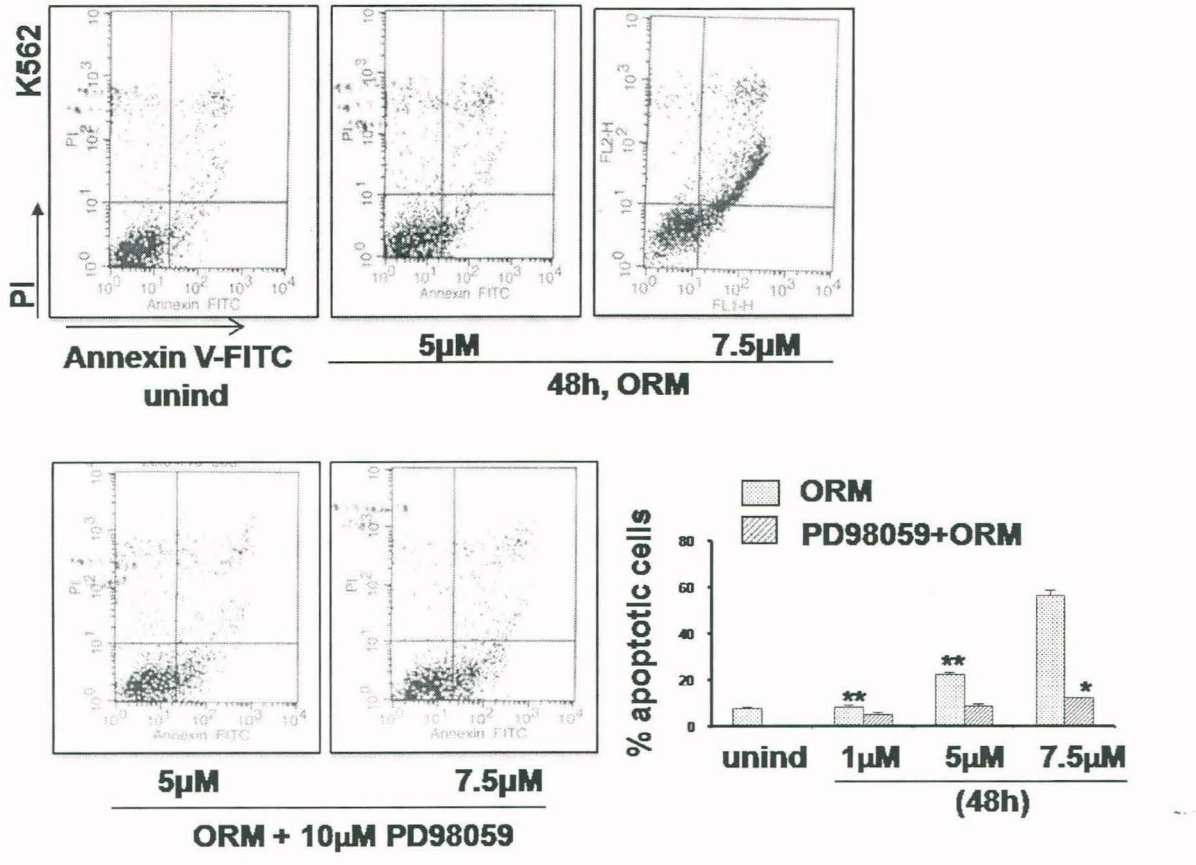

Figure 15: Percentage of Annexin V-PI double positive cells were assessed by FACS flow cytometer in the presence of ORM alone and together treated with ERK inhibitor. Lower panel graphically depicts the percentage of double positive cells. Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

Furthermore, because PD98059 treatment substantially reduced the apoptotic effects of ORM, we further determined the expression of cleaved PARP by pre treating K562 cells with PD98059 before induction with ORM. PARP cleavage in ORM treated cells pretreated with PD98059 was dramatically inhibited as seen in figure 16.

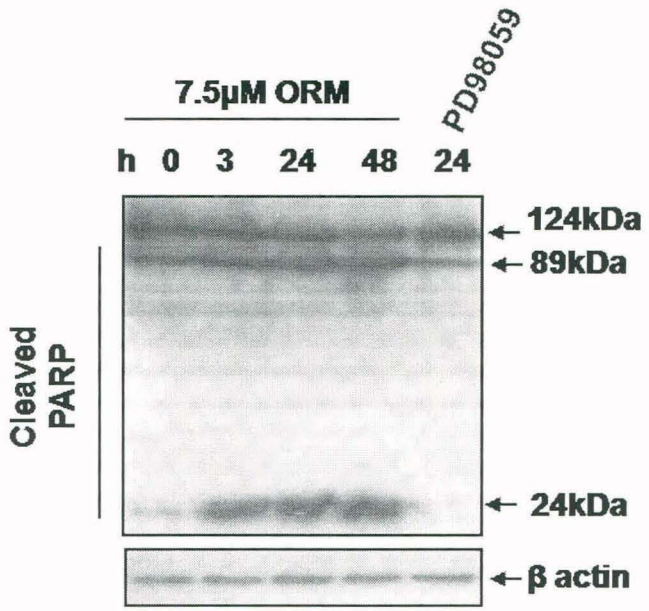

Figure 16: Immunoblot against cleaved PARP with ORM alone and after 1h pretreatment with ERK inhibitor. β actin is used as a loading control.

Taken together, these data affirm that ORM induced apoptosis is mediated by activation of ERK and subsequent release of cytochrome-c leading to caspase-3 activation and thus culminating in increased apoptosis. Importantly these effects could substantially be reversed by ERK inhibitor PD98059.

3.2.3 ORM inhibits cell proliferation and induces G0-G1 growth arrest

3.2.3.1 ORM inhibits cells cycle at G0-G1 phase

ORM efficiently induced cytotoxicity in K562 cells. In addition we showed that this cytotoxicity is via induction of apoptosis. However, since K562 is a highly proliferative due to presence of the activated oncogenic Bcr-Abl fusion protein, we sought to examine if ORM also inhibits proliferation by inducing growth arrest in these cells. To address this, K562 cells were treated with different doses of ORM for indicated time points and were assessed by PI staining for the distribution of cells in different phases of cell cycle using FACS Flow Cytometer. Marked increase in the no. of cells (62%) in G0-G1 phase of the cell cycle as compared to control (38%)

was observed suggesting ORM induced cytotoxicity to be coupled with growth arrest in this phase of cell cycle (figure 17).

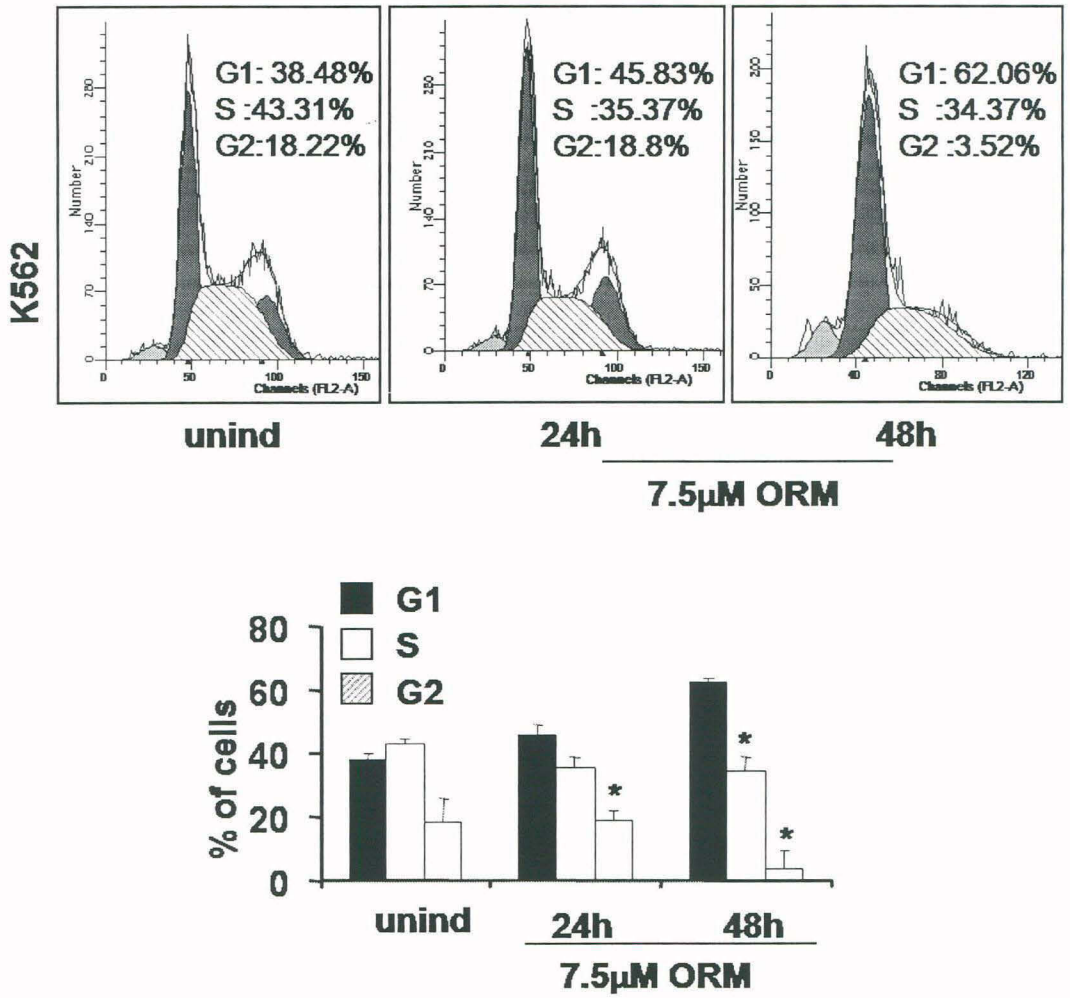

Figure 17: Distribution of cells in different phases of cell cycle in K562 cells as analyzed by Flow cytometry after 24 and 48h of culture is depicted. Sub-G1 designates the fraction of cells that is undergoing apoptosis. The percentage of cells in each phases of cycle is also depicted in the graph (lower panel). Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

Note that no. of cells after 48h in sub-G1 state is also enhanced which indicates marked increase in the apoptotic cells and further strengthens our apoptosis related finding in this study.

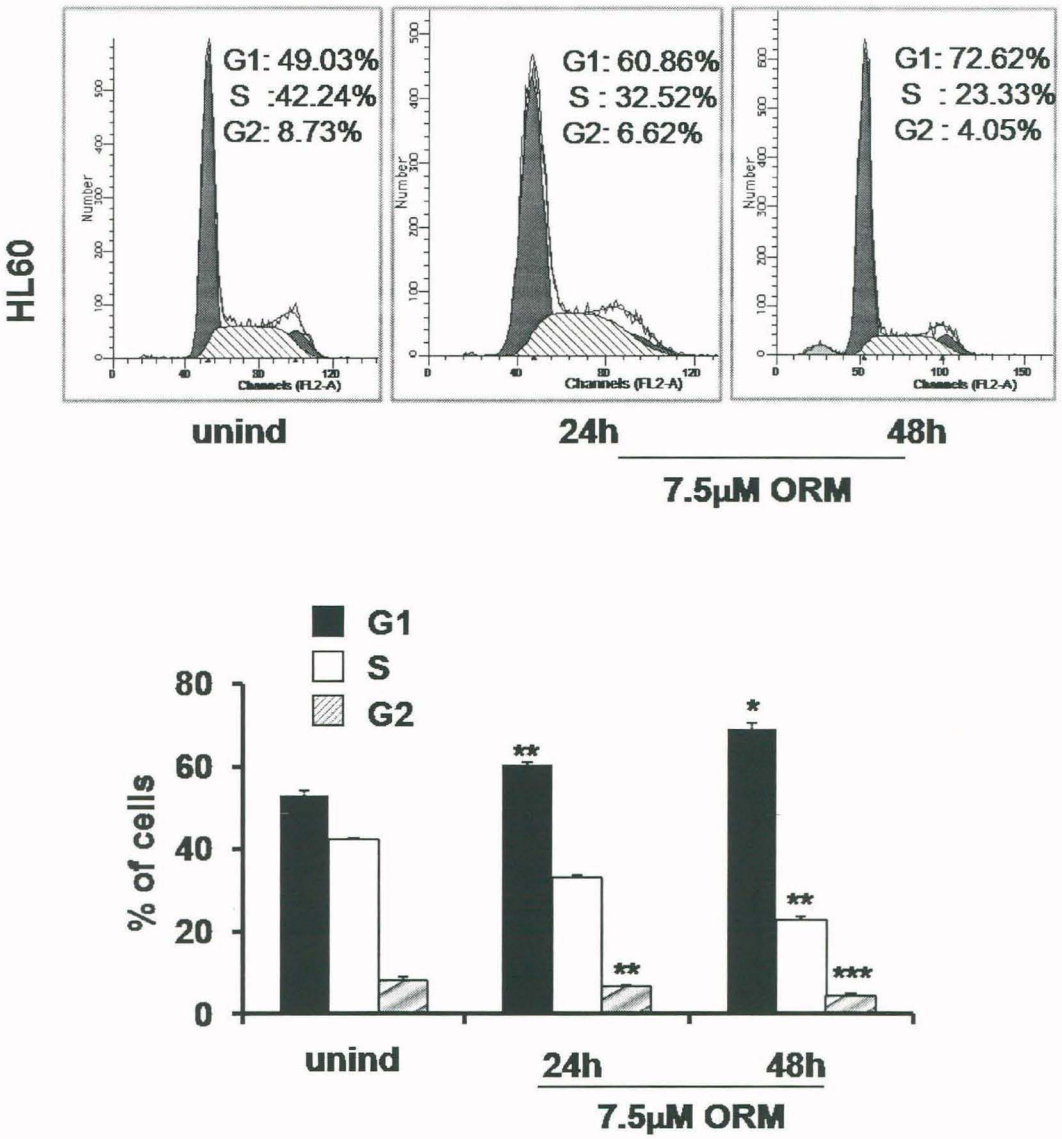

Figure 18: Distribution of cells in different phases of cell cycle in HL60 cells as analyzed by Flow cytometry after 24 and 48h of culture is depicted. Sub-G1 designates the fraction of cells that is undergoing apoptosis. The percentage of cells in each phases of cycle is also depicted in the graph (lower panel). Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

Furthermore, HL60 and U937 cells were also treated with ORM for 24 and 48h for the cell cycle analysis. After induction with ORM, cells were stained with PI solution and analyzed in flow cytometer. As observed with K562, marked increase in the percentage of cells in the G0-G1 phase of the cell cycle as compared to the control was observed in these cells as well.

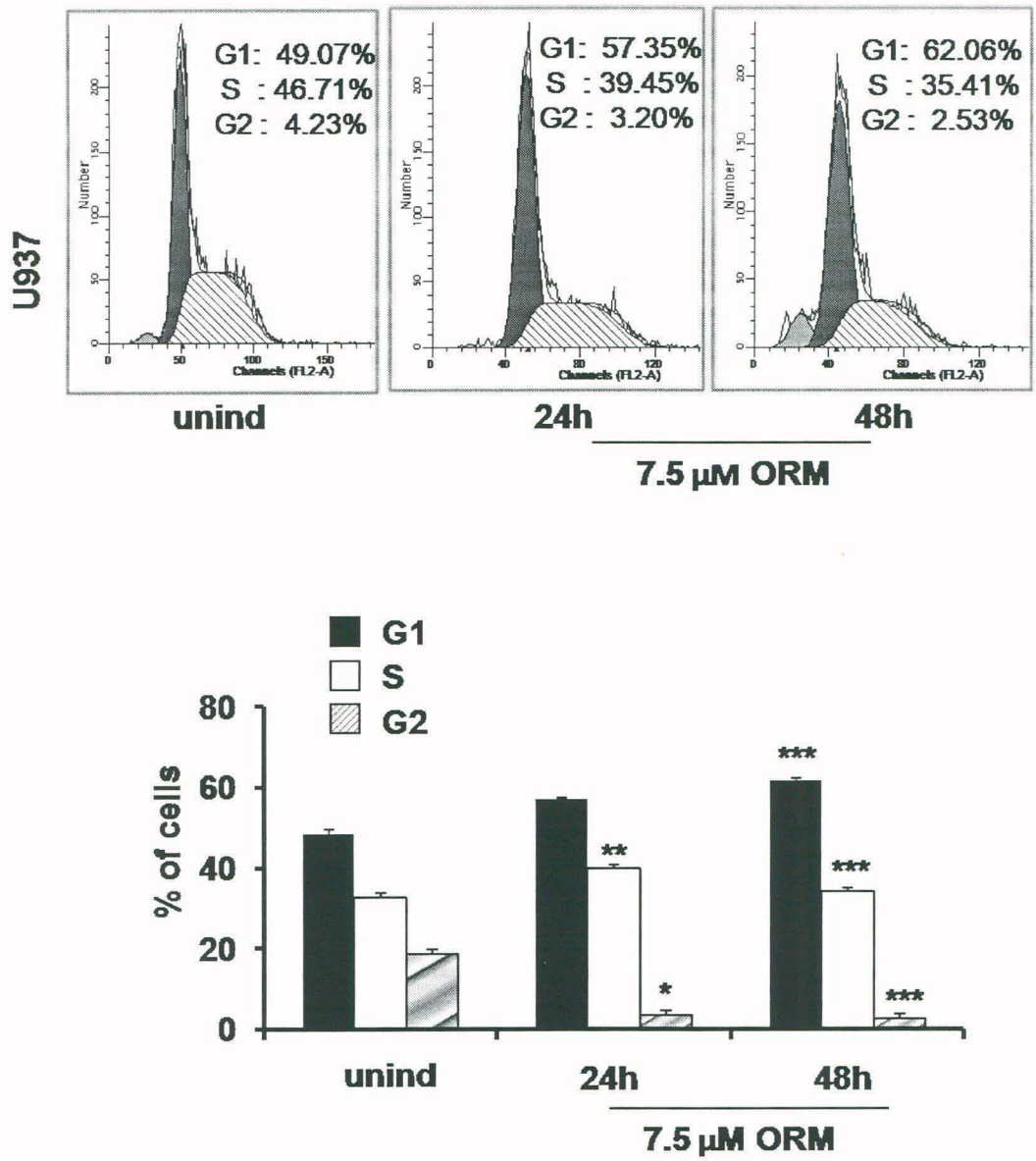

Figure 19: Distribution of cells in different phases of cell cycle in U937 cells as analyzed by Flow cytometry after 24 and 48h of culture is depicted. Sub-G1 designates the fraction of cells that is undergoing apoptosis. The percentage of cells in each phases of cycle is also depicted in the graph (lower panel). Data represents mean ± SEM from three independent experiments. *P < 0.05, **P < 0.01 and ***P<0.001 compared to control.

Ormeloxifene treatment in HL60 and U937 cells inhibits cell cycle proliferation which validates our previous data that ORM induction leads to cytotoxicity and apoptosis in these cell lines.

Based on our finding, we can conclude that like K562, ORM treatment is equally effective in the acute myeloid leukemia cells HL60 and U937.

3.2.3.2 p21 up regulation leads to cell cycle inhibition

To elucidate the mode of ORM mediated growth arrest, changes in the molecular proteins associated with block of proliferation and G0-G1 arrest were assessed. As K562 is highly proliferative cell line, we analyzed the changes in the mRNA expression level of c-Myc, a well known marker of proliferation (205, 206) and p21, a cyclin dependent kinase inhibitor known to be upregulated during G0-G1 growth arrest (207, 208) with RT-PCR and quantitative PCR (real time PCR).

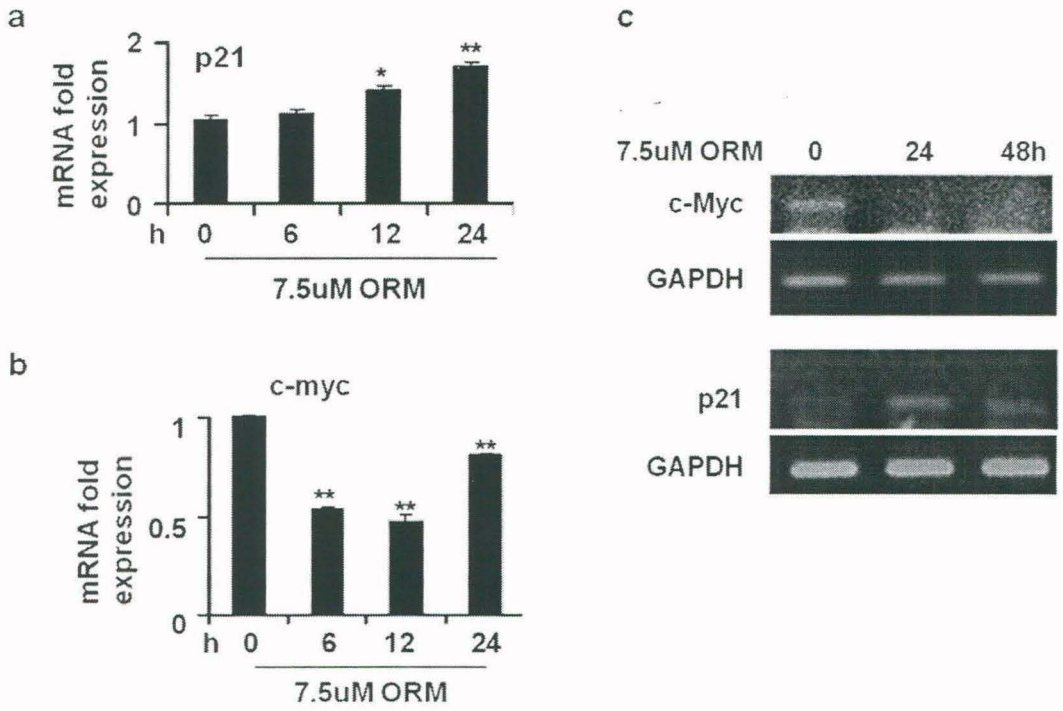

Figure 20: (a, b) Real Time PCR shows relative mRNA expression of p21 and c-myc upon ORM treatment after indicated time points. **(c)** RT-PCR analysis shows the downregulation of c-Myc and upregulation of p21 after ORM treatment in K562 cells.

The analysis revealed dramatic decrease in the mRNA level of c-Myc and increase in p21. In addition, concomitant decrease in cdk2 expression was observed with increase in p21 protein levels which again is associated with transition of cells from G1 to S phase.

We further corroborated our finding by assessing the changes in the protein expression of c-Myc and p21 upon 7.5μM ORM treatment for 24 and 48hrs compared to control cells.

Significant decrease in the protein levels of c-Myc upon ORM treatment was observed which corresponded well to the changes observed in the mRNA expression levels. p21 protein levels were also enhanced upon ORM treatment which approved the cell cycle inhibitory effects of ormeloxifene.

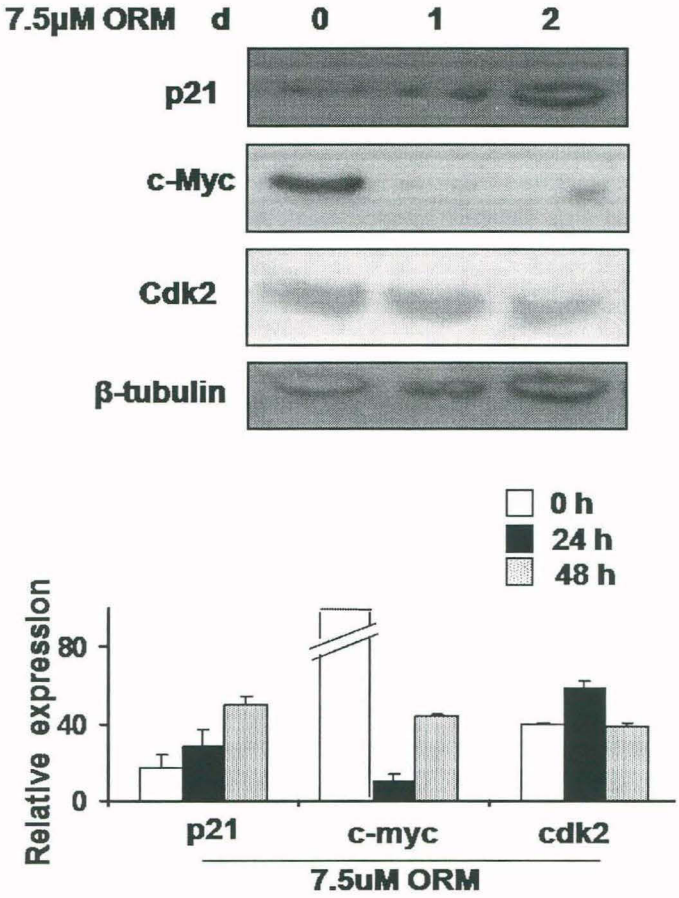

Figure 21: Immunoblot with p21, c-Myc and cdk2 antibody showing reciprocal protein expression of p21 (upregulated) and c-Myc, cdk2 (down regulated). Lower panel graphically depicts relative expression levels (densitometry) in the immunoblots.

Thus, taken together these data suggest that ORM inhibits K562 cell proliferation by inducing G0-G1 growth arrest via modulating the expression levels of molecules associated with it.

3.2.4 Ormeloxifene inhibits Bcr-Abl phosphorylation

The antiapoptotic activity of Bcr-Abl significantly contributes to the development of CML. Bcr-Abl may function either by enhancing the proliferation potential of hematopoietic progenitors or

by protecting these progenitors from apoptosis. Imatinib mesylate (STI571, Gleevec®) developed by Novartis is the first successful, rationally developed, receptor-targeted agent for chronic myelogenous leukemia (CML). Imatinib inhibits the constitutively active Bcr-Abl tyrosine kinase protein encoded by the fusion gene generated by the Philadelphia chromosome translocation, which actually is responsible for the pathogenesis of CML. Moreover, pre-clinical and clinical studies have shown that imatinib specifically inhibits the proliferation of cells expressing Bcr-Abl both *in-vivo* and *in-vitro* (209).

Based on this, we hypothesized that ability of ormeloxifene to induce apoptosis in K562 cells may also involve inhibition of Bcr-Abl. To investigate this, K562 cells were treated with 7.5μM ORM and were harvested after indicated time points (figure 22). Protein lysates were separated on 10% SDS PAGE and were probed with c-abl antibody. Like with Imatinib (IM) treated cells, marked decrease in Bcr-Abl protein expression after 48hrs was observed.

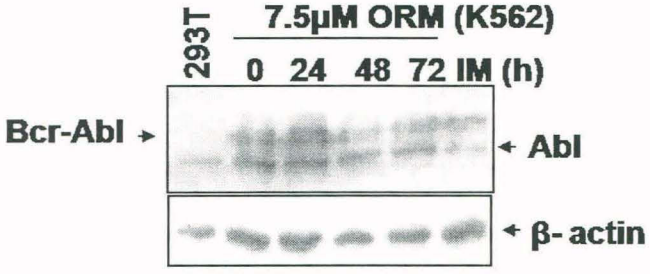

Figure 22: Whole cell extracts of ORM treated K562 cells after indicated time points were separated on 10% SDS PAGE and probed with c-Abl antibody, 293T alone served as negative control for Bcr-Abl (upper panel).(IM- Imatinib mesylate, 10μM)

Furthermore, since constitutive tyrosine phosphorylation of Bcr-Abl is required for its leukemogenic function, we assessed if ORM can inhibit tyrosine phosphorylation of Bcr-Abl. To answer this, cells were again treated with 7.5μM of ORM and protein lysates were prepared after indicated time points (figure 23). Whole cell extracts were separated on 10% SDS PAGE and were probed with phosphotyrosine antibody.

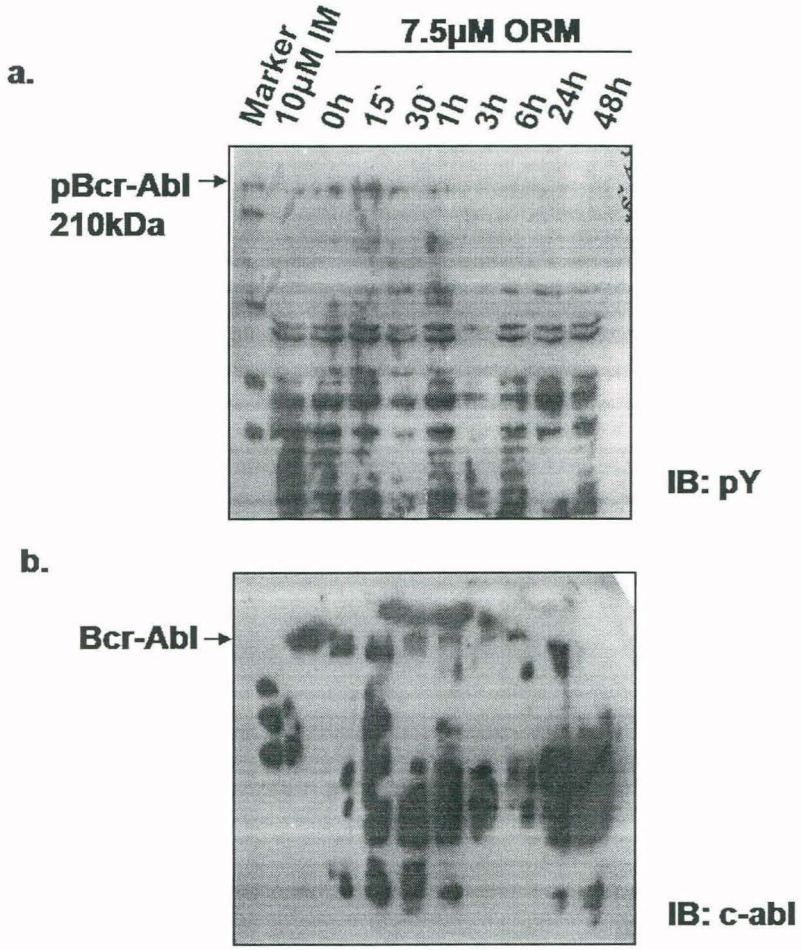

Figure 23: (a) Whole cell extracts of ORM treated K562 cells after indicated time points were separated on 10% SDS PAGE and probed with phosphotyrosine antibody.(b) Same membrane was striped and reprobed with c-Abl antibody (Lower panel).

As shown in figure 23a dramatic decrease in phosphorylation of Bcr-Abl was observed. Reprobing of same membrane after stripping with Bcr-Abl again showed marked decrease in BCR-ABL protein expression (Figure 23b).

Taken together, these data suggest that ORM induced apoptosis in K562 cells is apparently associated with inhibition of both phosphorylation as well as protein expression of Bcr-Abl leading to inactivation of survival pathways.

3.2.5 Ormeloxifene mediated apoptosis is translatable in CML patients

Peripheral blood from two CML patients after their prior consent was kindly provided by Dr. Bhatt (CSMMU, Lucknow). Mononuclear cells were isolated from these cells using ficol hypaque density gradient centrifugation as described in materials and methods.

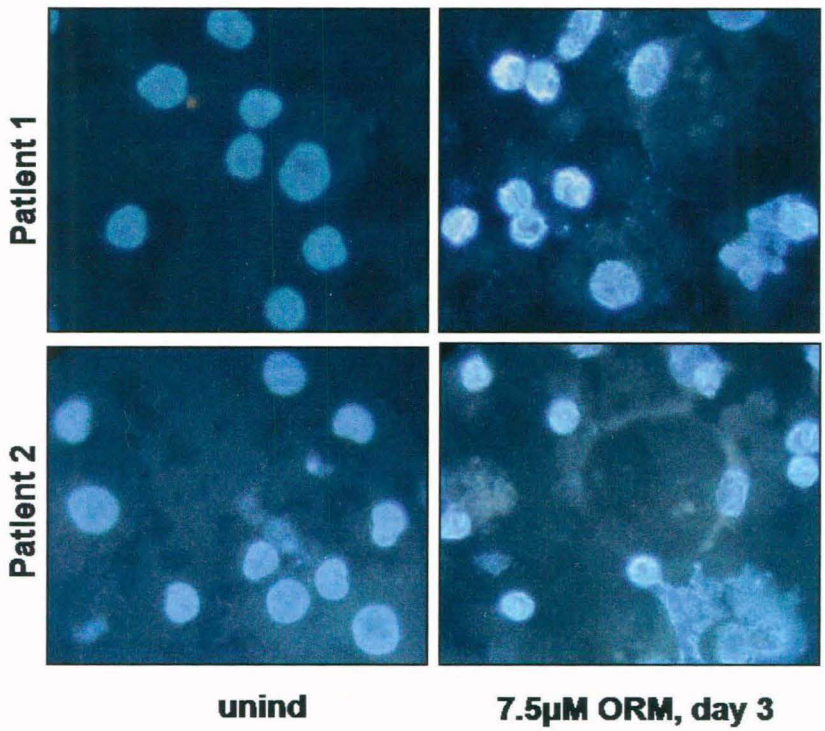

Figure 24: Mononuclear cells isolated from two CML patients were cultured in RPMI and treated with 7.5μM ORM for three days. Hoechst staining shows visible apoptosis like features in treated cells as compared to control untreated cells.

Isolated mononuclear cells were cultured for 24h in RPMI-1640 supplemented with 10% FBS. Post 24h culture; cells were treated with 7.5μM ORM and cytospun on slides after 3 days. Cytospun cells were stained with Hoechst and visualized under microscope for morphological changes. Significant chromatin condensation and nuclear membrane blebbing were observed in ORM treated cells as compared to control cells.

3.3 Discussion

In the present study, for the first time we show that ORM, reportedly first non-steroidal SERM induces growth arrest and apoptosis in myeloid leukemia cells, K562 in particular. Like in breast cancer cells, 50% growth inhibitory concentration of ORM using MTT assay for myeloid leukemia cells was calculated at around 7.5μM. Note that, to induce similar extent of growth inhibition in HEK293 (considered as control) cells, more than 30μM of ORM was needed. The ability to evade apoptosis through a gain of antiapoptotic function or loss of proapoptotic signal is a hallmark of cancer. Therefore, studies involving therapeutic agents focus on selective growth inhibition and induction of apoptosis in cancer cells. Consistent with this, we also show that ORM induces apoptosis in myeloid leukemia cells U937, HL60 and K562. Interestingly, the induction of apoptosis was more prominent in K562 cells, therefore, we used it as a model cell line for our further studies. The rationale was that these cells harbor a fusion protein Bcr-Abl produced due to a reciprocal translocation between chromosomes 9 and 22 which imparts excessive proliferation and higher survival rate in these cells and hence anticancer effects of ORM would be more prominent.

Apoptosis is characterized by morphological and biochemical parameters such as chromatin condensation, membrane blebbing, membrane permeabilization, the formation of apoptotic bodies, chromatin fragmentation and phosphatidylserine exposure (203, 210). Consistent with this, flow cytometry analysis of TUNEL positive cells further confirmed the apoptosis inducing potential of ORM in K562 cells. TUNEL assay detects the presence of a multitude of DNA strand breaks and is considered to be the gold standard for identification of apoptotic cells (201). Studies demonstrating loss of mitochondrial membrane potential and caspase 9 and 3 activation further substantiated ORM mediated apoptosis in these cells.

Several studies indicate that apoptosis induced by a therapeutic agent in various cell types requires activation of the caspase pathways (209, 211). Therefore, we assumed that ORM induced apoptosis in myeloid leukemia cells, K562 in particular might also be mitochondria mediated. In line with this, we show that the loss of mitochondrial membrane potential in ORM induced cells is coupled with concomitant increase in Bax expression and release of cytochrome-c. A differential regulation of Bcl2 expression was also seen which may be attributed to K562 cell survival response against ORM. In addition, activation of Caspase 3; main executer of

apoptotic process and subsequent cleavage of PARP further confirmed that ORM induced apoptosis is indeed mitochondria mediated.

Our data nicely correlates the activation of ERK phosphorylation with release of cytochrome-c which initiates mitochondria mediated caspase activation. Note that ERK activation by various compounds is reported to be followed by release of cytochrome-c leading to apoptosis and this can be inhibited by blocking the ERK activity (204, 212, 213). Consistent with this, ORM treatment enhanced phosphorylation of ERK which is followed by increase in cytochrome c release. Furthermore, our data shows that inhibition of ERK phosphorylation by PD98059 reversed the ORM mediated apoptotic effects as shown in immunoblot showing inhibition of cytochrome-c release and reduced formation of cleaved PARP products. This is also obvious in FACS flow cytometer which shows dramatic reduction in annexinV-PI stained cells. Notably, apoptosis can be initiated by extracellular (non-mitochondrial) or intracellular (mitochondrial) signals and as such two major pathways can be distinguished, they are not isolated, and in fact have considerable overlap. Therefore, the possibility of apoptosis induction in these cells through non-mitochondrial mediated pathways may not be ruled out (214).

Significant advances made in cancer therapy during the last decade as our understanding of molecular biology and leukemogenesis evolved, suggests that leukemic cell proliferation and apoptosis should be targeted by antileukemic agents. In line with this ORM mediated inhibition of proliferation is also associated with arrest of cells in G0-G1 phase of cell cycle with reciprocal expression of p21 and c-Myc regulatory proteins known to be involved in progression of cells from G0-G1 to S phase. In addition, our finding that Ormeloxifene induces growth arrest and apoptosis in chronic myeloid leukemia cells is in accordance with inhibition of both phosphorylation and protein expression of oncogenic protein Bcr-Abl which is a hallmark signature protein of these cancer cells. Since Bcr-Abl provides proliferative and subsequent survival advantage to these cells, it makes sense that inhibition of this protein is a primary requirement. In agreement with this, we show that ORM does inhibit BCR-ABL protein which may further support the notion that ORM induces apoptosis and growth arrest in these myeloid leukemia cells.

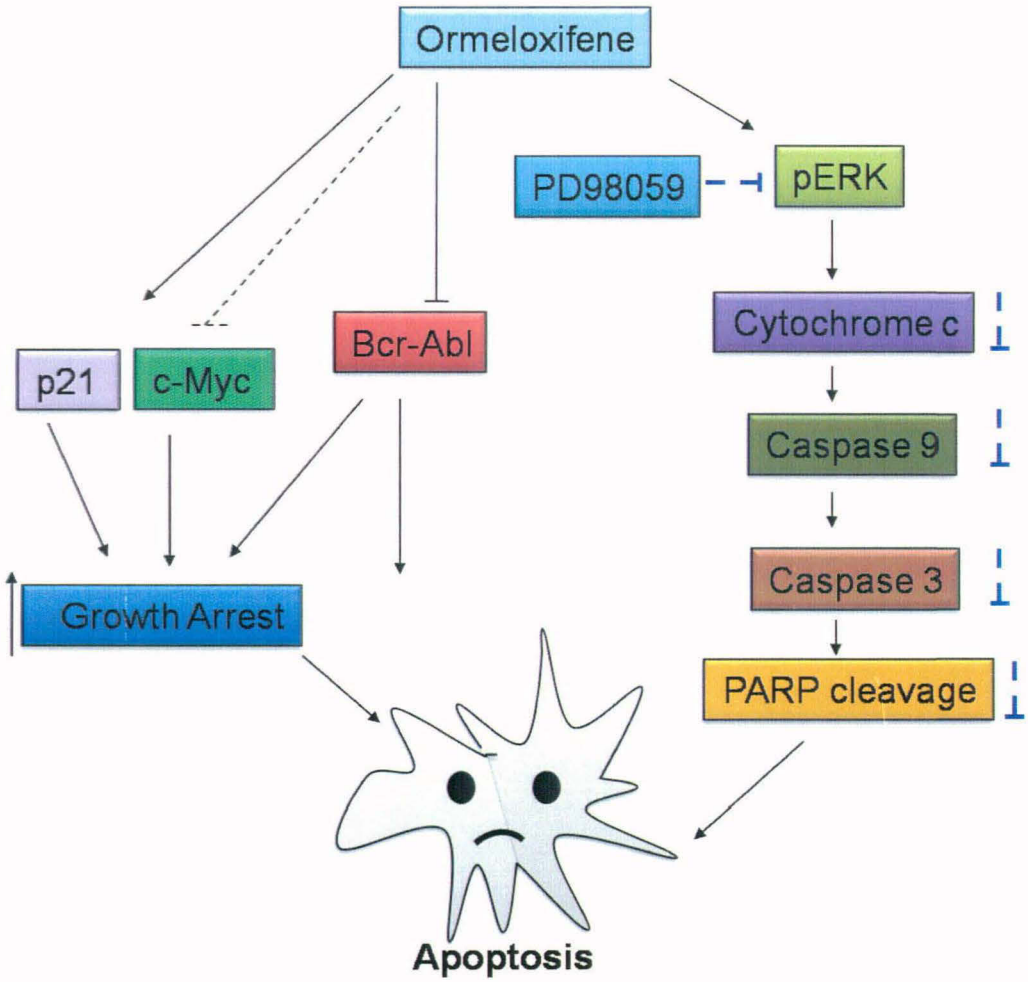

Figure 25: Hypothetical model - Based on our findings we propose a hypothetical model of ORM mode of apoptosis induction in K562 cells.

Based on our findings, we propose a hypothetical model where treatment of K562 cells with ORM activates ERK leading to cytochrome-c release, caspase 9, 3 activation and subsequent PARP cleavage culminating in enhanced apoptosis of these cells. This ERK induced mitochondria mediated apoptosis can be substantially inhibited by use of ERK inhibitor. In addition, ORM also promotes G0-G1 growth arrest of these cells by reciprocally modulating the expression molecular signature proteins of G0-G1 to S phase cell cycle transition.

In conclusion, we propose a hypothetical model which shows that in addition to inducing G0-G1 growth arrest, ORM induces apoptosis in chronic myeloid leukemia cells K562 via phosphorylation of ERK leading to mitochondria mediated caspase activation. Although the

precise mechanistic details of ormeloxifene mediated effects may need intensive investigation, it offers significant hope for progress both on its own and in conjunction with classical cytotoxic chemotherapy for the treatment of leukemogenic malignancies.

Chapter 4

E6AP, an E3 ubiquitin ligase inhibits tumor suppressor C/EBPα via ubiquitin mediated proteasomal degradation

4.1 Introduction

4.1.1 CCAAT/enhancer binding protein alpha (C/EBPα)

CCAAT/enhancer binding protein alpha (C/EBPα) was originally identified as a heat stable protein present in soluble extract of rat liver nuclei (215, 216). C/EBPα was the first member cloned in the C/EBP family transcription factor (217). Since it bound to the CCAAT box present in several promoters as well as to the core homology sequence [TGTGG (A/T)(A/T)(A/T)G] of certain viral enhancers, it was termed CCAAT/enhancer binding protein. Expression patterns of C/EBPα mRNA are similar in mice and humans with measurable levels in liver, adipose, intestine, lung, adrenal gland, peripheral blood mononuclear cells, and placenta (217, 218). In liver and adipose, highest levels of C/EBPα mRNA are detected only in differentiated cells.

CCAAT/enhancer binding proteins (C/EBPs) are members of the basic leucine zipper (bZIP) class of transcription factors which are characterized by a DNA binding basic rich and a leucine rich dimerization domain aptly termed as "leucine zipper". The leucine zipper region is a heptad repeat of leucines within a 35 amino acid sequence that forms an amphipathic α-helix with the leucines being located on one side of the helix per every two turns. These leucines allow the two α-helices of separate monomers to dimerize by hydrophobic interactions (zipping) (219).

Member of C/EBP family

The CCAAT/enhancer-binding proteins (C/EBPs) encompass a family of transcription factors with structural as well as functional homologies (220). Since the cloning of the family's original member, C/EBPα, five other C/EBPs have been identified that interact with each other and transcription factors in other protein families to regulate mRNA transcription. The six C/EBP proteins are designated as C/EBPα, β, γ, δ, ε and ζ (221-223). C/EBPβ expression is highest in liver, intestine, lung, adipose, spleen, kidney, epidermis, and myelomonocytic cells while C/EBPδ is expressed in adipose, lung, and intestine. C/EBPγ and C/EBPζ are expressed ubiquitously and C/EBPε is highly expressed in myeloid and lymphoid cells.

Similarities between C/EBP family members suggest an evolutionary history of genetic duplications with subsequent pressure to diversify. The resulting family of proteins vary in tissue specificity and their transactivation ability. The pleiotropic effects of C/EBPs are in part because

of tissue- and stage-specific expression, leaky ribosomal reading, post-transcriptional modifications, and variable DNA binding specificities. These mechanisms result in variable amounts of the C/EBP isoforms, available to dimerize and bind to cognate sites in different tissues. Each isoform, however, shows distinct but overlapping patterns of tissue-or stage-restricted expression. Modifications of a transcription factor, such as by phosphorylation, glycosylation, and reduction-oxidation affects its binding activity and function. C/EBP family members bind to specific DNA sequences as dimers. The basic leucine zipper (bZIP) regions of C/EBP isoforms show high similarity, and some cis-elements are recognized by each of the C/EBP isoform.

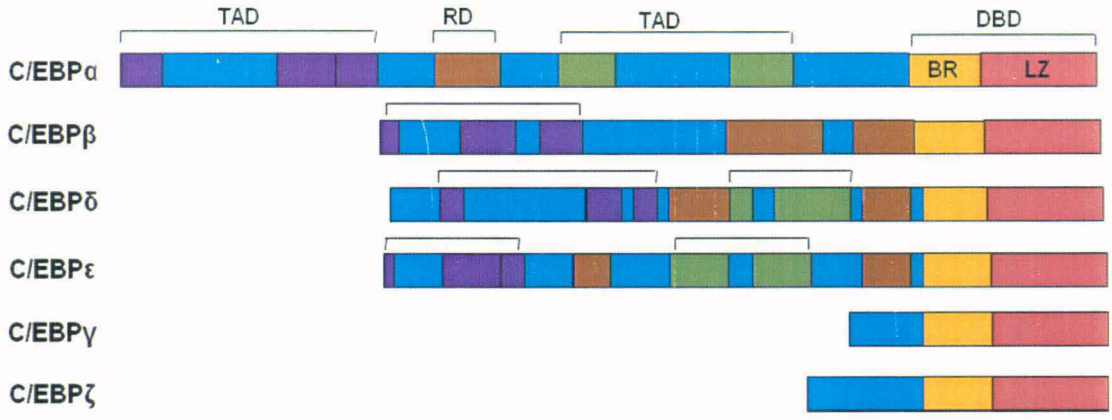

Figure 1: Domain organization of C/EBP-family members. Each C/EBP protein contains a functionally related leucine zipper dimerization domain (LZ) at its C-terminus. All family members, with the exception of C/EBPζ, share an adjacent highly conserved basic region (BR) that mediates sequence-specific DNA binding. C/EBPα, C/EBPβ, C/EBPδ and C/EBPε each contain transactivation domains (TADs) and a regulatory domain (RD) located in their N terminal regions. A tripartite TAD at the N-terminus displays homology among several of the family members (Adapted from Peter F. Johnson, Journal of Cell Science, 2005).

Domains of C/EBPα

C/EBPα, like many other transcription factors, is a modular protein, consisting of an activation domain, a DNA-binding basic region, and a leucine rich dimerization domain.

The activation domain

This domain is responsible for transcriptional activation and/or repression and is located in the N-terminal end of the protein. Two isoforms of C/EBPα are generated from its mRNA by a ribosomal scanning mechanism (224, 225). The full-length 42kDa protein contains three transactivation domains (TEI, II and III) (226-228). TEI and TEII mediate cooperative binding of C/EBPα to TBP (TATA-binding protein) and TFIIB which are the two components of the RNA polymerase II basal transcriptional apparatus. A fraction of ribosomes ignore the first two AUG codons and initiate translation at the third AUG, 351 nucleotides downstream of the first AUG. This shorter 30kDa protein retains its dimerization and DNA-binding domains; however, it possesses an altered transactivation potential compared with the 42kDa isoform (224). TE-III contains a negative regulatory domain, the function of which is alleviated when C/EBPα is bound to the albumin promoter (226).

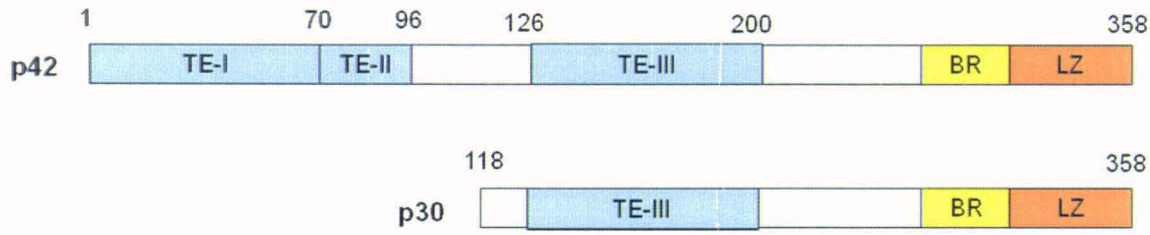

Figure 2: C/EBPα functional domains, protein interaction regions and phosphorylation sites. The two major translational isoforms, p42 and p30, are shown. Three distinct transactivation elements (TE-I, TE-II and TE-III) have been identified.

The leucine zipper domain:

The dimerization domain, aptly termed the "leucine zipper", is a heptad of leucine repeats that intercalate with repeats of the dimmer partner, forming a coiled coil of alpha-helices in parallel orientation (229-231). Leucine zipper (LZ) dimerization segment contributes to DNA-binding specificity by determining which subunits form stable dimers, and by appropriately positioning the basic region helices over the binding site. The primary sequence of the leucine zipper is a repeating heptad (a-b-c-d-e-f-g) with hydrophobic and apolar residues predominating at positions a and d, and polar and charged amino acids dominating the other positions of the repeat (232).

There is formation of heterodimers within a transcription factor family as well as transcription factors from different families that contain similar as well as unrelated DNA-binding domains. This suggests cell-type specific cooperation with other transcription factors as a basis for differential activation of genes by C/EBPα in different cell types. Protein-protein interactions between C/EBP transcription factors and other classes of protein molecules have been reported, some of the examples are C/EBPα-NFκB, C/EBPβ-NFκB, C/EBPδ-NFκB, C/EBPβ-glucocorticoid receptor, C/EBPβ-Sp1, C/EBPβ-Myb, and C/EBPα-AML1 interactions (233-236). For C/EBPs interaction with the NFκB or glucocorticoid receptor, it has been shown that the leucine zipper domain in C/EBPs mediates this interaction (237).

The DNA-binding domain

C/EBP dimerization is a prerequisite to DNA binding. DNA binding specificity, however, is determined by the DNA contact surface; the "basic" region of approximately 20 amino acids upstream of the leucine zipper specifically by 3 amino acids lying along the protein-DNA interface (238). The bZIP domain comprises a carboxyl-terminal dimerization segment and an amino-terminal DNA-binding segment (the basic region) consisting of two extended alpha-helices rich in basic residues. Residues along the 'inner' surface of the basic region helix contact the edges of base-pairs in the major groove, while neighbouring residues contact the phosphodiester backbone of the binding site. Transcription factors sharing a common DNA-binding motif may associate as homodimers or heterodimers with distinct DNA-binding and transcriptional activities. These alternative, dimeric combinations of proteins explain the regulatory potential of each family. The overall appearance of the bZIP is that of an alpha helical fork. The bZIP subunits diverge at the amino-terminus of the leucine zipper and position a relatively straight basic region helix in the major groove over each half-site, where it contacts the edges of base-pairs and the phosphodiester backbone (239).

Biological functions of C/EBPα

C/EBPα in adipocytes

Preadipocyte differentiation into functional adipocytes results from a highly regulated cascade of C/EBPs isoform expression (222). C/EBP transcription factors have an important role in

preadipocyte differentiation (240, 241). Promoter regions of many adipocyte-specific genes contain C/EBP binding sites (242, 243). C/EBPα expression is highest in terminally differentiated adipocytes and the induction of C/EBPβ and C/EBPδ mRNA precedes C/EBPα mRNA expression during preadipocyte differentiation. C/EBPα promoter is activated by all three C/EBP isoforms (C/EBPα, C/EBPβ, and C/EBPδ), C/EBPβ and C/EBPδ have been proposed to function in early preadipocyte differentiation by inducing C/EBPα transcription, which in turn induces transcription of adipocyte-specific genes (244). Overexpression of C/EBPα or C/EBPβ may promote preadipocyte differentiation while inhibition of C/EBPα by antisense RNA blocks preadipocyte differentiation. Furthermore, embryonic fibroblasts from C/EBPβ and C/EBPδ deficient mice were unable to differentiate in response to hormonal stimulus and C/EBPα deficient mice had dramatically reduced lipid accumulation in adipose tissue (245). In addition, ectopic expression of C/EBPα in 3T3-L1 cells arrests mitotic growth (246). C/EBPα interacts with known regulators of cell cycle progression; it activates transcription and induces post-transcriptional stabilization of p21 (WAT-1/CIP- 1/SDI-1) protein, an inhibitor of cyclin-dependent kinase (138). Additionally, c-Myc and C/EBPα share a reciprocal relationship, balancing proliferation versus growth arrest via C/EBPα-transactivated expression of gadd45 (growth arrest-associated gene), a target of p53 tumor suppressor protein at G1 (247, 248). Transient modulation of C/EBP levels in response to insulin and dexamethasone suggests a dynamic role in adipocyte metabolism (247-249).

C/EBPα in haematopoiesis

C/EBPα predominantly induces granulocytic differentiation during hematopoiesis. Targeted disruption of C/EBPα in blood cells results in a selective early block in granulocyte maturation without affecting other hematopoietic lineages. Profound abnormalities of the hematopoietic system are seen in C/EBPα deficient mice. Mice deficient in C/EBPα display an early block in the maturation of granulocytes. Peripheral blood and bone marrow smears show only myeloblastic cells of the myeloid lineage. The G-CSF receptor expression is undetectable in these cells, suggesting a loss of G-CSF signal-directed maturation. In addition, upregulation of C/EBPα function results in induction of granulocytic development and inhibition of monocytic development from bipotential precursors. Ectopic expression of C/EBPα prevented 12-O-

tetradecanoylphorbol-13- acetate (TPA) induced monocytic differentiation of bipotential myeloid progenitor cells (250). In addition, TPA treatment of HL60 cells led to a decrease of C/EBPα mRNA levels by almost 90% (250). C/EBPα protein interacts with the p21 and cdk2 proteins, resulting in decreased cdk2 activity and inhibition of cell proliferation (138). C/EBPα protein disrupts E2F complexes in several cell lines leading to growth arrest. Interaction of C/EBPα with E2F appears to be the mechanism by which it downregulates c-myc expression during granulocytic differentiation (251). These protein-protein interactions appear to involve several regions of the C/EBPα protein, including the bZIP domain. Perhaps the duplication and deletion mutations in the bZIP domain may affect these interactions, thereby contributing to leukemogenesis (138).

Role of C/EBPα in cell proliferation

C/EBPα negatively regulates cell proliferation. C/EBPα expression is highly elevated in terminally differentiated adipocytes/hepatocytes/keratinocytes while it is down-regulated after partial hepatectomy (proliferation). Overexpression of C/EBPα results in strong growth arrest in cultured cells and abnormal cell proliferation is observed in liver and lung of C/EBPα deficient mice (252, 253). Furthermore, the expression levels of C/EBPα are reduced in some tumors/tumor cell lines. However, the importance of C/EBPα in the regulation of growth arrest and differentiation is exemplified by recent studies in which dominant-negative mutations in C/EBPα were found in some patients with human acute myeloid leukemia (AML). Such mutations in C/EBPα are thought to result in a differentiation block of the granulocytic blasts and have implicated C/EBPα as a tumor suppressor gene (254). In addition, it has been suggested that AML1-ETO, a fusion protein resulting from t(8;21) translocation in AML, suppresses C/EBPα expression indirectly by inhibiting positive autoregulation of the C/EBPα promoter (255). The growth and differentiation regulatory functions of C/EBPα are complex and multifaceted. C/EBPα regulates p21 expression and interacts with Rb family proteins (138). C/EBPα also directly represses E2F function through its physical associations with E2F and this repression is necessary for growth arrest and adipocyte and granulocyte differentiation. However, recent studies indicate that C/EBPα can block cell growth independent of its DNA binding and transcriptional activity by forming a complex with cdk2 (cyclin-dependent kinase 2) and cdk4

and thereby blocking cyclin-cdk interactions and cell cycle progression. C/EBPα has also been shown to interact with Max, a heterodimeric partner of Myc in Myc/Max/Mad link (256). Also, C/EBPα cooperates with p21 to inhibit cdk2 activity (138). Thus, in addition to its DNA binding/transcription factor activity, C/EBPα can modulate growth arrest and differentiation by protein/protein interactions with cell cycle regulatory proteins independent of its transcription activity (257).

C/EBPα in Myeloid Leukemia

Transcription factor C/EBPα is crucial for neutrophil differentiation and this makes it vulnerable to mutational defects leading to acute myeloid leukemia (AML) (258). The highest percentage of *CEBPA* mutations were found in patients with AML subtype M2, a myeloblastic leukaemia characterized by an early block in the neutrophil differentiation (259, 260). It has been reported that loss of C/EBPα expression and function in myeloid blast cell contributes to a block in myeloid cell differentiation leading to leukemia. In these leukemic cells, C/EBPα function was disrupted either through mutation of one allele of the *CEBPA* or by transcriptional repression (261). *CEBPA* mutations were observed in leukemic cells lacking any chromosomal translocations. These mutations resulted in truncated forms of C/EBPα protein that retained the DNA-binding domain. Unexpectedly, these proteins failed to bind DNA but inhibited DNA binding of wild-type C/EBPα suggesting that these mutants act as dominant inhibitors. The t(8;21) creates a fusion protein between the amino-terminal DNA binding domain of the acute myeloid leukaemia-1(AML1) transcription factor and the eight-twenty-one (ETO) corepressor, suggesting that repression of AML1 target genes stimulates leukemogenesis. The different phenotypes associated with these translocations suggest that AML-1/ETO has a 'modifier' effect that causes the down-regulation of C/EBPα and blockade at a distinct stage of differentiation (255).

Differentiation blocks are thought to contribute to tumorigenesis along with mutations that affect cellular proliferation, inactivate cell-cycle checkpoints, and block apoptosis. Thus, the loss of C/EBPα could represent one 'hit' in multi-step leukemogenesis (260). The observation that C/EBPα function can be deregulated by two separate mechanisms highlights the importance of this factor. Disrupting C/EBPα activity either by creating dominant negative forms or

repressing its transcription may have the same effects of block in myeloid progenitor-cell differentiation. The importance of C/EBPα in cell growth and differentiation suggests it may play act as a tumour suppressor.

There are many transcriptional modulations of C/EBPα which occur in acute myeloid leukemia (262). Termination of translation by introducing a nonsense codon; (The nonsense mutations would introduce termination codons before the bZIP domains. This would create polypeptides that are unable to localize to the nucleus, dimerize, and bind to the DNA) (263). Alteration of amino acid sequence by introducing a missense codon; (the mutation of Arg305Pro occurs in the fork region of bZIP domain). Frame-shift by either deletion or duplication of nucleotides and an eventual termination (264); and inframe deletion or duplication that removes or inserts additional amino acid residues (The inframe deletion and insertion mutations occur within the first conserved leucine finger) (52). In chronic myeloid leukemia, mutations in *C/EBPA* are not reported, however, In CML blast crisis cells, protein levels of C/EBP alpha are undetectable but its mRNA is present. Expression of C/EBPα is found to be suppressed at the translational level by interaction of the poly (rC)-binding protein hnRNP E2 with C/EBPα mRNA.

4.1.2 Ubiquitin/Proteasome-Mediated Proteolytic System

The cellular integrity is maintained by dynamic equilibrium between synthesis and degradation of each protein. Cells must control the rates of both synthesis and destruction of their proteins in order to control proper cellular function. For proper degradation of target proteins, cells contain two major degradation machineries: i) proteases (cathepsin B, D, H, and L, calpain I and II) and ii) multicatalytic, multisubunit ubquitin/proteasome in nucleus/cytoplasm. While proteases make several cuts in its target proteins without energy input resulting in several fragments of polypeptides, ubiquitin/proteasome system requires energy expenditure and cleaves every peptide bond in the target protein. Many cellular proteins involved in the critical cellular processes, such as proliferation, differentiation, DNA repair, and apoptosis, have been discovered to be degraded by the ubiquitin/proteasome pathway (265, 266). Furthermore, deregulated proteasomal degradation has been implicated in a number of human diseases,

including cancer, neurodegenerative diseases and myodegenerative diseases; therefore, proteasome inhibitors are being developed as a therapeutic agent.

Structure of Proteasome

The proteasome holoenzyme (also known as the 26S proteasome) is a 2.5MDa complex made up of two copies each of at least 32 different subunits that are highly conserved among all eukaryotes. The overall structure can be divided into two major sub complexes: the 20S core particle that contains the protease subunits and the 19S regulatory particle that regulates the function of the former (figure 3). One of the two regulatory particles attach to the surface of the outer rings of the core particle to form the 26S proteasome holoenzyme.

The core proteasome consists of the 20S core unit (~ 700kD) and the structure of the 20S proteasome is well conserved in all organisms from archaebacteria to yeast to human (267-269). The 20S core unit forms the hollow barrel shape in which four heptameric rings are stacked forming central chamber where proteolysis occurs. Two outer rings are composed of seven α-subunits and two inner rings are composed of seven β-subunits. Eukaryotic proteasomes have two additional 19S regulatory units which bind to the 20S proteasome forming a lid and a base in the 26S proteasome (270). 19S units contain binding sites for ubiquitinated proteins, enzymes that depolymerize ubiquitin chain, and six distinct ATPases. The ATPase function of the 19S unit unfolds target protein and facilitates the entry of target protein into the 20S proteasome (271). To ensure that every peptide bond in target protein is susceptible for cleavage, 20S proteasome has multiple proteolytic activities: i) chymotrypsin-like, ii) trypsin-like, iii) caspase-like, iv) branched-amino acid preferring, and v) small neutral amino acid-preferring (272-274). Based on mutational studies in yeast, these distinct proteolytic activities are mapped to different subunits in 20S proteasome (275-279). Different subunits and auxiliary factors of 26S proteasome are depicted in figure 3.

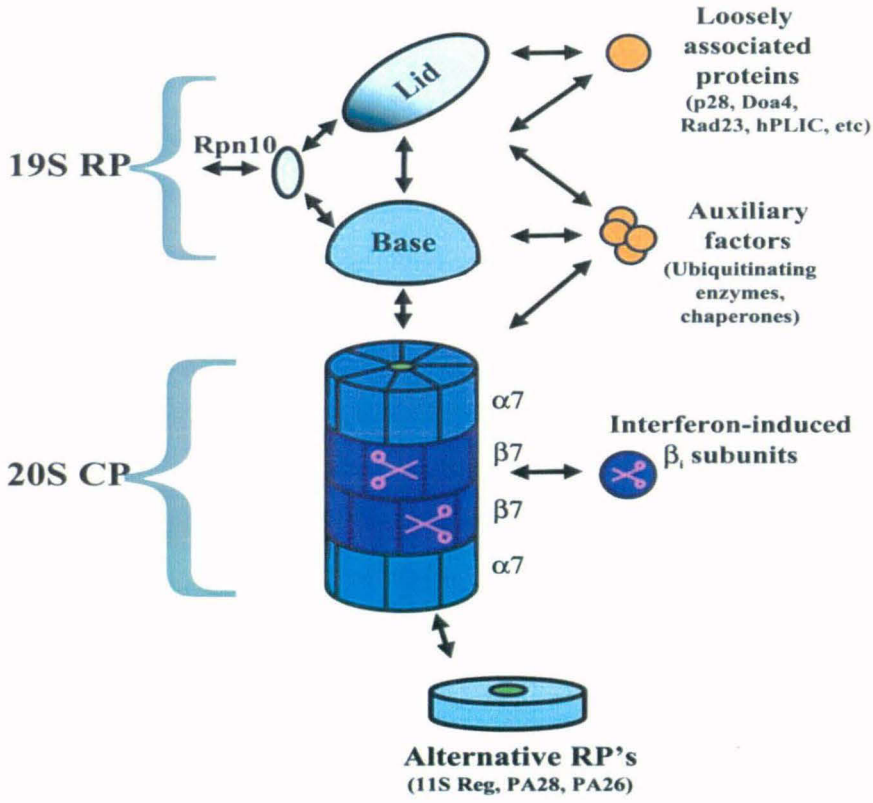

Figure 3: Structure of 26s proteasome (Adapted from Gickman,M.H., Ciechanover, A., Physiol Rev, 2002)

The ubiquitin-conjugation cascade

Ubiquitination (also referred to as "ubiquitin conjugation" or "ubiquitylation") is the post-translational modification of proteins by the covalent attachment of ubiquitin, a 76 amino-acid protein. "Monoubiquitination", the attachment of a single ubiquitin moiety involves isopeptide bond formation between the α-carboxy group of the C-terminal Glycine residue of ubiquitin and the ε-amino group of a lysine residue in the target protein. In addition to being modified by single ubiquitin moieties, proteins can also be modified by chains of ubiquitin ("polyubiquitination"). Polyubiquitin chain formation takes place by subsequent attachment of ubiquitin moieties linked to one of the lysine residues present in the previously added ubiquitin. Of all the known consequences of ubiquitination, the targeting of proteins for degradation has been best characterized. Substrates tagged with a polyubiquitin chain are selectively degraded by a multi-subunit ATP-dependent protease known as the 26S proteasome (280).

Free ubiquitin (Ub) is activated in an ATP-dependent manner by the activity of an ubiquitin-activating enzyme (E1), which hydrolyses ATP and forms a thioester bond with ubiquitin (281). Subsequently, ubiquitin is transferred to one of many distinct ubiquitin-conjugating enzymes (E2). In some reactions, E2s can directly ubiquitinate substrates, whereas others require the help of a ligase (E3) (282). While some E3s have the ability to form ubiquitin thioester during the transfer of ubiquitin to the substrate, others support ubiquitination by recruiting substrates to E2 enzymes (283). Usually, several ubiquitin molecules, in the form of a polyubiquitin chain, are conjugated to a substrate. In some cases, this requires a specific polyubiquitin chain-assembly factor (E4). Based on the number of ubiquitins (monoubiquitin versus polyubiquitin) and the type of chain linkage (e.g. K48 or K63), the fate of the substrate protein is decided.

Ubiquitin contains seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) and each of these lysine residues can form an isopeptide bond with C-terminal glycine of next ubiquitin, resulting in polyubiquitination (284). However, the most common type of ubiquitin chain is the K-48 linked type chain which signals the target protein for proteasomal degradation (285). Ubiquitin modification can simultaneously be removed by deubiquitinating enzymes (DUB). The attachment of ubiquitin to the substrate could also act as signal for the recruitment of ubiquitin-binding proteins (UBP), which could either protect the substrate from deubiquitination and/or act as a bridging factor to transfer polyubiquitinated substrates to the proteasome (286). To be fully active, the E1 must non-covalently bind to, and adenylate a second ubiquitin molecule. Secondly, the thioester-linked ubiquitin is transferred from E1 onto the active site cysteine residue of one of a number of E2s, where it is again linked by a thioester bond.

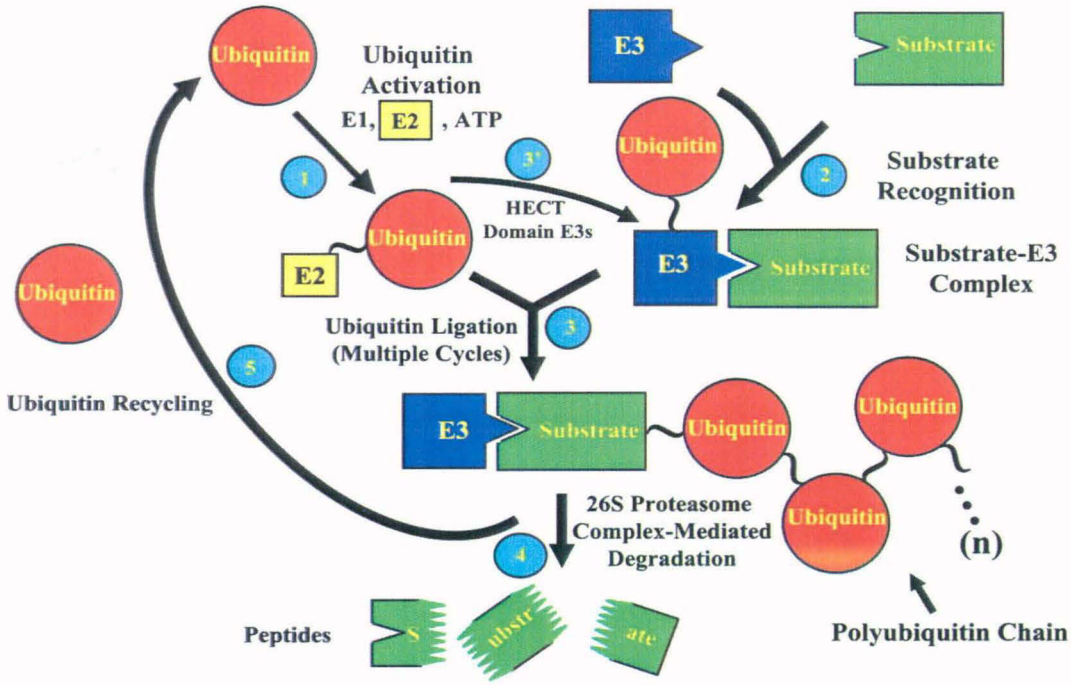

Figure 4: The ubiquitin proteolytic pathway: 1: Activation of ubiquitin by the ubiquitin-activating enzyme E1, a ubiquitin-carrier protein, E2 (ubiquitin-conjugating enzyme, UBC), and ATP. The product of this reaction is a high-energy E2~ubiquitin thiol ester intermediate. 2: Binding of the protein substrate, via a defined recognition motif, to a specific ubiquitin-protein ligase, E3. 3: Multiple (n) cycles of conjugation of ubiquitin to the target substrate and synthesis of a polyubiquitin chain. E2 transfers the first activated ubiquitin moiety directly to the E3-bound substrate, and in following cycles, to previously conjugated ubiquitin moiety. Direct transfer of activated ubiquitin from E2 to the E3-bound substrate occurs in substrates targeted by RING finger E3s. 3′: The activated ubiquitin moiety is transferred from E2 to a high-energy thiol intermediate on E3, before its conjugation to the E3-bound substrate or to the previously conjugated ubiquitin moiety. This reaction is catalyzed by HECT domain E3s. 4: Degradation of the ubiquitin-tagged substrate by the 26S proteasome complex with release of short peptides. 5: Ubiquitin is recycled via the activity of deubiquitinating enzymes (DUBs). (Adapted from Gickman, M.H., Ciechanover, A., Physiol Rev, 2002).

Finally, with the help of a third enzyme, the E3 ligase, ubiquitin is transferred from the E2 to a lysine residue of a substrate protein. This final transfer of ubiquitin to the substrate results in an isopeptide bond between a substrate lysine and ubiquitin. In addition, E3s can also catalyze ubiquitin-ubiquitin conjugation to form a polyubiquitin chain. Although the precise mechanisms vary among the different E3s, they all promote the transfer of ubiquitin, either directly or indirectly, from an E2 to a substrate or another ubiquitin. Complete cycle of proteolytic pathway showing involvement of different enzymes and proteins is depicted in figure 4.

In addition to the aforementioned components of the ubiquitin conjugation cascade, other conjugation factors, or accessory factors have also been identified to play a role in either polyubiquitin chain assembly or in delivering ubiquitinated substrates to the proteasome. Furthermore, concurrent to the conjugation of ubiquitin to substrate proteins, the action of deubiquitinating enzymes (DUBs) antagonizes polyubiquitin chain assembly thus adding another layer of selectivity and regulation (286). In contrast to polyubiquitination, monoubiquitination of proteins targets them for endocytosis and lysosomal degradation (287). In addition, monoubiquitination can also act as a regulatory modification such as in protein sorting, histone function and transcription (287).

E3 Ubiquitin Ligase

During protein ubquitination, the E3 ubiquitin ligases (E3s) play a critical role in the ubiquitin conjugation cascade by recruiting ubiquitin-loaded E2s, recognizing specific substrates, and facilitating or directly catalyzing ubiquitin transfer to either the Lys residues (in most cases) or the N terminus of their molecular targets. E3s modify protein substrates by either monoubiquitylation or sequential attachment of ubiquitin molecules to form polyubiquitin chains. Based on the sequence homology of their E2-binding domains, E3s can generally be classified into three subfamilies: the homologous to E6-AP carboxyl terminus (HECT) domain containing E3s, RING (Really Interesting New Gene) finger domain- containing E3s, and the U box E3s.

HECT E3

The family of HECT (Homologous to E6-AP C-Terminus) domain E3s has been named after their founding member E6-AP (288). Members of this family of E3 ligases are characterized by the presence of a conserved catalytically active C-terminal region of approximately 350 amino acid residues, called the HECT domain. This catalytic activity is mediated by a conserved cysteine residue positioned approximately 35 residues upstream of the C-terminus within the HECT domain, which acts as a site of ubiquitin thioester formation (288, 289). HECT domain proteins have a modular structure consisting of the C-terminal HECT domain and a variable N-terminal extension. While the HECT domain is required for interactions with their cognate E2,

the N-terminus is presumably involved in determining the substrate specificity of the individual E3. Even though the crystal structure of HECT E3s in complex with their cognate E2s are available (290-292) the mechanism of how exactly ubiquitin is transferred from E2 to E3 and from E3 to the substrate remains ill-defined.

RING E3

The RING (Really Interesting New Gene) family of E3s is the largest family of E3s identified so far (293). Members of this family contain a RING finger or RING finger-like domain and are assumed to function predominantly as molecular scaffolds that bring other proteins together rather than as chemical catalysts. The RING finger domain consists of a series of histidine and cysteine residues with a characteristic spacing that allows for the coordination of two zinc ions in a cross brace structure. They contain a classic C3H2C3 or C3HC4 RING finger domain. This domain has four pairs of metal binding residues with a characteristic linear sequence of Cys-X2-Cys-X9-39-Cys-X1-3-His-X2-3-Cys/His-X2-Cys-X4-48-Cys-X2-Cys, where X can be any amino acid, although there are distinct preferences for particular types of amino acid at a particular position. The RING finger domain binds to two zinc atoms per molecule in a cross-braced system where the first and third pairs of cysteines/histidine form the first binding site and the second and forth pairs of cysteines/histidine form the other.

RING E3s can further be classified into two subtypes; a) single-subunit RING E3s and b) multi-subunit RING E3s. The single-subunit E3s consist of a single polypeptide that possesses the capacity to recognize the ubiquitination signals in their specific substrates through domains that, in general, are structurally distinct from the RING finger. Single-subunit RING E3s include Mdm2, which ubiquitinates p53 and c-Cbl, which is involved in down-regulation of growth factor receptors (294). In the multi-subunit RING E3s, the substrate recognition and the RING finger are on separate subunits. The SCF (Skp1-Cullin-F-box protein), the APC, and the VCB types belong to this class of multi-subunit RING E3s (295).

U Box E3s

In addition to the RING finger domain, two closely related domains the U-box (296) and the PHD/LAP (plant homeodomain or leukemia-associated protein) domains have been shown to

confer E3 activity (297). Though these motifs cannot bind zinc, they take up a RING-finger like conformation and are maintained by salt bridges and hydrogen bonds (298).

4.1.3 E6-AP (E6-Associated Protein)

The founding member of the HECT E3 family is E6-AP, a 100kDa polypeptide that interacts with the E6 protein of the cervical cancer-related human papillomavirus (HPV) (299). E6-AP forms a stable complex with the adaptor protein E6. The dimeric complex binds to and targets p53 for ubiquitin-mediated proteolysis, thus eventually interfering with the negative growth-regulating activities of this tumor suppressor protein (300-302). The gene encoding E6-AP (*UBE3A*) has been localized to the region q11-q13 of chromosome 15 (303). Five mRNA subtypes of E6-AP encoding three potential isoforms have been identified (304). These isoforms are approximately 100 kDa in size and vary at their N-terminal region. Difference in functionality of these isoforms is not known. In addition to its E3 activity E6-AP has also been reported to serve as a transcriptional coactivator for steroid hormone receptors (141). However, these functions are separable and independent of one another.

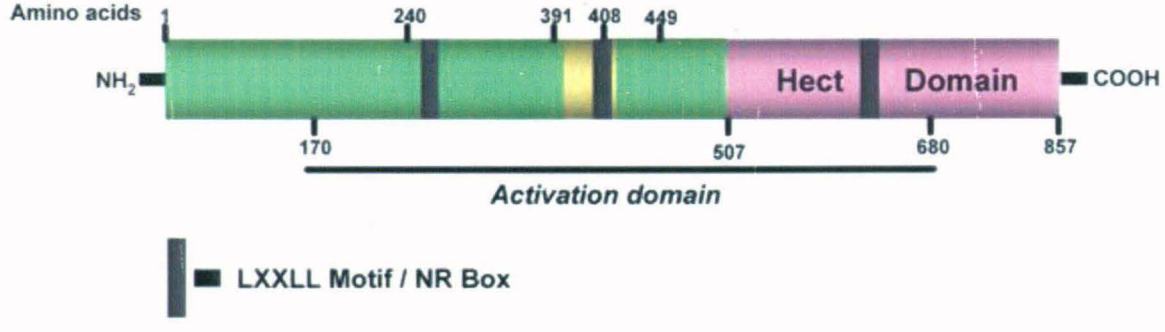

Figure 5: E6-AP possesses five well-characterized functional domains: (i) a *hect* domain, (ii) an E6 binding domain, (iii) a p53 binding domain, (iv) two nuclear receptor interaction domains and (v) an activation domain, which have been represented with the amino acid numbers they correspond to. The three LXXLL motifs (NR box) are shown dispersed throughout the protein. Two of these motifs are located in the amino terminus and the third one is located within the carboxy terminus of the protein. (Adapted from Ramamoorthy, S. and Nawaz, Z., NRS, 2008)

Understanding the physiological role of E6-AP is of interest because inactivation of *UBE3A* gene has been associated with Angelman Syndrome, a hereditary neurological disorder. Moreover, in the case of cervical cancer, the E6/E6-AP complex not only targets p53 for ubiquitin-mediated

degradation, but also targets other proteins, which is necessary for HPV-induced cervical carcinogenesis. Figure 5 depicts different functional domains of E6AP protein.

E6 and p53

The p53 tumour suppressor protein plays vital roles in cellular responses to various stress signals (305). In normal cells (HPV-negative cells), p53 is predominantly regulated by a negative feed-back loop with Mdm2, a RING finger ligase that targets p53 for proteasomal degradation (306, 307). However under stress conditions inactivation of the Mdm2 feed-back loop leads to stabilization of p53 levels and increased transcriptional activity, the outcome of this leads to cell-cycle dependent growth arrest, senescence or apoptosis. In contrast to most cancers, wherein the p53 gene is mutated (greater than 40%), it is rarely mutated in HPV-positive cervical carcinomas (308). Thus it has been hypothesized that the inactivation of the normal functions of p53 by E6 is similar to the inactivation of p53 by mutation and is a key step in HPV-induced cervical carcinogenesis. In contrast to E7 wherein the biochemical mechanism of pRB degradation remains poorly understood, E6 mediated degradation of p53 has been shown to involve the recruitment of E6-AP (301).

Biological role of E6-AP

The physiological role of E6-AP was examined using mice lacking the E6-AP gene (E6-APKO) (309). E6-APKO mice are viable, but exhibit several interesting phenotypic changes. Postnatal viability is reduced in E6-AP null homozygotes. Heterozygous mice lacking a maternal copy of E6-AP exhibits numerous adverse symptoms such as motor dysfunction and inducible seizures, reminiscent of AS in humans. In addition, in these mice, the cytoplasmic levels of p53 are grossly elevated in Purkinje cells and some hippocampal pyramidal neurons which may be responsible for the observed deficiencies in context-dependent learning and hippocampal long-term potentiation. These results suggest that E6-AP is required for the proper development of neuronal synapses in the central nervous system (309, 310). E6-AP KO mice of both sexes are less fertile than wild type mice apparently due to reduced size of their gonads. Consistent with this observation, defects in sperm production and ovulation were also seen. Moreover, the

hormonal induction of growth of the prostate and uterine glands by testosterone and estradiol was significantly reduced in these mice (311).

Proteasomal Defects Associated with Human Diseases

Since proteasome regulates the cellular levels of numerous proteins, defects in the proteasomal system can result in human diseases. Defects in the gene encoding human E3 ligase (UBE3A) is identified in Angelman's syndrome; a neurological disorder characterized by developmental delay, speech impairment, and movement or balance disorder (310). Another type of inherited proteasomal defect is Liddle syndrome which is characterized by abnormally high blood pressure due to incapability to maintain the balance of salt and water (312). In these patients, increased half-life of sodium channel due to deletion or mutation of ubiquitination site in sodium channel protein allows excessive reabsorption of sodium and water.

Skeletal muscle wasting in some pathological states also results from accelerated ubiquitin-mediated proteolysis (313). US2 and US11, the proteins encoded by human cytomegalovirus (CMV), target MHC class I heavy chain molecule for degradation by directing it from the ER to the cytoplasm, thereby evading immune system (314). Accumulation of ubiquitin conjugates is observed in senile plaques, lysosomes, endosomes, and intracellular inclusion bodies in neurological disorders such as Alzheimer's and Parkinson's disease although it is not known whether this accumulation is a direct result of defects in the proteasomal pathway (315).

The expression of E6-AP protein is decreased in human invasive breast and prostate carcinomas compared with their adjacent normal tissues. This down-regulation of E6-AP is accompanied by the elevation of ER in breast and AR in prostate carcinomas (316). Furthermore, *in vivo* data from E6-AP-knockout animals indicated that the expression levels of ER and AR are increased in E6-AP-null mammary and prostate glands, respectively, when compared with that of normal control animals, suggesting that E6-AP modulates the protein levels of ER in breast and AR in prostate glands (317). However, there are also published contradictory reports wherein E6AP has been shown to be upregulated in breast tumors (318, 319).

p53 is targeted for proteasomal degradation by mdm2 which is a p53 target gene containing E3 ubiquitin ligase activity (306). While mdm2 targets p53 for degradation, mdm2 is self-

ubiqutinated and degraded. Cyclin dependent kinase inhibitor p21waf/cip, another p53 target gene, is degraded by proteasome and GSK3 (glycogen synthase kinase 3) mediated phosphorylation (320). Rb (Retinoblastoma) protein is a tumor suppressor and negatively regulates G1/S transition by interacting with E2F transcription factor. Rb protein is degraded in an ubiquitin dependent manner (321). In addition, free E2F is also degraded in ubiquitin dependent manner by the 26S proteasome. Thus, HECT domain containing E3 ligases are important for homeostasis of protein levels and defects in their function may lead to various diseases including cancer. Table 1 depicts some of the HECT Family E3 ligsaes and their involvement in cancer.

Table 1: The HECT family of E3s and their involvement in cancer (322).

E3	Substrate (s)	Outcome of Substrate Ubiquitylation	Adaptors/ Regulators	Biological Function	Alterations in Cancer
E6-AP	p53	proteasomal degradation	E6	apoptosis	infection by high-risk HPV in cervical carcinomas
Huwe1	p53	proteasomal degradation	ARF	apoptosis, growth arrest	overexpression in breast, lung, and colorectal carcinomas
EDD	TopBP1	proteasomal degradation	unknown	DNA damage	amplification and overexpression in breast cancers
Nedd4-1	PTEN, Hgs, Eps15	proteasomal degradation, cytoplasmic/nuclear shuffling	unknown	Apoptosis, genome ntegrity endocytosis	overexpression in bladder and prostate carcinomas
Nedd4-2	Smad2,Smad4, TbR-I/III	proteasomal degradation	Smad6, Smad7	apoptosis, growth arrest	unknown
Itch	p63,p73, Notch1,c-Jun	proteasomal degradation	Numb	apoptosis, differentiatio n	unknown
WWP1	p53, Notch1, KLF2,KLF5, Smad2,Smad4, TbR I/II	proteasomal degradation, nuclear export	Smad2, Smad6, Smad7	apoptosis, growth arrest	amplification and overexpression in breast cancers
Smurf1	Smad1, Smad4, Smad5, TbR-I/II, BMP-RI/II	proteasomal/ lysosomal degradation	Smad6, Smad7	apoptosis, growth arrest	amplification and overexpression in pancreatic cancers
Smurf2	Smad1, Smad2, Smad4, Smad5, TbR-I/II	proteasomal/ lysosomal degradation	Smad2, Smad7	apoptosis, growth arrest	overexpression in esophageal squamous cell carcinomas

Mutations in the tumor suppressor gene APC is associated with the development of colon cancer. Mutations in the APC gene lead to accumulation of β-catenin which is normally degraded via the proteasome pathway. This increased level of β-catenin is related to carcinogenesis (323). Bcr-Abl fusion protein targets abl interactor proteins (ABI1 and ABI2) for proteasomal degradation (324). These abl interactor proteins are inhibitors of abl tyrosine kinase activity. Increased activity of the NFκB transcription factor promotes cell survival in some tumors and as a matter of fact NFκB is activated by the ubiquitin/proteasome pathway. IκBα which normally makes complex with NFκB is phosphorylated by IκB kinase and is ubiquitinated by SCF ubiquitin ligase for proteasomal degradation (325).

Thus, the ubiquitin-proteasome degradation plays an important role in the degradation of cellular proteins which are involved in regulating various cellular process, including cell cycle regulation, differentiation and apoptosis.

4.2 Ubiquitination of C/EBPα

Ubiquitination is a highly ordered multistep enzymatic process carried out by the ubiquitin pathway (figure 4) which is required for maintaining appropriate levels and cellular functions of various proteins. This targeted regulation of ubiquitination regulates various cellular processes including cell proliferation, apoptosis, differentiation, signal transduction and transcriptional regulation. C/EBPα is an important transcription factor involved in the various cellular processes like adipogenesis, granulopoiesis and cell cycle regulation. It undergoes various posttranslational modifications in the cell which regulates its cellular functions (326). Ubiquitination is among one such posttranslational modification which also regulates the activity of C/EBPα. In recent years, we and others have shown that C/EBPα can be ubiquitinated and degraded via proteasomal pathway (142, 327). However, the identities of E3 ligases required for C/EBPα ubiquitination has thus far remained elusive and only one such E3 ligase is know; Fbwx7 degrades C/EBPα and negatively regulates adipogenesis (328). Thus, the hunt for identification of new E3 ligases for C/EBPα is on.

Recently, E6AP has been shown to ubiquitinate a wide range of proteins including p53, p27 and PML-RARα which play an important role as a tumor suppressors and cell proliferation inhibitors (139, 329, 330). In one of our previously published study, we identified E6AP as a

molecular target of tamoxifen (331). Interestingly, Tamoxifen has also been shown to induce C/EBPα expression in HeLa cells leading to induction of apoptosis (332). In addition, it is noteworthy to mention that we and others have previously shown that C/EBPα, a tumor suppressor can be regulated via ubiquitination. Since Tamoxifen downregulates E3 ligase E6AP and induces expression of C/EBPα, we hypothesized that E6AP might be an E3 ligase for C/EBPα.

Thus, in the present study we have explored whether E6AP is an E3 ligase for C/EBPα. Further, through a number of experimental proofs; we demonstrate that E6AP indeed promotes C/EBPα ubiquitination leading to its proteasome mediated degradation and thereby inhibits its functional activity. In contrary, we show that E6AP knock down in C/EBPα stably transfected K562 cells (K562-p42 C/EBPα-ER cells) by siE6AP induces granulopoiesis. Taken together, our data suggests that E6AP may negatively regulate granulopoiesis by targeting C/EBPα for degradation via ubiquitin-proteasome pathway.

4.3 Results

4.3.1 E6AP degrades C/EBPα

In order to evaluate if E6AP targets C/EBPα for degradation, we used a heterogeneous system i.e HEK293T cells where there is no endogenous expression of C/EBPα.

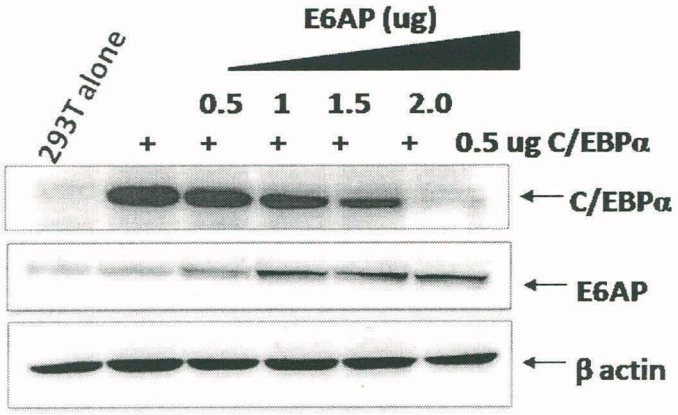

Figure 6: 293T cells were transfected with C/EBPα (0.5μg) along with E6AP (0.5μg-2.0μg). This was followed by immunoblotting with C/EBPα, E6AP and β actin antibodies.

These cells were transfected with 0.5μg C/EBPα together with increasing amounts of E6AP (0.5μg-2.0μg). 24h post transfection, whole cell extracts were prepared and resolved on 10% SDS-PAGE. Immunoblot with C/EBPα and E6AP antibody shows a drastic decrease in C/EBPα protein expression (figure 6). Similar transfections were performed with expression plasmid for dominant negative form of E6AP i.e. E6AP-C843A [A catalytically inactive form of E6AP where active site cysteine residue is substituted with alanine (C843A)]. This cysteine residue present in the catalytic domain transfers ubiquitin directly to the substrate via ubiquitin-enzyme cascade leading to their degradation.

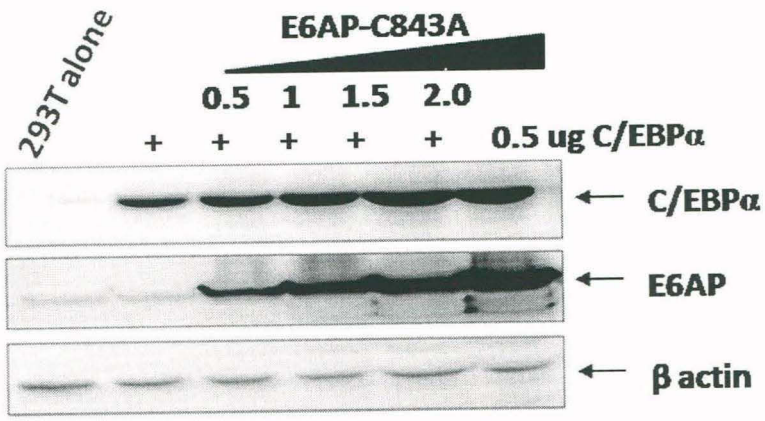

Figure 7: 293T cells were transfected with C/EBPα (0.5μg) along with increasing amounts of E6AP-C843A (0.5μg-2.0μg) and were followed by immunoblotting with C/EBPα, E6AP and β actin antibodies.

Unlike with E6AP transfection, no downregulation of C/EBPα protein was observed with E6AP-C843A (figure 7). In fact, E6AP-C843A rather stabilized C/EBPα protein due to its dominant negative nature over endogenous E6AP.

Thus, these results suggest that E6AP may lead to downregulation of C/EBPα protein expression in a dose dependent manner. Because E6AP is an E3 ligase, this downregulation of C/EBPα protein expression may apparently be via its ubiquitination.

4.3.2 E6AP and C/EBPα physically interact with each other

Proteins control all biological systems in a cell, and while many proteins perform their functions independently, the vast majority of proteins interact with others for proper biological activity. The biological activities are controlled by protein-protein interactions which is required for: (i) to

alter the kinetic properties of enzymes which may be the result of subtle changes in substrate binding or allosteric effects and allows moving a substrate between domains or subunits resulting ultimately in an intended end product (ii) to create a new binding site, typically for small effector molecules to inactivate or destroy a protein (ubiquitination) (iii) to change the specificity of a protein for its substrate through the interaction with different binding partners; e.g., to demonstrate a new function that neither protein can exhibit alone and to serve a regulatory role in either an upstream or a downstream event.

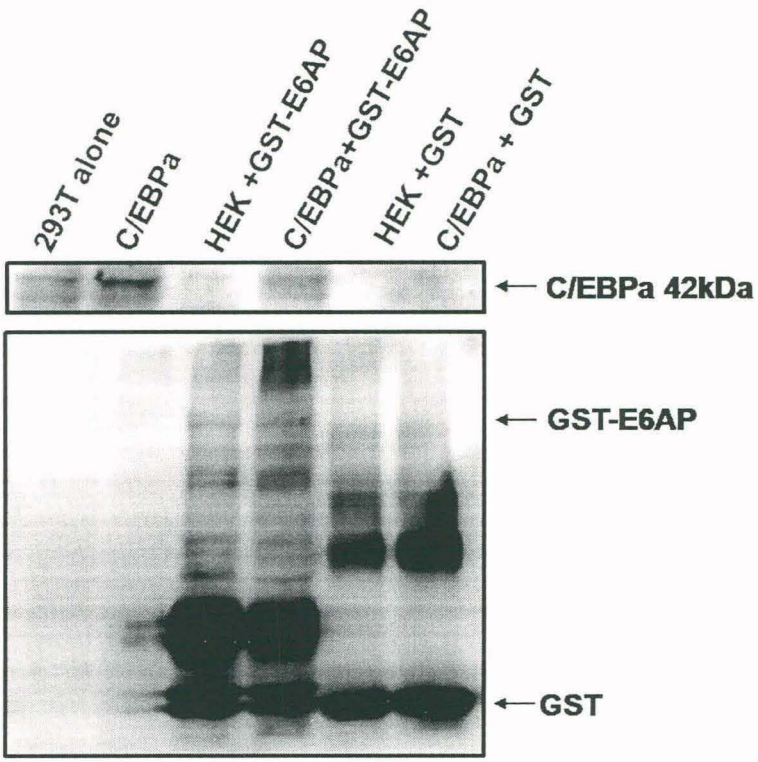

Figure 8: GST-Pull down using GST-E6AP and C/EBPα transfected 293T protein lysate was performed. Washed beads were harvested with SDS loading buffer and resolved on 10% SDS-PAGE, followed by immunoblotting with C/EBPα and E6AP antibody.

Because E6AP inhibits C/EBPα protein expression presumably by promoting its ubiquitination, we hypothesized that these two proteins may physically interact. To address this, we performed *in-vitro* GST pull down assay using a bacterially purified GST-E6AP fusion protein and nuclear extracts of 293T transfected with C/EBPα. After pull down, bead bound GST-E6AP with its interacting proteins from the lysates were resolved on 10% SDS-PAGE and probed with C/EBPα

antibody. As shown in figure 8 C/EBPα does interact with GST-E6AP while no interaction is observed with GST alone.

To further assess the *in-vivo* interaction, we performed C/EBPα co-immunoprecipitation from nuclear extracts of 293T cells transfected with C/EBPα and E6AP together.

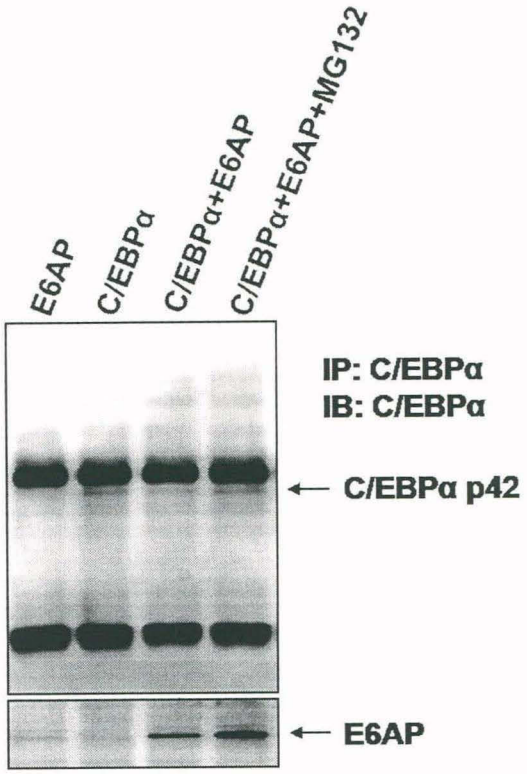

Figure 9: Coimmunoprecipitation using C/EBPα antibody was performed from lysates prepared from C/EBPα and E6AP transfected 293T cells as indicated. In one of the C/EBPα and E6AP transfected condition, cells were treated with MG132 3h prior to lysate preparation.

Co-immunoprecipitates were lysed in SDS buffer and resolved on 10% SDS-PAGE. Immunoblot with C/EBPα followed by E6AP antibody confirmed the *in vivo* interaction between C/EBPα and E6AP. For co-immunoprecipitation studies, we also treated C/EBPα and E6AP transfected cells with proteasome inhibitor (25μg MG132, 3h prior to harvest). As expected in MG132 treated condition, a prominent interaction between C/EBPα and E6AP was observed which is apparently due inhibition of C/EBPα degradation (figure 9).

4.3.3 E6AP colocalizes with C/EBPα in the nucleus

C/EBPα, a nuclear protein is a transcription factor. In previous results we show that E6AP inhibits C/EBPα and does physically associates with it. To further corroborate our findings we asked if these two proteins colocalize together, therefore, we first assessed the status of C/EBPα and E6AP in nuclear and cytoplasmic fractions by transfecting 293T with indicated expression plasmids [C/EBPα alone (0.5ug) or together with E6AP (2.0ug) or E6AP-C843A (2.0ug)]. 24h post transfection, nuclear extracts were prepared and resolved on 10% SDS-PAGE. Immunoblot with C/EBPα and E6AP antibody shows that E6AP does inhibit C/EBPα protein expression in the nucleus suggesting them to be co-localizing together. As expected, E6AP-C843A did not affect the C/EBPα protein expression in the nucleus.

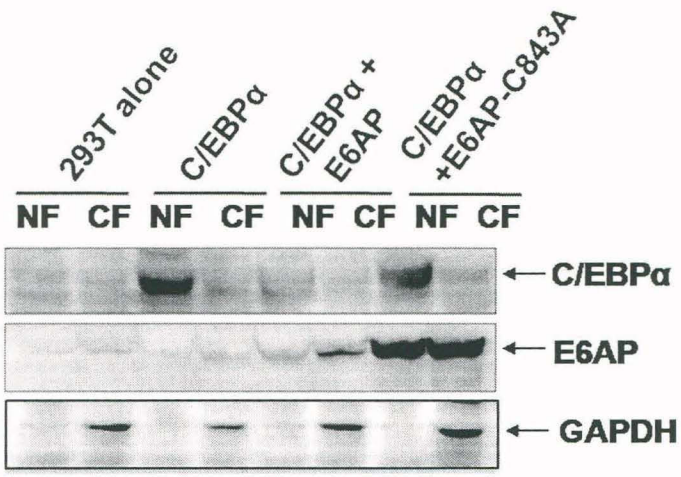

Figure 10: 293T cells were transfected with C/EBPα (0.5μg), E6AP (2.0μg) and E6AP-C843A (2.0μg) as indicated. 24h post transfection, nuclear extracts were prepared, resolved on 10%SDS PAGE and probed with C/EBPα, E6AP and GAPDH antibodies. GAPDH was used a control for cytoplasmic protein extract.

In order to further examine the co-localization of C/EBPα and E6AP, we performed immunofluorescence assay in 293T cells. C/EBPα was transfected in 293T cells and slides were prepared 24h post transfections for confocal microscopy. In untransfected 293T cells, E6AP is rather more in cytoplasm while no C/EBPα is seen at all. However, in C/EBPα transfected 293T cells, both C/EBPα and E6AP seem to be localized in the nucleus which is also obvious from

appearance of greenish yellow colour in merged panel of C/EBPα transfected 293T cells compared to 293T alone (figure 11).

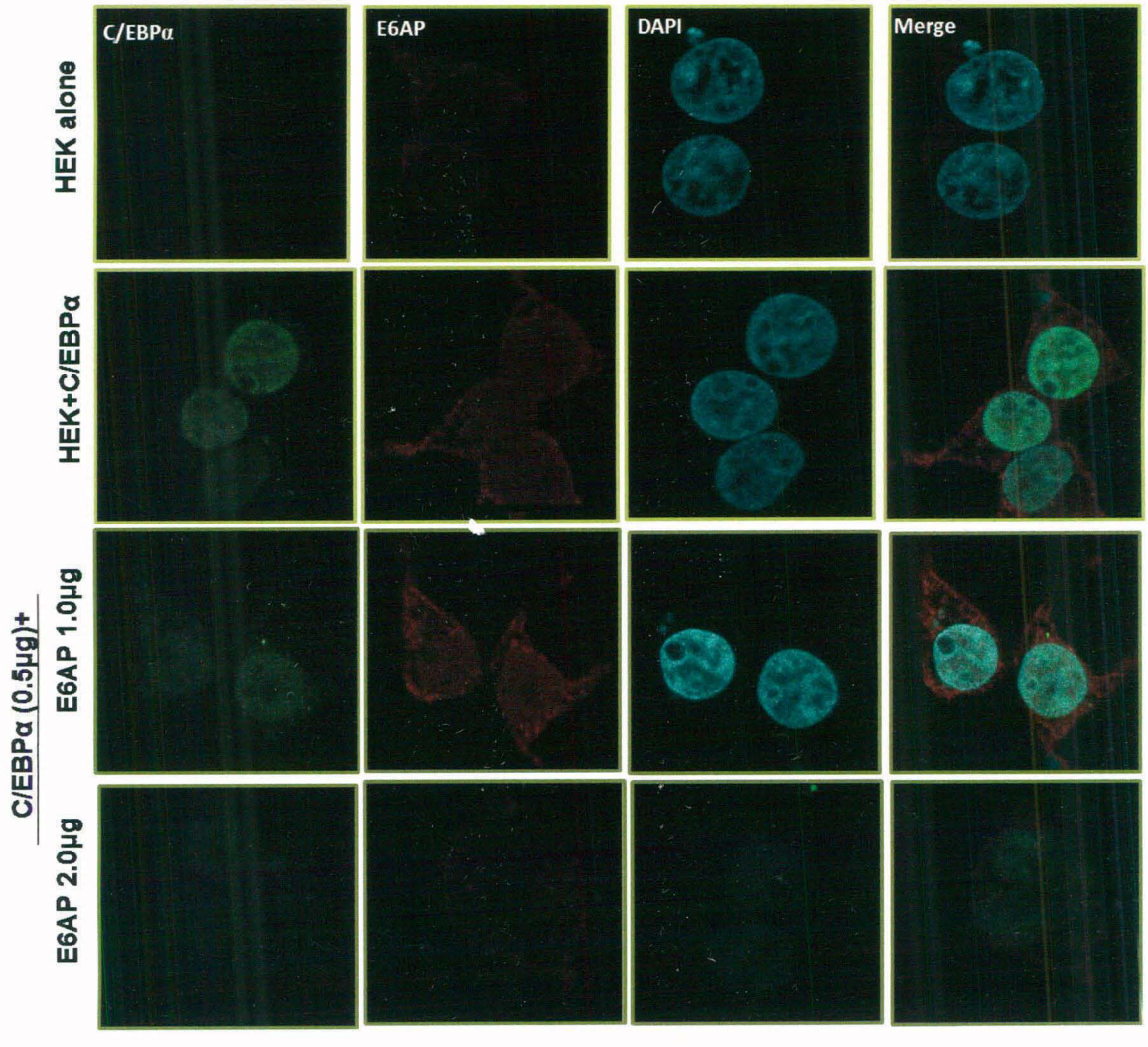

Figure 11: 293T cells were transfected with C/EBPα; 24h post transfection cells were fixed and subjected to immunofluorescent staining. Slides were visualized with Leica confocal microscope (100 X). Co-localization is shown in greenish yellow in the merge pictures in the lower panel.

Further, in 293T cells co-transfected with C/EBPα and E6AP together, two proteins colocalize with obvious degradation of C/EBPα in the nucleus with the increasing concentrations of E6AP (1.0μg and 2.0μg). Taken together, these data suggest that C/EBPα and E6AP colocalize together in the nucleus.

4.3.4 E6AP degrades C/EBPα via ubiquitin-proteasome pathway

For the degradation of a target protein, cells utilize ubiquitin/proteasome system which requires energy expenditure and cleaves every peptide bond in target protein. Many cellular proteins involved in the critical cellular processes, such as proliferation, differentiation, DNA repair, and apoptosis, have been discovered to be degraded by the ubiquitin/proteasome pathway.

Because E6AP, being an E3 ligase inhibits C/EBPα protein expression, we asked whether this E6AP mediated inhibition of C/EBPα is due ubiquitin mediated degradation of C/EBPα involving proteasome pathway. To address this, 293T cells were transfected either with C/EBPα or together with E6AP expression plasmids. 24h post transfection, cells were treated with MG132 (25μM) for 3h in C/EBPα and E6AP co-transfected condition followed by lysate preparation. Immunoblot with C/EBPα and E6AP antibody nicely shows that C/EBPα protein expression is dramatically restored upon MG132 treatment (figure 12) which strongly suggests that E6AP promotes proteasomal degradation of C/EBPα. Note that MG132 is a potent inhibitor of protasome pathway.

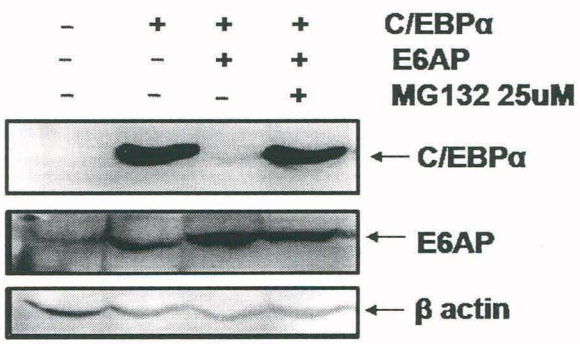

Figure 12: 293T cells were transfected with C/EBPα (0.5μg) and E6AP (2.0μg); cells were treated with 25μM MG132 3h prior to cell harvesting. Lysates were resolved on 10% SDS PAGE and probed with C/EBPα, E6AP and β-actin antibodies after stripping and reprobing the same blot.

However, in order to further support the notion that E6AP mediated degradation of C/EBPα involves ubiquitin-proteasome pathway, we performed in-vivo ubiquitination assay by transfecting 293T cells with expression plasmids for C/EBPα-His, Ubiquitin, E6AP and E6AP-C843A. Post 24h transfection, cells were harvested, C/EBPα was co-immunoprecipitated and probed with ubiquitin antibody. As shown in figure 13, there is mild ubiquitination in C/EBPα

alone transfected condition while a prominent ladder pattern is observed in C/EBPα, ubiquitin and E6AP co-transfected condition. Also, a very prominent ladder pattern is seen in MG132 treated cells which is due to inhibition of proteasome mediated degradation of ubiquitinated C/EBPα. Interestingly, very faint ladder pattern was observed in E6AP-C843A transfected condition which is expected owing to its lack of ligase activity.

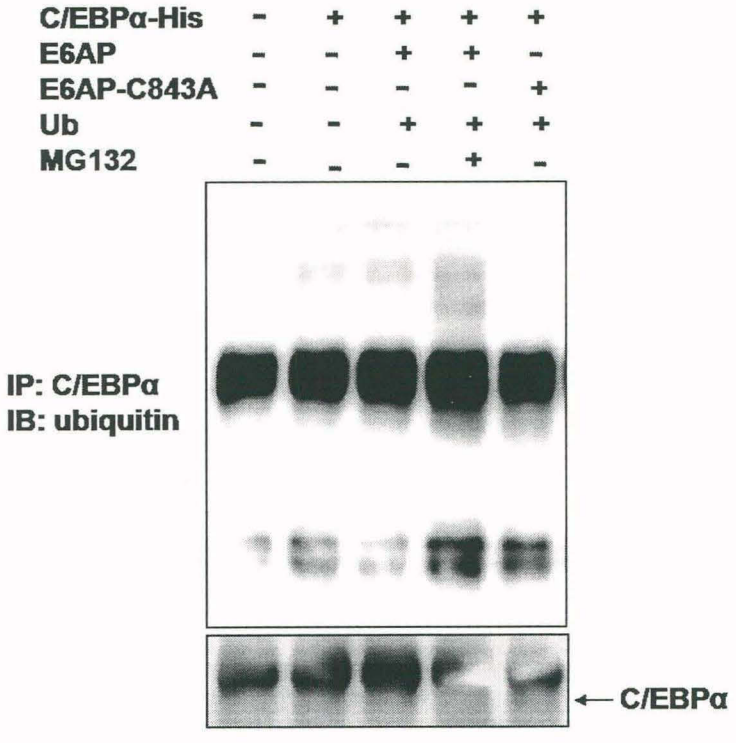

Figure 13: 293T cells were transfected with expression plasmids for C/EBPα (1.0μg), E6AP (2.0μg), E6AP-C843A (2.0μg) and ubiquitin (1.0μg) as indicated. 48h post transfection, cells were treated with 25μM MG132 3h prior to cell harvest in the indicated lanes. C/EBPα was co-immunoprecipitated and probed with ubiquitin antibody. Same membrane was stripped and reprobed with C/EBPα antibody (lower panel).

In order to further strengthen our finding that E6AP affects stability of C/EBPα at protein level, we sought to inhibit new protein synthesis by treating cells with cycloheximide (Inhibits new protein synthesis in eukaryotes by inhibiting peptidyl transferase).

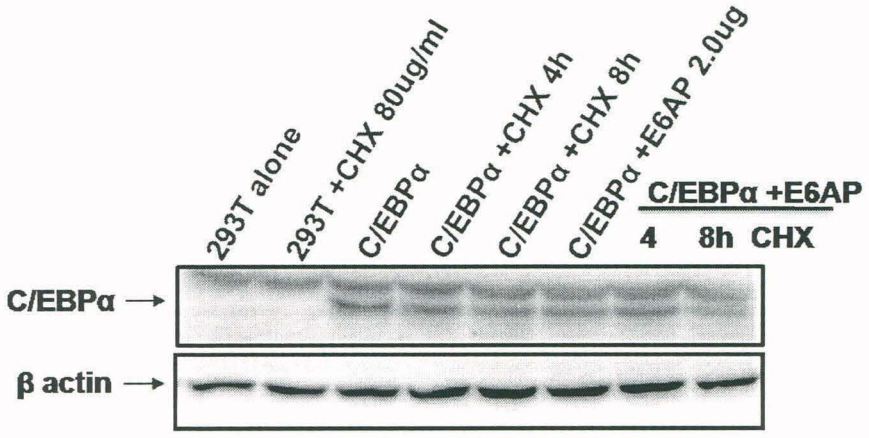

Figure 14: Half life of C/EBPα was measured with cycloheximide in the presence of E6AP. 293T cells were transfected with C/EBPα and E6AP. Cells were treated with Cycloheximide (80μg/ml) post 24h transfection for 4h and 8h. Lysates were resolved on 10% SDS PAGE and probed with C/EBPα antibody.

Therefore, 293T cells were transfected either with C/EBPα alone or together with E6AP. 24h post transfection, de novo protein synthesis was inhibited by addition of cycloheximide for 4h and 8h. Subsequently, lysates were prepared and resolved on 10% SDS-PAGE. In figure 14 immunoblot with C/EBPα shows that stability of C/EBPα protein is dramatically reduced in the presence of E6AP. Taken together, these data strongly suggest that E6AP modulates C/EBPα protein stability by promoting its degradation by ubiquitin-proteasome pathway.

4.3.5 E6AP mediated degradation of C/EBPα negatively affects its transactivation potential

Since E6AP targets C/EBPα for degradation, we asked if E6AP mediated C/EBPα degradation has any effect on C/EBPα transactivation potential. To answer this, we performed luciferase reporter assay on a minimal pTK promoter containing two C/EBP binding sites.

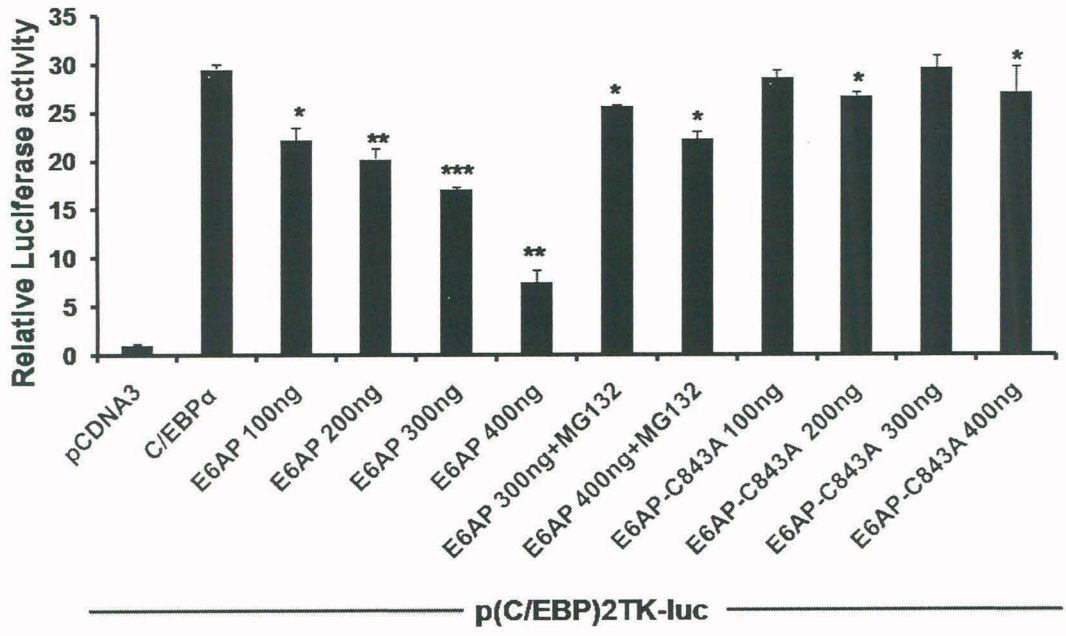

Figure 15: 293T cells were transiently transfected with pTK-C/EBP-luc reporter and expression plasmids for C/EBPα, E6AP and E6AP-C843A. 24h post transfection, luciferase activity was measured. MG132 treatment was given 3h prior to cell harvesting for luciferase activity measurement. Data are representative of three independent experiments. Results are given as standard error of mean (± s.e.m.); *p<0.05; **p<0.001, ***<0.0001.

Indicated amounts of reporter vector and expression plasmids for C/EBPα, E6AP and E6AP-C843A were transfected in 293T cells. Post 24h transfection, luciferase activity was measured which showed that co-transfection of E6AP with C/EBPα significantly inhibited C/EBPα transactivation capacity in a dose dependent manner. Further, MG132 treatment efficiently restored C/EBPα transactivation potential even in the presence of E6AP. Additionally, co-transfection of E6AP-C843A with C/EBPα did not inhibit transactivation potential of C/EBPα. This data indicates that catalytically active E6AP promotes C/EBPα degradation and thereby has inhibitory effects over C/EBPα functions.

4.3.6 Preparation and verification of C/EBPα-ER stably transfected K562 (K562-p42-C/EBPα-ER) cells

β -estradiol inducible stable clones were generated in K562 cell line. The vector construct used for the stable clone preparation was pBabe-Puro-p42 C/EBPα-ER and pBabe-Puro empty vector (143). For this K562 cells were transfected with pBabe-Puro-p42 C/EBPα-ER and empty vector,

selection of cells was performed in RPMI1640 supplemented with 10% FBS, 1X antibiotic solution and 2.0μg puromycin. Cells resistant to puromycin (2.0μg) were cultured in puromycin supplemented medium for two further weeks. In this C/EBPα-ER, 42kDa isoform is linked to estradiol receptor ligand binding domain generating a fusion protein of 66kDa, once stably transfected, this fusion protein stays in the cytoplasm bound with heat shock proteins (HSPs). Stimulation with β -estradiol activates it by binding to ER of this fusion protein, this ligand bidning relieves the HSPs bound to C/EBPα-ER. This relieved C/EBPα-ER then migrates to the nucleus and binds to its target genes. It is noteworthy to mention that β -estradiol binding to this fusion protein also promotes its degradation. In total, six clones were selected by the serial dilution of cells. These six clones were cultured for another two weeks in RPMI 1640 supplemented with puromycin (2.0μg/ml) and subsequently C/EBPα-ER expression level was confirmed by immunoblotting.

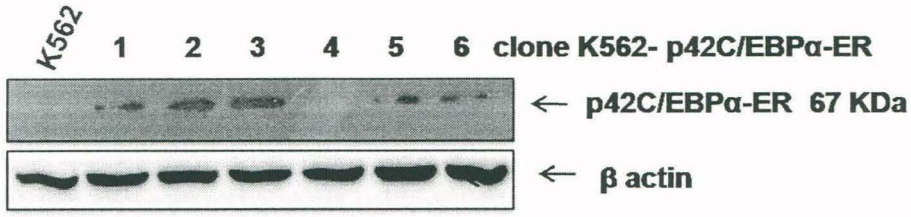

Figure 16: Expression of C/EBPα was confirmed in the K562 p42C/EBPα stable clones. Lysates were resolved on 10% SDS-PAGE and blot was probed with C/EBPα and β-actin antibody.

These clones were subjected to the western blot analysis and blot was probed with C/EBPα antibody. Five out of six clones were positive for the expression of C/EBPα-ER (figure 16). Expression levels of C/EBPα-ER were most prominent in clone 2 and 3, therefore, for our further study we selected clone2, K562- p42C/EBPα-ER.

Further, in order to confirm the functional activity of fusion protein in Clone 2 we performed series of experiments. Expression of C/EBPα-ER in clone 2 was further confirmed by immunoblotting (figure 16) and was then assessed for its ability to undergo granulocytic differentiation upon induction with β-estradiol for this. K562-p42C/EBPα-ER stable cells (clone 2) were stimulated with 5μM β-estradiol, post 5 days of induction; cells were cytospun and

stained with May-Grunwald Giemsa stain which nicely shows the apprearance of granulocyte like differentiated cells while most of blast cells were seen in the untreated cells (figure17). The granulocytic differentiation in K562- p42C/EBPα-ER cell line verified that the clone is functional and hence was used for further studies.

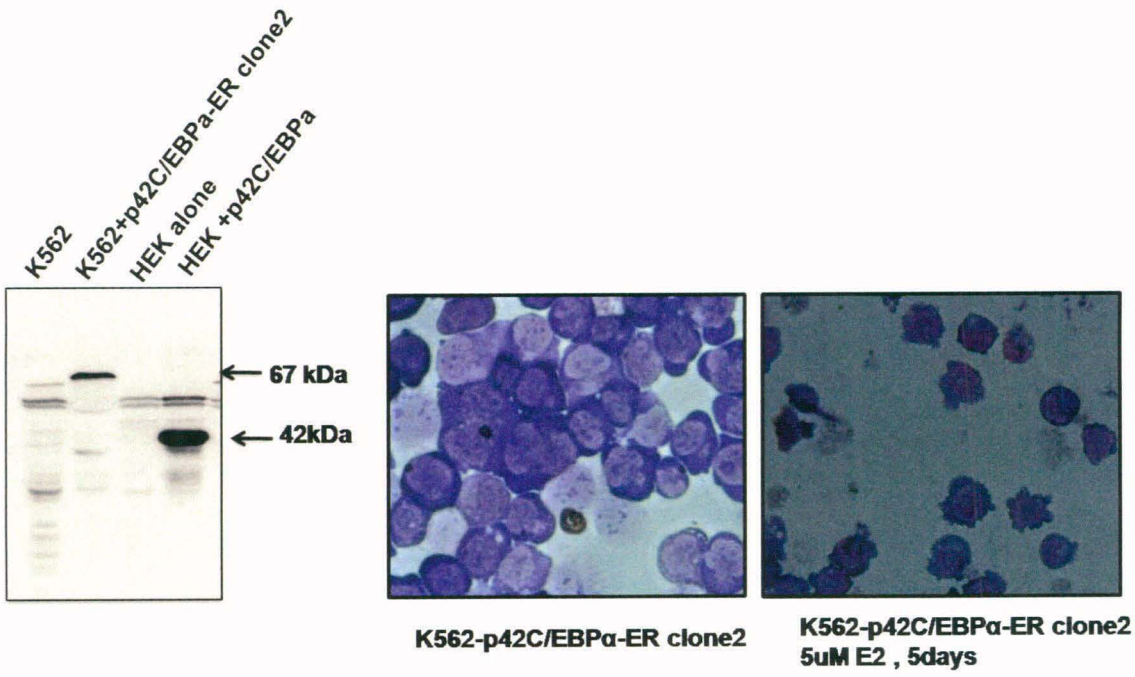

Figure 17: C/EBPα expression in K562-p42C/EBPα-ER stable clone 2 was confirmed with western blot analysis. Cell lysate was resolved on 10% SDS-PAGE and probed with C/EBPα antibody. 293T cells transfected with C/EBPα plasmid was used as positive control. May-Gruenwald Giemsa staining was performed in the K562-p42C/EBPα-ER stable clone after induction with 5μM estradiol for 5 days.

4.3.7 Downregulation of E6AP promotes granulopoiesis

E6AP promotes degradation of C/EBPα leading to its functional inactivation. This was further validated in the β-estradiol inducible stable cell line of K562 expressing C/EBPα-ER. In this system C/EBPα becomes functionally active when induced with estradiol however, there is rapid degradation of C/EBPα. This rapid degradation of C/EBPα starts within 1hr of induction with estradiol (figure 18a). Since E6AP also promotes degradation of C/EBPα, we presumed this rapid degradation of C/EBPα in these clones can be inhibited by E6AP- C843A. To address this, K562-p42C/EBPα-ER stable cells were transfected with the expression plasmid for E6AP-

C843A. 24h post transfection, cells were stimulated with β-estradiol and were harvested after indicated time points.

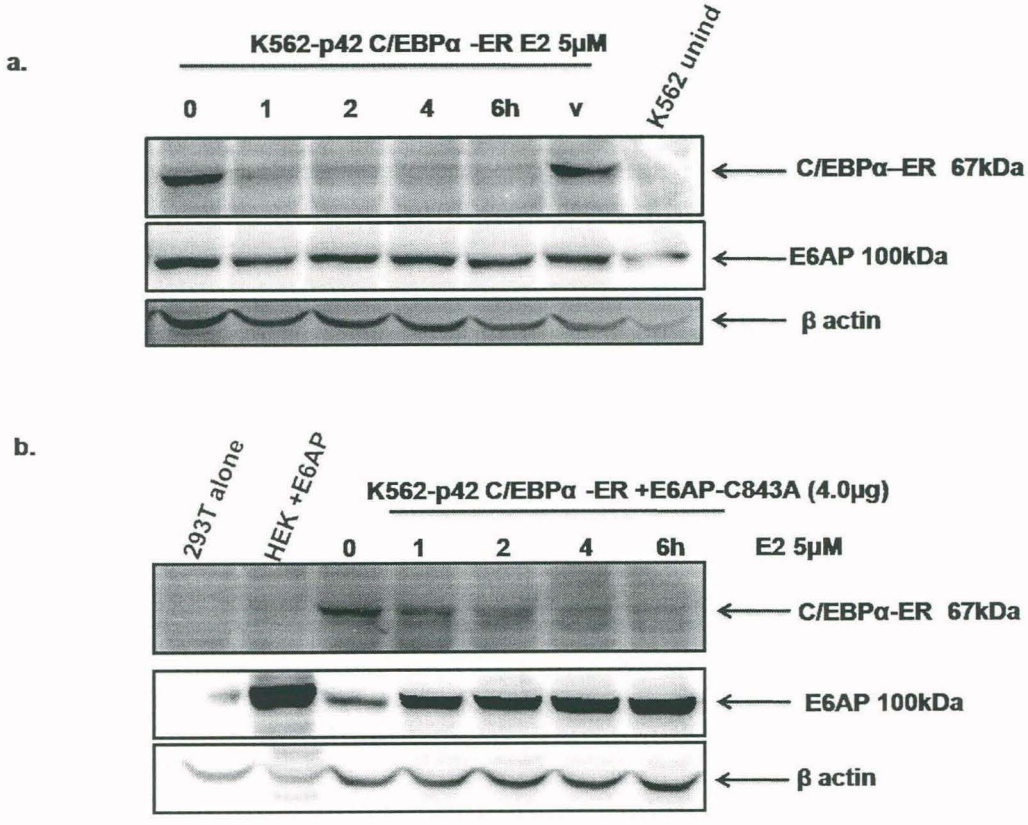

Figure 18: (a) K562-p42 C/EBPα-ER stable cell line was induced with 5uM E2 for the indicated time points. Lysates were resolved on 10% SDS PAGE and probed with C/EBPα antibody. Same immunoblot was stripped and reprobed with E6AP and β actin antibody.

As shown in figure 18b, the rate of degradation of C/EBPα is substantially inhibited in the presence of E6AP-C843A and protein is stabilized for the longer duration (figure 18b). This clearly indicates that C/EBPα protein degradation is inhibited in the presence of E6AP-C843A.

Since transfection of E6AP-C843A in K562 p42-C/EBPα-ER cells inhibited the rate of degradation of C/EBPα-ER, we assumed that E6AP knock down by siE6AP should enhance myeloid differentiation in these cells upon β-estradiol induction. To address this, K562 p42-C/EBPα-ER cells were transfected with siE6AP and control siRNA. 24h post transfection, cells were induced with β-estradiol for 72h and then subjected to FACS analysis for the expression of

cd11b after labeling with cd11b-PE conjugated antibody; a surface marker for myeloid differentiation.

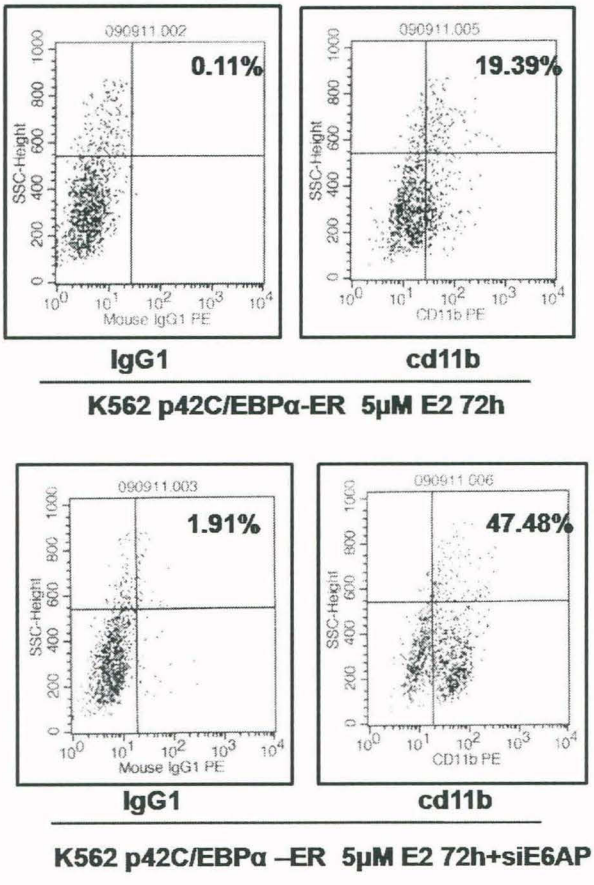

Figure 19: K562 p42-C/EBPα-ER stable clones were transfected with siE6AP and were induced with 5uM estradiol. After 72h of induction cells were washed and labeled with cd11b-PE conjugated antibody for the FACS analysis.

The percentage of cells undergoing differentiation increased nearly two fold in the cells transfected with siE6AP as compared to the cells treated with estradiol alone (figure 19). Thus, these results indicate that inhibition of E6AP may stabilize C/EBPα leading to its enhanced functional activity.

4.4 Discussion

Most cancers are caused by activating mutations in proto-oncogenes and/or inactivating mutations in tumor suppressor genes which render the function of corresponding proteins inactive. In addition, perturbed stability of these regulatory proteins is also a leading cause for their impaired function. The target proteins are usually degraded by proteasomal degradation system in a controlled fashion. This proteasome mediated degradation of target proteins involves ubiquitin attachement to the target proteins through a series of enzymatic reactions where E3 ligase is one of the crucial enzymes. E6AP is one such E3 ubiquitin ligase that has been implicated in degradation of tumor suppressor protein p53 in conjunction with HPV viral E6 protein in some of the cancers. This degradation of p53 affects the apoptosis and cell cycle as it is involved in the cellular regulation of these pathways. Like p53, there are many more cellular proteins reported to be regulated by E6AP through ubiquitin mediated protein degradation (333-337).

C/EBPα is an important transcription factor involved in the regulation of various cellular processes including granulopoiesis. We and others have previously shown that C/EBPα can be degraded via ubiquitin proteasome pathway, however, except Fbwx7 (328), the E3 ligases involved in its degradation has largely remained elusive. In the present study, we for the first time report that HECT domain containing E3 ligase E6AP can also target C/EBPα for proteasomal degradation.

We explored the role played by E6AP in the ubiquitination of C/EBPα, their interaction and consequences of this interaction in granulopoesis. Here we provide several evidences that E6AP is an E3 ligase for C/EBPα. First, E6AP promotes the proteasomal degradation of C/EBPα (figure 6, 12 and 13). Second, E6AP shortens the half life of C/EBPα protein (figure 14). Third, a catalytically inactive E6AP-C843A inhibits degradation of C/EBPα (figure 7 and 13). Fourth, we have shown that C/EBPα and E6AP physically associate (figure 8 and 9) and co-localize together in the nucleus (figure 10 and 11).These data together indicate that E6AP has a direct role in the C/EBPα proteasomal degradation.

As a consequence of C/EBPα protein degradation mediated by E6AP, there was decrease in the C/EBPα transactivation capacity as shown in figure 15. Thus as expected, E6AP mediated destabilization of C/EBPα has direct effects on its functional activity. E6AP mediated negative

effect on the functional activity of C/EBPα is again addressed in K562-p42 C/EBPα-ER cells which stably expresses C/EBPα protein. As shown in figure 16 and 17 there is expression of p42C/EBPα in the K562 stable clones which induces granulocytic differentiation after estradiol induction.

C/EBPα is required for granulocytic differentiation, and restoration of its proper function can enable leukemic stem and progenitor cells in AML and CML myeloid blast crisis to overcome the block of differentiation and undergo maturation into functional effector cells. The down regulation and functional inactivation of C/EBPα is involved in the tumor development. There is increasing evidence that alterations of the key myeloid transcription factor C/EBPα have an important role in the pathogenesis of acute myeloid leukemia. Importantly, different mechanisms mediate decreased C/EBPα function in different subgroups of patients with AML. Therefore, enhancing/stabilizing C/EBPα protein expression or function in myeloid leukemia cells can be very beneficial from therapeutic perspective. Since reintroduction of C/EBPα in K562 cells promotes granulocytic differentiation (338), we assumed that the stabilization of C/EBPα protein in these stable cells should have similar effects. To examine this, we knocked down E6AP in these cells using siE6AP and 24h post transfection these cells were induced with β-estradiol. siE6AP mediated knock down of E6AP in K562-p42 C/EBPα-ER stable cells substantially increased the percentage of cells undergoing differentiation in the presence of estradiol. As shown in figure 19, the percentage of cells expressing cell surface differentiation marker cd11b is enhanced. This further strengthens our finding that E6AP degrades C/EBPα and knock down of E6AP may stabilize C/EBPα and enhance its functions.

Taken together, these studies suggest that E6AP, a HECT domain containing E3 ligase targets C/EBPα for degradation via ubiquitin-proteasome pathway. Further, inhibition of E6AP via siE6AP knock down may stabilize C/EBPα protein leading to enhanced granulopoiesis. Thus, understanding the role of E6AP in normal differentiation can have therapeutic implications for patients with myeloid leukemia and various other cancers.

Summary

Summary

Leukemia is a malignant hematological disorder characterized by proliferation of abnormal white cells that infiltrate the bone marrow, peripheral blood and other important organs. Leukemia arising from myeloid cells is known as myeloid leukemia which may either be chronic myeloid leukemia (CML) and/or acute myeloid leukemia (AML). CML is a clonal myeloproliferative disorder of hematopoietic stem cells consistently associated with the Philadelphia chromosome (Ph) resulting from reciprocal translocation of the long arms of chromosome 9 and 22 which generates a 210kDa hybrid oncoprotein (Bcr/Abl). The development of the Bcr-Abl tyrosine kinase inhibitor imatinib mesylate (Gleevec, formerly known as STI571) is the treatment of choice for chronic phase CML and its remarkable therapeutic effects suggest that the blast crisis transition will be postponed for several years in most patients with CML. AML is a malignant disease of hematopoietic system in which cells accumulate in an undifferentiated state due to mutations that prevent their normal differentiation and allow undifferentiated cells to survive and proliferate. The molecular changes that occur in AML usually lead to either abnormal cell proliferation (FLT3 and Ras mutations) or block in differentiation (AML1/ETO, PML/RAR alpha, C/EBPα mutations) or suppression of apoptosis (Bcl2 overexpression). Agents like ATRA can induce leukemic cell differentiation in other AML subtypes, although these effects may differ between patients. Therefore, screening of compounds having potential to induce apoptosis and differentiation in myeloid blasts is required that may have substantial impact in the development of better therapeutics for leukemia. The increased understanding of apoptosis and differentiation pathways has directed attention to components of these pathways as potential targets for therapeutic agents. Therefore, future studies in leukemia should focus on (i) the identification of new agents with more predictable effects on differentiation and apoptosis; (ii) the use of clinical and laboratory parameters to define new subsets of leukemic patients in which differentiation/apoptosis induction has a predictable and beneficial effect, and (iii) further characterization of how blast cells sensitivity to drug-induced apoptosis is modulated by differentiation induction. This prompted us to screen for natural/synthetic compounds for their antileukemic property. From this screening we found ormeloxifene as a potential antileukemic agent.

Ormeloxifene is a non-steroidal selective estrogen receptor modulator (SERM) and has been shown to possess potential anticancer activities in breast and uterine cancer. In the present study, we have assessed the effects of ormeloxifene in myeloid leukemia cells. We show that ormeloxifene induces apoptosis in a dose dependent manner in a variety of myeloid leukemia cells, more strikingly in K562. Our data demonstrates that Ormeloxifene induced apoptosis in K562 cells involves activation of Extracellular Signal-Regulated Kinases (ERK) and subsequent cytochrome-c release leading to mitochondria mediated caspase-3 activation. Ormeloxifene induced apoptosis via ERK activation was drastically inhibited by prior treatment of K562 cells with ERK inhibitor PD98059. Ormeloxifene also inhibits proliferation of K562 cells by blocking them in G0-G1 phase by inhibiting the expression of c-myc and enhancing the expression of cell cycle inhibitor protein p21. K562 is a chronic myeloid leukemia cell line having Bcr-Abl fusion oncoprotein which makes it highly proliferative so we sought to investigate the effect of ormeloxifene on this fusion protein. We further show that ormeloxifene inhibits the phosphorylation as well as protein expression of Bcr-Abl leading to inactivation of survival pathway. Ormeloxifene induced apoptosis is translatable to mononuclear cells isolated from CML patients. Thus, ormeloxifene induces apoptosis in K562 cells via phosphorylation of ERK and mitochondria mediated caspase activation and arrest them in G0-G1 phase of cell cycle by reciprocal regulation of p21 and c-myc. Because chronic myeloid leukemia is characterized by low apoptosis and high proliferation/survival rate, our data suggest that inclusion of ormeloxifene in the therapy of CML can be of potential utility.

Lineage differentiation in blood cells is a highly regulated process. This regulation includes multiple factors and pathways. Various transcription factors are involved in the commitment of lineage differentiation at the different stages of blood cell development. C/EBPα is one such key transcription factor required for differentiation of various cell types including myeloid cells. In addition, it is also a tumor suppressor protein involved in cell cycle regulation and apoptosis. Being a crucial factor in the neutrophil differentiation it is highly vulnerable to mutational defects that often observed in myeloid leukemia. Thus, loss of C/EBPα expression and function in myeloid cells contributes to block in differentiation leading to leukemia. Understanding the regulation of C/EBPα in myeloid cell differentiation can provide better insights in to the pathophysiology of leukemia. Post translational modifications of protein regulate its functional activity; phosphorylation, sumoylation and ubiquitination are some of the

post-translational modifications which are reported to modulate the activity of C/EBPα protein at various stages. Ubiquitination is a post translational modification where E3 ubiquitin ligases attach an activated ubiquitin to a target protein leading to its subsequent proteasome degradation. C/EBPα is reported to be ubiquitinated and subsequently degraded, however, so far Fbxw7 is the only reported E3 ligase for C/EBPα. In the present study, for the first time we demonstrate that E6AP, an E3 ubiquitin ligase also serves as an E3 ligase for C/EBPα. E6AP is a known E3 ligase involved in the ubiquitination of various cellular proteins like p53, p21 and PML-RARα. We show that E6AP degrades C/EBPα in a dose dependent manner by ubiquitin mediated proteasome degradation; however, catalytically inactive E6AP-C843A rather stabilizes it. E6AP, an E3 ubiquitin ligase degrades C/EBPα and thereby modulates its transcriptional activity. E6AP interacts with C/EBPα and co-localizes together in the nucleus. We further show that E6AP mediated degradation of C/EBPα inhibits its transactivation potential thereby modulating its functional activity. This clearly indicates that E6AP has a direct role in the C/EBPα proteasomal degradation. E6AP mediated negative effects on the functional activity of C/EBPα are again exemplified in K562-p42C/EBPα-ER cells which stably expresses estradiol receptor ligand binding domain linked C/EBPα protein. The rate of degradation of C/EBPα in K562-p42C/EBPα-ER cells upon β-estradiol induction is decreased in the presence of E6AP-C843A. In addition, knock down of E6AP in K562-p42C/EBPα-ER cells enhances the granulocytic differentiation of these cells upon β-estradiol induction which is evident from FACS analysis for cell surface differentiation marker cd11b. Taken together, these studies substantiate the role of E6AP in the degradation and functional activity of C/EBPα. This makes E6AP an interesting protein in the study of regulation of myeloid cell development and leukemogenesis. Also, due to their target specificity these E3 ligases represent an attractive target for the cancer therapy. Therapeutic strategies that inhibit the E3 ligases activity in the degradation of tumor suppressor protein can provide a new scenario in the cancer treatment.

References

References

1. Orkin SH. Diversification of haematopoietic stem cells to specific lineages. *Nat Rev Genet* 2000 Oct; **1**(1): 57-64.

2. Quesenberry PJ, Dooner G, Dooner M, Colvin G. The stem cell continuum: considerations on the heterogeneity and plasticity of marrow stem cells. *Stem Cell Rev* 2005; **1**(1): 29-36.

3. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. *Cell* 2000 Jan 7; **100**(1): 157-168.

4. Lemischka I. Stem cell dogmas in the genomics era. *Rev Clin Exp Hematol* 2001 Mar; **5**(1): 15-25.

5. Eaves C, Miller C, Cashman J, Conneally E, Petzer A, Zandstra P, *et al.* Hematopoietic stem cells: inferences from in vivo assays. *Stem Cells* 1997; **15 Suppl 1**: 1-5.

6. Jones RJ, Collector MI, Barber JP, Vala MS, Fackler MJ, May WS, *et al.* Characterization of mouse lymphohematopoietic stem cells lacking spleen colony-forming activity. *Blood* 1996 Jul 15; **88**(2): 487-491.

7. Turpen JB, Knudson CM, Hoefen PS. The early ontogeny of hematopoietic cells studied by grafting cytogenetically labeled tissue anlagen: localization of a prospective stem cell compartment. *Dev Biol* 1981 Jul 15; **85**(1): 99-112.

8. Dieterlen-Lievre F. On the origin of haemopoietic stem cells in the avian embryo: an experimental approach. *J Embryol Exp Morphol* 1975 Jun; **33**(3): 607-619.

9. Cumano A, Dieterlen-Lievre F, Godin I. Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. *Cell* 1996 Sep 20; **86**(6): 907-916.

10. Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* 1996 Sep 20; **86**(6): 897-906.

11. Peault B, Tavian M. Hematopoietic stem cell emergence in the human embryo and fetus. *Ann N Y Acad Sci* 2003 May; **996**: 132-140.

12. Kumaravelu P, Hook L, Morrison AM, Ure J, Zhao S, Zuyev S, *et al.* Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development* 2002 Nov; **129**(21): 4891-4899.

13. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. *Cell* 2008 Feb 22; **132**(4): 631-644.

14. Mikkola HK, Gekas C, Orkin SH, Dieterlen-Lievre F. Placenta as a site for hematopoietic stem cell development. *Exp Hematol* 2005 Sep; **33**(9): 1048-1054.

15. Decotto E, Spradling AC. The Drosophila ovarian and testis stem cell niches: similar somatic stem cells and signals. *Dev Cell* 2005 Oct; **9**(4): 501-510.

16. Dzierzak E, Medvinsky A, de Bruijn M. Qualitative and quantitative aspects of haematopoietic cell development in the mammalian embryo. *Immunol Today* 1998 May; **19**(5): 228-236.

17. Delassus S, Cumano A. Circulation of hematopoietic progenitors in the mouse embryo. *Immunity* 1996 Jan; **4**(1): 97-106.

18. Cain D, Kondo M, Chen H, Kelsoe G. Effects of acute and chronic inflammation on B-cell development and differentiation. *J Invest Dermatol* 2009 Feb; **129**(2): 266-277.

19. Akashi K, Reya T, Dalma-Weiszhausz D, Weissman IL. Lymphoid precursors. *Curr Opin Immunol* 2000 Apr; **12**(2): 144-150.

20. Zhu J, Emerson SG. Hematopoietic cytokines, transcription factors and lineage commitment. *Oncogene* 2002 May 13; **21**(21): 3295-3313.

21. Rane SG, Reddy EP. JAKs, STATs and Src kinases in hematopoiesis. *Oncogene* 2002 May 13; **21**(21): 3334-3358.

22. Crooks GM, Hao QL, Petersen D, Barsky LW, Bockstoce D. IL-3 increases production of B lymphoid progenitors from human CD34+CD38- cells. *J Immunol* 2000 Sep 1; **165**(5): 2382-2389.

23. Haylock DN, Horsfall MJ, Dowse TL, Ramshaw HS, Niutta S, Protopsaltis S, *et al.* Increased recruitment of hematopoietic progenitor cells underlies the ex vivo expansion potential of FLT3 ligand. *Blood* 1997 Sep 15; **90**(6): 2260-2272.

24. Kinashi T, Springer TA. Steel factor and c-kit regulate cell-matrix adhesion. *Blood* 1994 Feb 15; **83**(4): 1033-1038.

25. Broxmeyer HE. Regulation of hematopoiesis by chemokine family members. *Int J Hematol* 2001 Jul; **74**(1): 9-17.

26. Christopherson K, 2nd, Hromas R. Chemokine regulation of normal and pathologic immune responses. *Stem Cells* 2001; **19**(5): 388-396.

27. Majka M, Ratajczak J, Baj-Krzyworzek M, Kijowski J, Reca R, Machalinski B, *et al.* Biological significance of chemokine receptor expression by normal human megakaryoblasts. *Folia Histochem Cytobiol* 2001; **39**(3): 235-244.

28. Porcher C, Swat W, Rockwell K, Fujiwara Y, Alt FW, Orkin SH. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell* 1996 Jul 12; **86**(1): 47-57.

29. Robb L, Rasko JE, Bath ML, Strasser A, Begley CG. scl, a gene frequently activated in human T cell leukaemia, does not induce lymphomas in transgenic mice. *Oncogene* 1995 Jan 5; **10**(1): 205-209.

30. Robb L, Begley CG. The helix-loop-helix gene SCL: implicated in T-cell acute lymphoblastic leukaemia and in normal haematopoietic development. *Int J Biochem Cell Biol* 1996 Jun; **28**(6): 609-618.

31. Shivdasani RA, Mayer EL, Orkin SH. Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature* 1995 Feb 2; **373**(6513): 432-434.

32. Yamada Y, Warren AJ, Dobson C, Forster A, Pannell R, Rabbitts TH. The T cell leukemia LIM protein Lmo2 is necessary for adult mouse hematopoiesis. *Proc Natl Acad Sci U S A* 1998 Mar 31; **95**(7): 3890-3895.

33. Pevny L, Simon MC, Robertson E, Klein WH, Tsai SF, D'Agati V, *et al.* Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* 1991 Jan 17; **349**(6306): 257-260.

34. Georgopoulos K, Bigby M, Wang JH, Molnar A, Wu P, Winandy S, *et al.* The Ikaros gene is required for the development of all lymphoid lineages. *Cell* 1994 Oct 7; **79**(1): 143-156.

35. Nichogiannopoulou A, Trevisan M, Neben S, Friedrich C, Georgopoulos K. Defects in hemopoietic stem cell activity in Ikaros mutant mice. *J Exp Med* 1999 Nov 1; **190**(9): 1201-1214.

36. Greenwood MJ, Lansdorp PM. Telomeres, telomerase, and hematopoietic stem cell biology. *Arch Med Res* 2003 Nov-Dec; **34**(6): 489-495.

37. Allsopp RC, Morin GB, Horner JW, DePinho R, Harley CB, Weissman IL. Effect of TERT over-expression on the long-term transplantation capacity of hematopoietic stem cells. *Nat Med* 2003 Apr; **9**(4): 369-371.

38. Allsopp RC, Morin GB, DePinho R, Harley CB, Weissman IL. Telomerase is required to slow telomere shortening and extend replicative lifespan of HSCs during serial transplantation. *Blood* 2003 Jul 15; **102**(2): 517-520.

39. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL. Physiological migration of hematopoietic stem and progenitor cells. *Science* 2001 Nov 30; **294**(5548): 1933-1936.

40. Abkowitz JL, Robinson AE, Kale S, Long MW, Chen J. Mobilization of hematopoietic stem cells during homeostasis and after cytokine exposure. *Blood* 2003 Aug 15; **102**(4): 1249-1253.

41. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, *et al.* Proposals for the classification of the acute leukaemias. French-American-British (FAB) co-operative group. *Br J Haematol* 1976 Aug; **33**(4): 451-458.

42. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DA, Gralnick HR, *et al.* Proposed revised criteria for the classification of acute myeloid leukemia. A report of the French-American-British Cooperative Group. *Ann Intern Med* 1985 Oct; **103**(4): 620-625.

43. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. *Blood* 2002 Sep 1; **100**(5): 1532-1542.

44. Reilly JT. FLT3 and its role in the pathogenesis of acute myeloid leukaemia. *Leuk Lymphoma* 2003 Jan; **44**(1): 1-7.

45. Reilly JT. Pathogenesis of acute myeloid leukaemia and inv(16)(p13;q22): a paradigm for understanding leukaemogenesis? *Br J Haematol* 2005 Jan; **128**(1): 18-34.

46. Ramsey H, Zhang DE, Richkind K, Burcoglu-O'Ral A, Hromas R. Fusion of AML1/Runx1 to copine VIII, a novel member of the copine family, in an aggressive acute myelogenous leukemia with t(12;21) translocation. *Leukemia* 2003 Aug; **17**(8): 1665-1666.

47. Elagib KE, Goldfarb AN. Oncogenic pathways of AML1-ETO in acute myeloid leukemia: multifaceted manipulation of marrow maturation. *Cancer Lett* 2007 Jun 28; **251**(2): 179-186.

48. Fu S, Consoli U, Hanania EG, Zu Z, Claxton DF, Andreeff M, *et al.* PML/RARalpha, a fusion protein in acute promyelocytic leukemia, prevents growth factor withdrawal-induced apoptosis in TF-1 cells. *Clin Cancer Res* 1995 Jun; **1**(6): 583-590.

49. Ammatuna E, Noguera NI, Zangrilli D, Curzi P, Panetta P, Bencivenga P, *et al.* Rapid detection of nucleophosmin (NPM1) mutations in acute myeloid leukemia by denaturing HPLC. *Clin Chem* 2005 Nov; **51**(11): 2165-2167.

50. Bower M, Parry P, Carter M, Lillington DM, Amess J, Lister TA, *et al.* Prevalence and clinical correlations of MLL gene rearrangements in AML-M4/5. *Blood* 1994 Dec 1; **84**(11): 3776-3780.

51. Baldus CD, Tanner SM, Ruppert AS, Whitman SP, Archer KJ, Marcucci G, *et al.* BAALC expression predicts clinical outcome of de novo acute myeloid leukemia patients

with normal cytogenetics: a Cancer and Leukemia Group B Study. *Blood* 2003 Sep 1; **102**(5): 1613-1618.

52. Gombart AF, Hofmann WK, Kawano S, Takeuchi S, Krug U, Kwok SH, *et al.* Mutations in the gene encoding the transcription factor CCAAT/enhancer binding protein alpha in myelodysplastic syndromes and acute myeloid leukemias. *Blood* 2002 Feb 15; **99**(4): 1332-1340.

53. Rucker FG, Schlenk RF, Bullinger L, Kayser S, Teleanu V, Kett H, *et al.* TP53 alterations in acute myeloid leukemia with complex karyotype correlate with specific copy number alterations, monosomal karyotype, and dismal outcome. *Blood* Mar 1; **119**(9): 2114-2121.

54. Mayer RJ, Davis RB, Schiffer CA, Berg DT, Powell BL, Schulman P, *et al.* Intensive postremission chemotherapy in adults with acute myeloid leukemia. Cancer and Leukemia Group B. *N Engl J Med* 1994 Oct 6; **331**(14): 896-903.

55. Castaigne S, Chomienne C, Daniel MT, Ballerini P, Berger R, Fenaux P, *et al.* All-trans retinoic acid as a differentiation therapy for acute promyelocytic leukemia. I. Clinical results. *Blood* 1990 Nov 1; **76**(9): 1704-1709.

56. Huang ME. [Treatment of acute promyelocytic leukemia with all-trans retinoic acid]. *Zhonghua Yi Xue Za Zhi* 1988 Mar; **68**(3): 131-133, 110.

57. Lehmann S, Bengtzen S, Paul A, Christensson B, Paul C. Effects of arsenic trioxide (As2O3) on leukemic cells from patients with non-M3 acute myelogenous leukemia: studies of cytotoxicity, apoptosis and the pattern of resistance. *Eur J Haematol* 2001 Jun; **66**(6): 357-364.

58. Fang J, Chen SJ, Tong JH, Wang ZG, Chen GQ, Chen Z. Treatment of acute promyelocytic leukemia with ATRA and As2O3: a model of molecular target-based cancer therapy. *Cancer Biol Ther* 2002 Nov-Dec; **1**(6): 614-620.

59. Cashman JD, Lapidot T, Wang JC, Doedens M, Shultz LD, Lansdorp P, *et al.* Kinetic evidence of the regeneration of multilineage hematopoiesis from primitive cells in normal human bone marrow transplanted into immunodeficient mice. *Blood* 1997 Jun 15; **89**(12): 4307-4316.

60. Giralt S, Estey E, Albitar M, van Besien K, Rondon G, Anderlini P, *et al.* Engraftment of allogeneic hematopoietic progenitor cells with purine analog-containing chemotherapy: harnessing graft-versus-leukemia without myeloablative therapy. *Blood* 1997 Jun 15; **89**(12): 4531-4536.

61. Slavin S, Nagler A, Naparstek E, Kapelushnik Y, Aker M, Cividalli G, *et al.* Nonmyeloablative stem cell transplantation and cell therapy as an alternative to

conventional bone marrow transplantation with lethal cytoreduction for the treatment of malignant and nonmalignant hematologic diseases. *Blood* 1998 Feb 1; **91**(3): 756-763.

62. Mutis T, Verdijk R, Schrama E, Esendam B, Brand A, Goulmy E. Feasibility of immunotherapy of relapsed leukemia with ex vivo-generated cytotoxic T lymphocytes specific for hematopoietic system-restricted minor histocompatibility antigens. *Blood* 1999 Apr 1; **93**(7): 2336-2341.

63. Dunussi-Joannopoulos K, Dranoff G, Weinstein HJ, Ferrara JL, Bierer BE, Croop JM. Gene immunotherapy in murine acute myeloid leukemia: granulocyte-macrophage colony-stimulating factor tumor cell vaccines elicit more potent antitumor immunity compared with B7 family and other cytokine vaccines. *Blood* 1998 Jan 1; **91**(1): 222-230.

64. Fialkow PJ, Jacobson RJ, Papayannopoulou T. Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. *Am J Med* 1977 Jul; **63**(1): 125-130.

65. Faderl S, Talpaz M, Estrov Z, O'Brien S, Kurzrock R, Kantarjian HM. The biology of chronic myeloid leukemia. *N Engl J Med* 1999 Jul 15; **341**(3): 164-172.

66. Sawyers CL. Chronic myeloid leukemia. *N Engl J Med* 1999 Apr 29; **340**(17): 1330-1340.

67. Nowell PC, Hungerford DA. Chromosome studies on normal and leukemic human leukocytes. *J Natl Cancer Inst* 1960 Jul; **25**: 85-109.

68. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. *Nature* 1973 Jun 1; **243**(5405): 290-293.

69. McLaughlin J, Chianese E, Witte ON. In vitro transformation of immature hematopoietic cells by the P210 BCR/ABL oncogene product of the Philadelphia chromosome. *Proc Natl Acad Sci U S A* 1987 Sep; **84**(18): 6558-6562.

70. Daley GQ, Van Etten RA, Baltimore D. Induction of chronic myelogenous leukemia in mice by the P210bcr/abl gene of the Philadelphia chromosome. *Science* 1990 Feb 16; **247**(4944): 824-830.

71. Elefanty AG, Hariharan IK, Cory S. bcr-abl, the hallmark of chronic myeloid leukaemia in man, induces multiple haemopoietic neoplasms in mice. *EMBO J* 1990 Apr; **9**(4): 1069-1078.

72. Goga A, McLaughlin J, Afar DE, Saffran DC, Witte ON. Alternative signals to RAS for hematopoietic transformation by the BCR-ABL oncogene. *Cell* 1995 Sep 22; **82**(6): 981-988.

73. Sawyers CL, McLaughlin J, Witte ON. Genetic requirement for Ras in the transformation of fibroblasts and hematopoietic cells by the Bcr-Abl oncogene. *J Exp Med* 1995 Jan 1; **181**(1): 307-313.

74. Skorski T, Bellacosa A, Nieborowska-Skorska M, Majewski M, Martinez R, Choi JK, *et al.* Transformation of hematopoietic cells by BCR/ABL requires activation of a PI-3k/Akt-dependent pathway. *EMBO J* 1997 Oct 15; **16**(20): 6151-6161.

75. Shuai K, Halpern J, ten Hoeve J, Rao X, Sawyers CL. Constitutive activation of STAT5 by the BCR-ABL oncogene in chronic myelogenous leukemia. *Oncogene* 1996 Jul 18; **13**(2): 247-254.

76. Ilaria RL, Jr., Van Etten RA. P210 and P190(BCR/ABL) induce the tyrosine phosphorylation and DNA binding activity of multiple specific STAT family members. *J Biol Chem* 1996 Dec 6; **271**(49): 31704-31710.

77. Castellanos A, Pintado B, Weruaga E, Arevalo R, Lopez A, Orfao A, *et al.* A BCR-ABL(p190) fusion gene made by homologous recombination causes B-cell acute lymphoblastic leukemias in chimeric mice with independence of the endogenous bcr product. *Blood* 1997 Sep 15; **90**(6): 2168-2174.

78. van Rhee F, Hochhaus A, Lin F, Melo JV, Goldman JM, Cross NC. p190 BCR-ABL mRNA is expressed at low levels in p210-positive chronic myeloid and acute lymphoblastic leukemias. *Blood* 1996 Jun 15; **87**(12): 5213-5217.

79. Melo JV. The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. *Blood* 1996 Oct 1; **88**(7): 2375-2384.

80. Traxler P, Bold G, Buchdunger E, Caravatti G, Furet P, Manley P, *et al.* Tyrosine kinase inhibitors: from rational design to clinical trials. *Med Res Rev* 2001 Nov; **21**(6): 499-512.

81. Manley PW, Cowan-Jacob SW, Buchdunger E, Fabbro D, Fendrich G, Furet P, *et al.* Imatinib: a selective tyrosine kinase inhibitor. *Eur J Cancer* 2002 Sep; **38 Suppl 5**: S19-27.

82. Buchdunger E, Cioffi CL, Law N, Stover D, Ohno-Jones S, Druker BJ, *et al.* Abl protein-tyrosine kinase inhibitor STI571 inhibits in vitro signal transduction mediated by c-kit and platelet-derived growth factor receptors. *J Pharmacol Exp Ther* 2000 Oct; **295**(1): 139-145.

83. Wang JY. Abl tyrosine kinase in signal transduction and cell-cycle regulation. *Curr Opin Genet Dev* 1993 Feb; **3**(1): 35-43.

84. Jackson P, Baltimore D. N-terminal mutations activate the leukemogenic potential of the myristoylated form of c-abl. *EMBO J* 1989 Feb; **8**(2): 449-456.

85. Hantschel O, Nagar B, Guettler S, Kretzschmar J, Dorey K, Kuriyan J, *et al.* A myristoyl/phosphotyrosine switch regulates c-Abl. *Cell* 2003 Mar 21; **112**(6): 845-857.

86. Franz WM, Berger P, Wang JY. Deletion of an N-terminal regulatory domain of the c-abl tyrosine kinase activates its oncogenic potential. *EMBO J* 1989 Jan; **8**(1): 137-147.

87. Gale RP, Grosveld G, Canaani E, Goldman JM. Chronic myelogenous leukemia: biology and therapy. *Leukemia* 1993 Apr; **7**(4): 653-658.

88. Chung SW, Daniel R, Wong BY, Wong PM. The ABL genes in normal and abnormal cell development. *Crit Rev Oncog* 1996; **7**(1-2): 33-48.

89. McWhirter JR, Wang JY. An actin-binding function contributes to transformation by the Bcr-Abl oncoprotein of Philadelphia chromosome-positive human leukemias. *EMBO J* 1993 Apr; **12**(4): 1533-1546.

90. Reuther GW, Fu H, Cripe LD, Collier RJ, Pendergast AM. Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. *Science* 1994 Oct 7; **266**(5182): 129-133.

91. Pendergast AM, Muller AJ, Havlik MH, Maru Y, Witte ON. BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent manner. *Cell* 1991 Jul 12; **66**(1): 161-171.

92. Skorski T, Kanakaraj P, Nieborowska-Skorska M, Ratajczak MZ, Wen SC, Zon G, *et al.* Phosphatidylinositol-3 kinase activity is regulated by BCR/ABL and is required for the growth of Philadelphia chromosome-positive cells. *Blood* 1995 Jul 15; **86**(2): 726-736.

93. Talpaz M, McCredie KB, Mavligit GM, Gutterman JU. Leukocyte interferon-induced myeloid cytoreduction in chronic myelogenous leukemia. *Blood* 1983 Sep; **62**(3): 689-692.

94. Interferon alfa-2a as compared with conventional chemotherapy for the treatment of chronic myeloid leukemia. The Italian Cooperative Study Group on Chronic Myeloid Leukemia. *N Engl J Med* 1994 Mar 24; **330**(12): 820-825.

95. Allan NC, Richards SM, Shepherd PC. UK Medical Research Council randomised, multicentre trial of interferon-alpha n1 for chronic myeloid leukaemia: improved survival irrespective of cytogenetic response. The UK Medical Research Council's Working Parties for Therapeutic Trials in Adult Leukaemia. *Lancet* 1995 Jun 3; **345**(8962): 1392-1397.

96. Ohnishi K, Ohno R, Tomonaga M, Kamada N, Onozawa K, Kuramoto A, *et al.* A randomized trial comparing interferon-alpha with busulfan for newly diagnosed chronic myelogenous leukemia in chronic phase. *Blood* 1995 Aug 1; **86**(3): 906-916.

97. Cotta CV, Bueso-Ramos CE. New insights into the pathobiology and treatment of chronic myelogenous leukemia. *Ann Diagn Pathol* 2007 Feb; **11**(1): 68-78.

98. O'Dwyer ME, Druker BJ. Chronic myelogenous leukaemia--new therapeutic principles. *J Intern Med* 2001 Jul; **250**(1): 3-9.

99. Buchdunger E, Zimmermann J, Mett H, Meyer T, Muller M, Druker BJ, *et al.* Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Res* 1996 Jan 1; **56**(1): 100-104.

100. Heinrich MC, Griffith DJ, Druker BJ, Wait CL, Ott KA, Zigler AJ. Inhibition of c-kit receptor tyrosine kinase activity by STI 571, a selective tyrosine kinase inhibitor. *Blood* 2000 Aug 1; **96**(3): 925-932.

101. Okuda K, Weisberg E, Gilliland DG, Griffin JD. ARG tyrosine kinase activity is inhibited by STI571. *Blood* 2001 Apr 15; **97**(8): 2440-2448.

102. Druker BJ, Tamura S, Buchdunger E, Ohno S, Segal GM, Fanning S, *et al.* Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. *Nat Med* 1996 May; **2**(5): 561-566.

103. le Coutre P, Mologni L, Cleris L, Marchesi E, Buchdunger E, Giardini R, *et al.* In vivo eradication of human BCR/ABL-positive leukemia cells with an ABL kinase inhibitor. *J Natl Cancer Inst* 1999 Jan 20; **91**(2): 163-168.

104. Hughes TP, Kaeda J, Branford S, Rudzki Z, Hochhaus A, Hensley ML, *et al.* Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. *N Engl J Med* 2003 Oct 9; **349**(15): 1423-1432.

105. Kaeda J, Chase A, Goldman JM. Cytogenetic and molecular monitoring of residual disease in chronic myeloid leukaemia. *Acta Haematol* 2002; **107**(2): 64-75.

106. Cortes J, O'Brien S, Kantarjian H. Discontinuation of imatinib therapy after achieving a molecular response. *Blood* 2004 Oct 1; **104**(7): 2204-2205.

107. Rousselot P, Huguet F, Rea D, Legros L, Cayuela JM, Maarek O, *et al.* Imatinib mesylate discontinuation in patients with chronic myelogenous leukemia in complete molecular remission for more than 2 years. *Blood* 2007 Jan 1; **109**(1): 58-60.

108. Bhatia R, Holtz M, Niu N, Gray R, Snyder DS, Sawyers CL, *et al.* Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment. *Blood* 2003 Jun 15; **101**(12): 4701-4707.

109. Chu S, Xu H, Shah NP, Snyder DS, Forman SJ, Sawyers CL, *et al.* Detection of BCR-ABL kinase mutations in CD34+ cells from chronic myelogenous leukemia patients in

complete cytogenetic remission on imatinib mesylate treatment. *Blood* 2005 Mar 1; **105**(5): 2093-2098.

110. Graham SM, Jorgensen HG, Allan E, Pearson C, Alcorn MJ, Richmond L, *et al.* Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro. *Blood* 2002 Jan 1; **99**(1): 319-325.

111. Goldman JM. Chronic myeloid leukemia-still a few questions. *Exp Hematol* 2004 Jan; **32**(1): 2-10.

112. Tauchi T, Ohyashiki K. Molecular mechanisms of resistance of leukemia to imatinib mesylate. *Leuk Res* 2004 May; **28 Suppl 1**: S39-45.

113. Gambacorti-Passerini C, Barni R, le Coutre P, Zucchetti M, Cabrita G, Cleris L, *et al.* Role of alpha1 acid glycoprotein in the in vivo resistance of human BCR-ABL(+) leukemic cells to the abl inhibitor STI571. *J Natl Cancer Inst* 2000 Oct 18; **92**(20): 1641-1650.

114. Gambacorti-Passerini C, le Coutre P, Zucchetti M, D'Incalci M. Binding of imatinib by alpha(1)-acid glycoprotein. *Blood* 2002 Jul 1; **100**(1): 367-368; author reply 368-369.

115. Dai H, Marbach P, Lemaire M, Hayes M, Elmquist WF. Distribution of STI-571 to the brain is limited by P-glycoprotein-mediated efflux. *J Pharmacol Exp Ther* 2003 Mar; **304**(3): 1085-1092.

116. Branford S, Rudzki Z, Walsh S, Grigg A, Arthur C, Taylor K, *et al.* High frequency of point mutations clustered within the adenosine triphosphate-binding region of BCR/ABL in patients with chronic myeloid leukemia or Ph-positive acute lymphoblastic leukemia who develop imatinib (STI571) resistance. *Blood* 2002 May 1; **99**(9): 3472-3475.

117. Branford S, Rudzki Z, Walsh S, Parkinson I, Grigg A, Szer J, *et al.* Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood* 2003 Jul 1; **102**(1): 276-283.

118. Hochhaus A, Kreil S, Corbin AS, La Rosee P, Muller MC, Lahaye T, *et al.* Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. *Leukemia* 2002 Nov; **16**(11): 2190-2196.

119. Shah NP, Nicoll JM, Nagar B, Gorre ME, Paquette RL, Kuriyan J, *et al.* Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell* 2002 Aug; **2**(2): 117-125.

120. Huntly BJ, Gilliland DG. Leukaemia stem cells and the evolution of cancer-stem-cell research. *Nat Rev Cancer* 2005 Apr; **5**(4): 311-321.

121. Al-Hajj M, Becker MW, Wicha M, Weissman I, Clarke MF. Therapeutic implications of cancer stem cells. *Curr Opin Genet Dev* 2004 Feb; **14**(1): 43-47.

122. Majumdar N, Datta JK. Effects of centchroman, a synthetic estrogen antagonist on the fertility of female hamsters. *Indian J Exp Biol* 1990 Aug; **28**(8): 717-720.

123. CDRI, inventor Centchroman: Contraceptive, antiestrogen. Drugs Fut 1994;19:684-685.

124. Grubb GS. Experimental methods of contraception. *Curr Opin Obstet Gynecol* 1991 Aug; **3**(4): 491-495.

125. Singh MM, Bhalla V, Wadhwa V, Kamboj VP. Effect of centchroman on tubal transport and preimplantation embryonic development in rats. *J Reprod Fertil* 1986 Jan; **76**(1): 317-324.

126. Singh MM, Sreenivasulu S, Kamboj VP. Duration of anti-implantation action of the triphenylethylene anti-oestrogen centchroman in adult female rats. *J Reprod Fertil* 1994 Mar; **100**(2): 367-374.

127. Trivedi RN, Chauhan SC, Dwivedi A, Kamboj VP, Singh MM. Time-related effects of a triphenylethylene antiestrogen on estrogen-induced changes in uterine weight, estrogen receptors, and endometrial sensitivity in rats. *Contraception* 1995 Jun; **51**(6): 367-379.

128. Giri AK, Mukhopadhyay A, Sun J, Hsie AW, Ray S. Antimutagenic effects of centchroman--a contraceptive and a candidate drug for breast cancer in multiple mutational assays. *Mutagenesis* 1999 Nov; **14**(6): 613-620.

129. Singh MM. Centchroman, a selective estrogen receptor modulator, as a contraceptive and for the management of hormone-related clinical disorders. *Med Res Rev* 2001 Jul; **21**(4): 302-347.

130. Roy S, Kumari GL, Madoiya K, Prakash V, Ray S. Induction of ovulation in the human with centchroman: a preliminary report. *Fertil Steril* 1976 Sep; **27**(9): 1108-1110.

131. Kamboj VP, Setty BS, Chandra H, Roy SK, Kar AB. Biological profile of Centchroman--a new post-coital contraceptive. *Indian J Exp Biol* 1977 Dec; **15**(12): 1144-1150.

132. Chauhan SC, Singh MM, Maitra SC, Kamboj VP. Inhibition of progesterone-induced development of giant mitochondria in uterine glandular epithelial cells by an antiestrogen in rat. *Contraception* 1996 Oct; **54**(4): 259-264.

133. Misra NC, Nigam PK, Gupta R, Agarwal AK, Kamboj VP. Centchroman--a non-steroidal anti-cancer agent for advanced breast cancer: phase-II study. *Int J Cancer* 1989 May 15; **43**(5): 781-783.

134. Kriplani A, Kulshrestha V, Agarwal N. Efficacy and safety of ormeloxifene in management of menorrhagia: a pilot study. *J Obstet Gynaecol Res* 2009 Aug; **35**(4): 746-752.

135. Mukhopadhyay A, Gupta S, Ray S, Giri AK. Anticlastogenic effects of centchroman and its enantiomers in Swiss albino mice. I. Acute study and their comparison with tamoxifen. *Cancer Lett* 1999 Oct 1; **144**(2): 137-143.

136. Kumar V, Lal J, Singh MM, Gupta RC. Effect of concurrently coadministered drugs on the pharmacokinetic/pharmacodynamic profile of centchroman, a nonsteroidal oral contraceptive, in rats. *Contraception* 2006 Aug; **74**(2): 165-173.

137. Kumar S, Khanduja KL, Verma N, Verma SC, Avti PK, Pathak CM. ATRA promotes alpha tocopherol succinate-induced apoptosis in freshly isolated leukemic cells from chronic myeloid leukemic patients. *Mol Cell Biochem* 2008 Jan; **307**(1-2): 109-119.

138. Harris TE, Albrecht JH, Nakanishi M, Darlington GJ. CCAAT/enhancer-binding protein-alpha cooperates with p21 to inhibit cyclin-dependent kinase-2 activity and induces growth arrest independent of DNA binding. *J Biol Chem* 2001 Aug 3; **276**(31): 29200-29209.

139. Mishra A, Godavarthi SK, Jana NR. UBE3A/E6-AP regulates cell proliferation by promoting proteasomal degradation of p27. *Neurobiol Dis* 2009 Oct; **36**(1): 26-34.

140. Shimoji T, Murakami K, Sugiyama Y, Matsuda M, Inubushi S, Nasu J, *et al.* Identification of annexin A1 as a novel substrate for E6AP-mediated ubiquitylation. *J Cell Biochem* 2009 Apr 15; **106**(6): 1123-1135.

141. Nawaz Z, Lonard DM, Smith CL, Lev-Lehman E, Tsai SY, Tsai MJ, *et al.* The Angelman syndrome-associated protein, E6-AP, is a coactivator for the nuclear hormone receptor superfamily. *Mol Cell Biol* 1999 Feb; **19**(2): 1182-1189.

142. Trivedi AK, Bararia D, Christopeit M, Peerzada AA, Singh SM, Kieser A, *et al.* Proteomic identification of C/EBP-DBD multiprotein complex: JNK1 activates stem cell regulator C/EBPalpha by inhibiting its ubiquitination. *Oncogene* 2007 Mar 15; **26**(12): 1789-1801.

143. Wang X, Scott E, Sawyers CL, Friedman AD. C/EBPalpha bypasses granulocyte colony-stimulating factor signals to rapidly induce PU.1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts. *Blood* 1999 Jul 15; **94**(2): 560-571.

144. Kumar R, Gupta L, Pal P, Khan S, Singh N, Katiyar SB, *et al.* Synthesis and cytotoxicity evaluation of (tetrahydro-beta-carboline)-1,3,5-triazine hybrids as anticancer agents. *Eur J Med Chem* Jun; **45**(6): 2265-2276.

145. Bortner CD, Oldenburg NB, Cidlowski JA. The role of DNA fragmentation in apoptosis. *Trends Cell Biol* 1995 Jan; **5**(1): 21-26.

146. Bratton DL, Fadok VA, Richter DA, Kailey JM, Guthrie LA, Henson PM. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *J Biol Chem* 1997 Oct 17; **272**(42): 26159-26165.

147. van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 1998 Jan 1; **31**(1): 1-9.

148. Lee DH, Choi SY, Ryu HM, Kim CD, Park SH, Chung HY, *et al.* 3,4-dideoxyglucosone-3-ene induces apoptosis in human peritoneal mesothelial cells. *Perit Dial Int* 2009 Jan-Feb; **29**(1): 44-51.

149. Cai J, Yang J, Jones DP. Mitochondrial control of apoptosis: the role of cytochrome c. *Biochim Biophys Acta* 1998 Aug 10; **1366**(1-2): 139-149.

150. Cohen GM. Caspases: the executioners of apoptosis. *Biochem J* 1997 Aug 15; **326 (Pt 1)**: 1-16.

151. Pal P, Kanaujiya JK, Lochab S, Tripathi SB, Bhatt ML, Singh PK, *et al.* 2-D gel electrophoresis-based proteomic analysis reveals that ormeloxifen induces G0-G1 growth arrest and ERK-mediated apoptosis in chronic myeloid leukemia cells K562. *Proteomics* Apr; **11**(8): 1517-1529.

152. J.F. Sambrook TM. *Molecular cloning: A laboratory manual.* Cold Spring Harbor laboratory Press: Cold Spring Harbor, New York,USA, 1989.

153. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972 Aug; **26**(4): 239-257.

154. Horvitz HR. Genetic control of programmed cell death in the nematode Caenorhabditis elegans. *Cancer Res* 1999 Apr 1; **59**(7 Suppl): 1701s-1706s.

155. Hacker G. The morphology of apoptosis. *Cell Tissue Res* 2000 Jul; **301**(1): 5-17.

156. Savill J, Fadok V. Corpse clearance defines the meaning of cell death. *Nature* 2000 Oct 12; **407**(6805): 784-788.

157. Kurosaka K, Takahashi M, Watanabe N, Kobayashi Y. Silent cleanup of very early apoptotic cells by macrophages. *J Immunol* 2003 Nov 1; **171**(9): 4672-4679.

158. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000 Oct 12; **407**(6805): 770-776.

159. Rai NK, Tripathi K, Sharma D, Shukla VK. Apoptosis: a basic physiologic process in wound healing. *Int J Low Extrem Wounds* 2005 Sep; **4**(3): 138-144.

160. Nakagawa T, Zhu H, Morishima N, Li E, Xu J, Yankner BA, *et al.* Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 2000 Jan 6; **403**(6765): 98-103.

161. Koenig U, Eckhart L, Tschachler E. Evidence that caspase-13 is not a human but a bovine gene. *Biochem Biophys Res Commun* 2001 Aug 3; **285**(5): 1150-1154.

162. Kang SJ, Wang S, Kuida K, Yuan J. Distinct downstream pathways of caspase-11 in regulating apoptosis and cytokine maturation during septic shock response. *Cell Death Differ* 2002 Oct; **9**(10): 1115-1125.

163. Hu S, Snipas SJ, Vincenz C, Salvesen G, Dixit VM. Caspase-14 is a novel developmentally regulated protease. *J Biol Chem* 1998 Nov 6; **273**(45): 29648-29653.

164. Fesus L, Madi A, Balajthy Z, Nemes Z, Szondy Z. Transglutaminase induction by various cell death and apoptosis pathways. *Experientia* 1996 Oct 31; **52**(10-11): 942-949.

165. Arur S, Uche UE, Rezaul K, Fong M, Scranton V, Cowan AE, *et al.* Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. *Dev Cell* 2003 Apr; **4**(4): 587-598.

166. Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, *et al.* Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. *Cell* 2005 Oct 21; **123**(2): 321-334.

167. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. *Nat Rev Cancer* 2002 Apr; **2**(4): 277-288.

168. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* 2001 Feb 23; **104**(4): 487-501.

169. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science* 1998 Aug 28; **281**(5381): 1305-1308.

170. Chicheportiche Y, Bourdon PR, Xu H, Hsu YM, Scott H, Hession C, *et al.* TWEAK, a new secreted ligand in the tumor necrosis factor family that weakly induces apoptosis. *J Biol Chem* 1997 Dec 19; **272**(51): 32401-32410.

171. Peter ME, Krammer PH. Mechanisms of CD95 (APO-1/Fas)-mediated apoptosis. *Curr Opin Immunol* 1998 Oct; **10**(5): 545-551.

172. Suliman A, Lam A, Datta R, Srivastava RK. Intracellular mechanisms of TRAIL: apoptosis through mitochondrial-dependent and -independent pathways. *Oncogene* 2001 Apr 19; **20**(17): 2122-2133.

173. Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell* 1995 May 19; **81**(4): 495-504.

174. Grimm S, Stanger BZ, Leder P. RIP and FADD: two "death domain"-containing proteins can induce apoptosis by convergent, but dissociable, pathways. *Proc Natl Acad Sci U S A* 1996 Oct 1; **93**(20): 10923-10927.

175. Wajant H. The Fas signaling pathway: more than a paradigm. *Science* 2002 May 31; **296**(5573): 1635-1636.

176. Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, *et al.* Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* 1995 Nov 15; **14**(22): 5579-5588.

177. Kataoka T, Schroter M, Hahne M, Schneider P, Irmler M, Thome M, *et al.* FLIP prevents apoptosis induced by death receptors but not by perforin/granzyme B, chemotherapeutic drugs, and gamma irradiation. *J Immunol* 1998 Oct 15; **161**(8): 3936-3942.

178. Hitoshi Y, Lorens J, Kitada SI, Fisher J, LaBarge M, Ring HZ, *et al.* Toso, a cell surface, specific regulator of Fas-induced apoptosis in T cells. *Immunity* 1998 Apr; **8**(4): 461-471.

179. Harman D. Role of free radicals in aging and disease. *Ann N Y Acad Sci* 1992 Dec 26; **673**: 126-141.

180. Saelens X, Festjens N, Vande Walle L, van Gurp M, van Loo G, Vandenabeele P. Toxic proteins released from mitochondria in cell death. *Oncogene* 2004 Apr 12; **23**(16): 2861-2874.

181. Dubin M, Stoppani AO. [Programmed cell death and apoptosis. The role of mitochondria]. *Medicina (B Aires)* 2000; **60**(3): 375-386.

182. van Loo G, Saelens X, van Gurp M, MacFarlane M, Martin SJ, Vandenabeele P. The role of mitochondrial factors in apoptosis: a Russian roulette with more than one bullet. *Cell Death Differ* 2002 Oct; **9**(10): 1031-1042.

183. Chinnaiyan AM. The apoptosome: heart and soul of the cell death machine. *Neoplasia* 1999 Apr; **1**(1): 5-15.

184. Schimmer AD, Welsh K, Pinilla C, Wang Z, Krajewska M, Bonneau MJ, *et al.* Small-molecule antagonists of apoptosis suppressor XIAP exhibit broad antitumor activity. *Cancer Cell* 2004 Jan; **5**(1): 25-35.

185. Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2002 Sep; **2**(9): 647-656.

186. Schuler M, Green DR. Mechanisms of p53-dependent apoptosis. *Biochem Soc Trans* 2001 Nov; **29**(Pt 6): 684-688.

187. Brunner T, Mueller C. Apoptosis in disease: about shortage and excess. *Essays Biochem* 2003; **39:** 119-130.

188. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* 2002 Oct; **2**(10): 735-747.

189. Pardo J, Bosque A, Brehm R, Wallich R, Naval J, Mullbacher A, *et al.* Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis. *J Cell Biol* 2004 Nov 8; **167**(3): 457-468.

190. Sakahira H, Enari M, Nagata S. Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* 1998 Jan 1; **391**(6662): 96-99.

191. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol* 2002 Jun; **2**(6): 401-409.

192. Goping IS, Barry M, Liston P, Sawchuk T, Constantinescu G, Michalak KM, *et al.* Granzyme B-induced apoptosis requires both direct caspase activation and relief of caspase inhibition. *Immunity* 2003 Mar; **18**(3): 355-365.

193. Fan Z, Beresford PJ, Oh DY, Zhang D, Lieberman J. Tumor suppressor NM23-H1 is a granzyme A-activated DNase during CTL-mediated apoptosis, and the nucleosome assembly protein SET is its inhibitor. *Cell* 2003 Mar 7; **112**(5): 659-672.

194. Slee EA, Adrain C, Martin SJ. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. *J Biol Chem* 2001 Mar 9; **276**(10): 7320-7326.

195. Kothakota S, Azuma T, Reinhard C, Klippel A, Tang J, Chu K, *et al.* Caspase-3-generated fragment of gelsolin: effector of morphological change in apoptosis. *Science* 1997 Oct 10; **278**(5336): 294-298.

196. Kerr JF, Winterford CM, Harmon BV. Apoptosis. Its significance in cancer and cancer therapy. *Cancer* 1994 Apr 15; **73**(8): 2013-2026.

197. Degterev A, Yuan J. Expansion and evolution of cell death programmes. *Nat Rev Mol Cell Biol* 2008 May; **9**(5): 378-390.

198. Ma WW, Adjei AA. Novel agents on the horizon for cancer therapy. *CA Cancer J Clin* 2009 Mar-Apr; **59**(2): 111-137.

199. Nigam M, Ranjan V, Srivastava S, Sharma R, Balapure AK. Centchroman induces G0/G1 arrest and caspase-dependent apoptosis involving mitochondrial membrane depolarization in MCF-7 and MDA MB-231 human breast cancer cells. *Life Sci* 2008 Mar 12; **82**(11-12): 577-590.

200. Samudio I, Konopleva M, Safe S, McQueen T, Andreeff M. Guggulsterones induce apoptosis and differentiation in acute myeloid leukemia: identification of isomer-specific antileukemic activities of the pregnadienedione structure. *Mol Cancer Ther* 2005 Dec; **4**(12): 1982-1992.

201. Darzynkiewicz Z, Galkowski D, Zhao H. Analysis of apoptosis by cytometry using TUNEL assay. *Methods* 2008 Mar; **44**(3): 250-254.

202. Wadia JS, Chalmers-Redman RM, Ju WJ, Carlile GW, Phillips JL, Fraser AD, *et al.* Mitochondrial membrane potential and nuclear changes in apoptosis caused by serum and nerve growth factor withdrawal: time course and modification by (-)-deprenyl. *J Neurosci* 1998 Feb 1; **18**(3): 932-947.

203. Green DR, Chipuk JE. Apoptosis: Stabbed in the BAX. *Nature* 2008 Oct 23; **455**(7216): 1047-1049.

204. Wang CL, Ng TB, Cao XH, Jiang Y, Liu ZK, Wen TY, *et al.* CLP induces apoptosis in human leukemia K562 cells through Ca(2+) regulating extracellular-related protein kinase ERK activation. *Cancer Lett* 2009 Apr 18; **276**(2): 221-227.

205. Trumpp A, Refaeli Y, Oskarsson T, Gasser S, Murphy M, Martin GR, *et al.* c-Myc regulates mammalian body size by controlling cell number but not cell size. *Nature* 2001 Dec 13; **414**(6865): 768-773.

206. Pelengaris S, Khan M, Evan G. c-MYC: more than just a matter of life and death. *Nat Rev Cancer* 2002 Oct; **2**(10): 764-776.

207. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature* 1993 Dec 16; **366**(6456): 701-704.

208. Mitra J, Dai CY, Somasundaram K, El-Deiry WS, Satyamoorthy K, Herlyn M, *et al.* Induction of p21(WAF1/CIP1) and inhibition of Cdk2 mediated by the tumor suppressor p16(INK4a). *Mol Cell Biol* 1999 May; **19**(5): 3916-3928.

209. Jacquel A, Herrant M, Legros L, Belhacene N, Luciano F, Pages G, *et al.* Imatinib induces mitochondria-dependent apoptosis of the Bcr-Abl-positive K562 cell line and its differentiation toward the erythroid lineage. *Faseb J* 2003 Nov; **17**(14): 2160-2162.

210. Green DR. Apoptotic pathways: ten minutes to dead. *Cell* 2005 Jun 3; **121**(5): 671-674.

211. Xia L, Chen D, Han R, Fang Q, Waxman S, Jing Y. Boswellic acid acetate induces apoptosis through caspase-mediated pathways in myeloid leukemia cells. *Mol Cancer Ther* 2005 Mar; **4**(3): 381-388.

212. Hoshino R, Tanimura S, Watanabe K, Kataoka T, Kohno M. Blockade of the extracellular signal-regulated kinase pathway induces marked G1 cell cycle arrest and apoptosis in tumor cells in which the pathway is constitutively activated: up-regulation of p27(Kip1). *J Biol Chem* 2001 Jan 26; **276**(4): 2686-2692.

213. Kim GS, Hong JS, Kim SW, Koh JM, An CS, Choi JY, *et al.* Leptin induces apoptosis via ERK/cPLA2/cytochrome c pathway in human bone marrow stromal cells. *J Biol Chem* 2003 Jun 13; **278**(24): 21920-21929.

214. Mao X, Yu CR, Li WH, Li WX. Induction of apoptosis by shikonin through a ROS/JNK-mediated process in Bcr/Abl-positive chronic myelogenous leukemia (CML) cells. *Cell Res* 2008 Aug; **18**(8): 879-888.

215. Johnson PF, Landschulz WH, Graves BJ, McKnight SL. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev* 1987 Apr; **1**(2): 133-146.

216. Landschulz WH, Johnson PF, Adashi EY, Graves BJ, McKnight SL. Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev* 1988 Jul; **2**(7): 786-800.

217. Antonson P, Xanthopoulos KG. Molecular cloning, sequence, and expression patterns of the human gene encoding CCAAT/enhancer binding protein alpha (C/EBP alpha). *Biochem Biophys Res Commun* 1995 Oct 4; **215**(1): 106-113.

218. Birkenmeier EH, Gwynn B, Howard S, Jerry J, Gordon JI, Landschulz WH, *et al.* Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes Dev* 1989 Aug; **3**(8): 1146-1156.

219. Osada S, Yamamoto H, Nishihara T, Imagawa M. DNA binding specificity of the CCAAT/enhancer-binding protein transcription factor family. *J Biol Chem* 1996 Feb 16; **271**(7): 3891-3896.

220. Lekstrom-Himes J, Xanthopoulos KG. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J Biol Chem* 1998 Oct 30; **273**(44): 28545-28548.

221. Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y, *et al.* A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J* 1990 Jun; **9**(6): 1897-1906.

222. Cao Z, Umek RM, McKnight SL. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev* 1991 Sep; **5**(9): 1538-1552.

223. Roman C, Platero JS, Shuman J, Calame K. Ig/EBP-1: a ubiquitously expressed immunoglobulin enhancer binding protein that is similar to C/EBP and heterodimerizes with C/EBP. *Genes Dev* 1990 Aug; **4**(8): 1404-1415.

224. Lin FT, MacDougald OA, Diehl AM, Lane MD. A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. *Proc Natl Acad Sci U S A* 1993 Oct 15; **90**(20): 9606-9610.

225. Ossipow V, Descombes P, Schibler U. CCAAT/enhancer-binding protein mRNA is translated into multiple proteins with different transcription activation potentials. *Proc Natl Acad Sci U S A* 1993 Sep 1; **90**(17): 8219-8223.

226. Nerlov C, Ziff EB. Three levels of functional interaction determine the activity of CCAAT/enhancer binding protein-alpha on the serum albumin promoter. *Genes Dev* 1994 Feb 1; **8**(3): 350-362.

227. Nerlov C, Ziff EB. CCAAT/enhancer binding protein-alpha amino acid motifs with dual TBP and TFIIB binding ability co-operate to activate transcription in both yeast and mammalian cells. *EMBO J* 1995 Sep 1; **14**(17): 4318-4328.

228. Friedman AD, McKnight SL. Identification of two polypeptide segments of CCAAT/enhancer-binding protein required for transcriptional activation of the serum albumin gene. *Genes Dev* 1990 Aug; **4**(8): 1416-1426.

229. Agre P, Johnson PF, McKnight SL. Cognate DNA binding specificity retained after leucine zipper exchange between GCN4 and C/EBP. *Science* 1989 Nov 17; **246**(4932): 922-926.

230. Vinson CR, Hai T, Boyd SM. Dimerization specificity of the leucine zipper-containing bZIP motif on DNA binding: prediction and rational design. *Genes Dev* 1993 Jun; **7**(6): 1047-1058.

231. Landschulz WH, Johnson PF, McKnight SL. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* 1988 Jun 24; **240**(4860): 1759-1764.

232. O'Shea EK, Klemm JD, Kim PS, Alber T. X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science* 1991 Oct 25; **254**(5031): 539-544.

233. LeClair KP, Blanar MA, Sharp PA. The p50 subunit of NF-kappa B associates with the NF-IL6 transcription factor. *Proc Natl Acad Sci U S A* 1992 Sep 1; **89**(17): 8145-8149.

234. Matsusaka T, Fujikawa K, Nishio Y, Mukaida N, Matsushima K, Kishimoto T, *et al.* Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc Natl Acad Sci U S A* 1993 Nov 1; **90**(21): 10193-10197.

235. Lee YM, Miau LH, Chang CJ, Lee SC. Transcriptional induction of the alpha-1 acid glycoprotein (AGP) gene by synergistic interaction of two alternative activator forms of AGP/enhancer-binding protein (C/EBP beta) and NF-kappaB or Nopp140. *Mol Cell Biol* 1996 Aug; **16**(8): 4257-4263.

236. Nishio Y, Isshiki H, Kishimoto T, Akira S. A nuclear factor for interleukin-6 expression (NF-IL6) and the glucocorticoid receptor synergistically activate transcription of the rat alpha 1-acid glycoprotein gene via direct protein-protein interaction. *Mol Cell Biol* 1993 Mar; **13**(3): 1854-1862.

237. Stein B, Cogswell PC, Baldwin AS, Jr. Functional and physical associations between NF-kappa B and C/EBP family members: a Rel domain-bZIP interaction. *Mol Cell Biol* 1993 Jul; **13**(7): 3964-3974.

238. Johnson PF. Identification of C/EBP basic region residues involved in DNA sequence recognition and half-site spacing preference. *Mol Cell Biol* 1993 Nov; **13**(11): 6919-6930.

239. Ellenberger T. Getting a grip on DNA recognition: structures of thebasic region leucine zipper, and the basic region helix-loop-helix DNAbindingdomains. *CurrOpinStrucBio* 1994; **4**: 12-21.

240. Darlington GJ, Ross SE, MacDougald OA. The role of C/EBP genes in adipocyte differentiation. *J Biol Chem* 1998 Nov 13; **273**(46): 30057-30060.

241. Lane MD, Tang QQ, Jiang MS. Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. *Biochem Biophys Res Commun* 1999 Dec 29; **266**(3): 677-683.

242. Morrison RF, Farmer SR. Insights into the transcriptional control of adipocyte differentiation. *J Cell Biochem* 1999; **Suppl 32-33**: 59-67.

243. Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. *Genes Dev* 2000 Jun 1; **14**(11): 1293-1307.

244. Christy RJ, Yang VW, Ntambi JM, Geiman DE, Landschulz WH, Friedman AD, *et al.* Differentiation-induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev* 1989 Sep; **3**(9): 1323-1335.

245. MacDougald OA, Lane MD. Adipocyte differentiation. When precursors are also regulators. *Curr Biol* 1995 Jun 1; **5**(6): 618-621.

246. Freytag SO, Paielli DL, Gilbert JD. Ectopic expression of the CCAAT/enhancer-binding protein alpha promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes Dev* 1994 Jul 15; **8**(14): 1654-1663.

247. Freytag SO, Geddes TJ. Reciprocal regulation of adipogenesis by Myc and C/EBP alpha. *Science* 1992 Apr 17; **256**(5055): 379-382.

248. Constance CM, Morgan JIt, Umek RM. C/EBPalpha regulation of the growth-arrest-associated gene gadd45. *Mol Cell Biol* 1996 Jul; **16**(7): 3878-3883.

249. MacDougald OA, Cornelius P, Liu R, Lane MD. Insulin regulates transcription of the CCAAT/enhancer binding protein (C/EBP) alpha, beta, and delta genes in fully-differentiated 3T3-L1 adipocytes. *J Biol Chem* 1995 Jan 13; **270**(2): 647-654.

250. Radomska HS, Huettner CS, Zhang P, Cheng T, Scadden DT, Tenen DG. CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Mol Cell Biol* 1998 Jul; **18**(7): 4301-4314.

251. Johansen LM, Iwama A, Lodie TA, Sasaki K, Felsher DW, Golub TR, *et al.* c-Myc is a critical target for c/EBPalpha in granulopoiesis. *Mol Cell Biol* 2001 Jun; **21**(11): 3789-3806.

252. Flodby P, Barlow C, Kylefjord H, Ahrlund-Richter L, Xanthopoulos KG. Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein alpha. *J Biol Chem* 1996 Oct 4; **271**(40): 24753-24760.

253. Friedman AD, Landschulz WH, McKnight SL. CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. *Genes Dev* 1989 Sep; **3**(9): 1314-1322.

254. Shih LY, Liang DC, Huang CF, Wu JH, Lin TL, Wang PN, *et al.* AML patients with CEBPalpha mutations mostly retain identical mutant patterns but frequently change in allelic distribution at relapse: a comparative analysis on paired diagnosis and relapse samples. *Leukemia* 2006 Apr; **20**(4): 604-609.

255. Pabst T, Mueller BU, Harakawa N, Schoch C, Haferlach T, Behre G, *et al.* AML1-ETO downregulates the granulocytic differentiation factor C/EBPalpha in t(8;21) myeloid leukemia. *Nat Med* 2001 Apr; **7**(4): 444-451.

256. Zada AA, Pulikkan JA, Bararia D, Geletu M, Trivedi AK, Balkhi MY, *et al.* Proteomic discovery of Max as a novel interacting partner of C/EBPalpha: a Myc/Max/Mad link. *Leukemia* 2006 Dec; **20**(12): 2137-2146.

257. Pal P, Lochab S, Kanaujiya J, Sanyal S, Trivedi AK. Ectopic expression of hC/EBPs in breast tumor cells induces apoptosis. *Mol Cell Biochem* Apr; **337**(1-2): 111-118.

258. Zhang DE, Zhang P, Wang ND, Hetherington CJ, Darlington GJ, Tenen DG. Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proc Natl Acad Sci U S A* 1997 Jan 21; **94**(2): 569-574.

259. Zhang DE, Hetherington CJ, Meyers S, Rhoades KL, Larson CJ, Chen HM, *et al.* CCAAT enhancer-binding protein (C/EBP) and AML1 (CBF alpha2) synergistically activate the macrophage colony-stimulating factor receptor promoter. *Mol Cell Biol* 1996 Mar; **16**(3): 1231-1240.

260. Hiebert S. Differentiation or leukemia: is C/EBPalpha the answer? *Nat Med* 2001 Apr; **7**(4): 407-408.

261. Pabst T, Mueller BU, Zhang P, Radomska HS, Narravula S, Schnittger S, *et al.* Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia. *Nat Genet* 2001 Mar; **27**(3): 263-270.

262. Preudhomme C, Sagot C, Boissel N, Cayuela JM, Tigaud I, de Botton S, *et al.* Favorable prognostic significance of CEBPA mutations in patients with de novo acute myeloid leukemia: a study from the Acute Leukemia French Association (ALFA). *Blood* 2002 Oct 15; **100**(8): 2717-2723.

263. Leroy H, Roumier C, Huyghe P, Biggio V, Fenaux P, Preudhomme C. CEBPA point mutations in hematological malignancies. *Leukemia* 2005 Mar; **19**(3): 329-334.

264. Pabst T, Mueller BU. Transcriptional dysregulation during myeloid transformation in AML. *Oncogene* 2007 Oct 15; **26**(47): 6829-6837.

265. Pajonk F, McBride WH. The proteasome in cancer biology and treatment. *Radiat Res* 2001 Nov; **156**(5 Pt 1): 447-459.

266. Myung J, Kim KB, Crews CM. The ubiquitin-proteasome pathway and proteasome inhibitors. *Med Res Rev* 2001 Jul; **21**(4): 245-273.

267. Tanaka K, Tamura T, Tanahashi N, Tsurumi C. Protein and gene structures of 20S and 26S proteasomes. *Adv Exp Med Biol* 1996; **389**: 187-195.

268. Koster AJ, Walz J, Lupas A, Baumeister W. Structural features of archaebacterial and eukaryotic proteasomes. *Mol Biol Rep* 1995; **21**(1): 11-20.

269. Tamura T, Nagy I, Lupas A, Lottspeich F, Cejka Z, Schoofs G, *et al.* The first characterization of a eubacterial proteasome: the 20S complex of Rhodococcus. *Curr Biol* 1995 Jul 1; **5**(7): 766-774.

270. Glickman MH, Rubin DM, Coux O, Wefes I, Pfeifer G, Cjeka Z, *et al.* A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. *Cell* 1998 Sep 4; **94**(5): 615-623.

271. Braun BC, Glickman M, Kraft R, Dahlmann B, Kloetzel PM, Finley D, *et al.* The base of the proteasome regulatory particle exhibits chaperone-like activity. *Nat Cell Biol* 1999 Aug; **1**(4): 221-226.

272. Orlowski M, Cardozo C, Michaud C. Evidence for the presence of five distinct proteolytic components in the pituitary multicatalytic proteinase complex. Properties of two components cleaving bonds on the carboxyl side of branched chain and small neutral amino acids. *Biochemistry* 1993 Feb 16; **32**(6): 1563-1572.

273. Cardozo C. Catalytic components of the bovine pituitary multicatalytic proteinase complex (proteasome). *Enzyme Protein* 1993; **47**(4-6): 296-305.

274. Wilk S, Orlowski M. Evidence that pituitary cation-sensitive neutral endopeptidase is a multicatalytic protease complex. *J Neurochem* 1983 Mar; **40**(3): 842-849.

275. Dick TP, Nussbaum AK, Deeg M, Heinemeyer W, Groll M, Schirle M, *et al.* Contribution of proteasomal beta-subunits to the cleavage of peptide substrates analyzed with yeast mutants. *J Biol Chem* 1998 Oct 2; **273**(40): 25637-25646.

276. Hilt W, Enenkel C, Gruhler A, Singer T, Wolf DH. The PRE4 gene codes for a subunit of the yeast proteasome necessary for peptidylglutamyl-peptide-hydrolyzing activity. Mutations link the proteasome to stress- and ubiquitin-dependent proteolysis. *J Biol Chem* 1993 Feb 15; **268**(5): 3479-3486.

277. Enenkel C, Lehmann H, Kipper J, Guckel R, Hilt W, Wolf DH. PRE3, highly homologous to the human major histocompatibility complex-linked LMP2 (RING12) gene, codes for a yeast proteasome subunit necessary for the peptidylglutamyl-peptide hydrolyzing activity. *FEBS Lett* 1994 Mar 21; **341**(2-3): 193-196.

278. Heinemeyer W, Gruhler A, Mohrle V, Mahe Y, Wolf DH. PRE2, highly homologous to the human major histocompatibility complex-linked RING10 gene, codes for a yeast proteasome subunit necessary for chrymotryptic activity and degradation of ubiquitinated proteins. *J Biol Chem* 1993 Mar 5; **268**(7): 5115-5120.

279. Salzmann U, Kral S, Braun B, Standera S, Schmidt M, Kloetzel PM, *et al.* Mutational analysis of subunit i beta2 (MECL-1) demonstrates conservation of cleavage specificity between yeast and mammalian proteasomes. *FEBS Lett* 1999 Jul 2; **454**(1-2): 11-15.

280. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* 2002 Apr; **82**(2): 373-428.

281. Hershko A, Heller H, Elias S, Ciechanover A. Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown. *J Biol Chem* 1983 Jul 10; **258**(13): 8206-8214.

282. Kalchman MA, Graham RK, Xia G, Koide HB, Hodgson JG, Graham KC, *et al.* Huntingtin is ubiquitinated and interacts with a specific ubiquitin-conjugating enzyme. *J Biol Chem* 1996 Aug 9; **271**(32): 19385-19394.

283. Passmore LA, Barford D. Getting into position: the catalytic mechanisms of protein ubiquitylation. *Biochem J* 2004 May 1; **379**(Pt 3): 513-525.

284. Johnson ES, Ma PC, Ota IM, Varshavsky A. A proteolytic pathway that recognizes ubiquitin as a degradation signal. *J Biol Chem* 1995 Jul 21; **270**(29): 17442-17456.

285. Thrower JS, Hoffman L, Rechsteiner M, Pickart CM. Recognition of the polyubiquitin proteolytic signal. *EMBO J* 2000 Jan 4; **19**(1): 94-102.

286. Wilkinson KD. Ubiquitination and deubiquitination: targeting of proteins for degradation by the proteasome. *Semin Cell Dev Biol* 2000 Jun; **11**(3): 141-148.

287. Hicke L, Zanolari B, Pypaert M, Rohrer J, Riezman H. Transport through the yeast endocytic pathway occurs through morphologically distinct compartments and requires an active secretory pathway and Sec18p/N-ethylmaleimide-sensitive fusion protein. *Mol Biol Cell* 1997 Jan; **8**(1): 13-31.

288. Huibregtse JM, Scheffner M, Beaudenon S, Howley PM. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. *Proc Natl Acad Sci U S A* 1995 Mar 28; **92**(7): 2563-2567.

289. Scheffner M, Nuber U, Huibregtse JM. Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade. *Nature* 1995 Jan 5; **373**(6509): 81-83.

290. Huang L, Kinnucan E, Wang G, Beaudenon S, Howley PM, Huibregtse JM, *et al.* Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science* 1999 Nov 12; **286**(5443): 1321-1326.

291. Verdecia MA, Joazeiro CA, Wells NJ, Ferrer JL, Bowman ME, Hunter T, *et al.* Conformational flexibility underlies ubiquitin ligation mediated by the WWP1 HECT domain E3 ligase. *Mol Cell* 2003 Jan; **11**(1): 249-259.

292. Zheng N. A closer look of the HECTic ubiquitin ligases. *Structure* 2003 Jan; **11**(1): 5-6.

293. Borden KL. RING domains: master builders of molecular scaffolds? *J Mol Biol* 2000 Feb 4; **295**(5): 1103-1112.

294. Pickart CM. Mechanisms underlying ubiquitination. *Annu Rev Biochem* 2001; **70:** 503-533.

295. Deshaies RJ. SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu Rev Cell Dev Biol* 1999; **15:** 435-467.

296. Hatakeyama S, Yada M, Matsumoto M, Ishida N, Nakayama KI. U box proteins as a new family of ubiquitin-protein ligases. *J Biol Chem* 2001 Aug 31; **276**(35): 33111-33120.

297. Boname JM, Stevenson PG. MHC class I ubiquitination by a viral PHD/LAP finger protein. *Immunity* 2001 Oct; **15**(4): 627-636.

298. Aravind L, Koonin EV. The U box is a modified RING finger - a common domain in ubiquitination. *Curr Biol* 2000 Feb 24; **10**(4): R132-134.

299. Narisawa-Saito M, Kiyono T. Basic mechanisms of high-risk human papillomavirus-induced carcinogenesis: roles of E6 and E7 proteins. *Cancer Sci* 2007 Oct; **98**(10): 1505-1511.

300. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* 1990 Dec 21; **63**(6): 1129-1136.

301. Huibregtse JM, Scheffner M, Howley PM. A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *EMBO J* 1991 Dec; **10**(13): 4129-4135.

302. Scheffner M, Huibregtse JM, Howley PM. Identification of a human ubiquitin-conjugating enzyme that mediates the E6-AP-dependent ubiquitination of p53. *Proc Natl Acad Sci U S A* 1994 Sep 13; **91**(19): 8797-8801.

303. Nakao M, Sutcliffe JS, Durtschi B, Mutirangura A, Ledbetter DH, Beaudet AL. Imprinting analysis of three genes in the Prader-Willi/Angelman region: SNRPN, E6-associated protein, and PAR-2 (D15S225E). *Hum Mol Genet* 1994 Feb; **3**(2): 309-315.

304. Yamamoto Y, Huibregtse JM, Howley PM. The human E6-AP gene (UBE3A) encodes three potential protein isoforms generated by differential splicing. *Genomics* 1997 Apr 15; **41**(2): 263-266.

305. Harris SL, Levine AJ. The p53 pathway: positive and negative feedback loops. *Oncogene* 2005 Apr 18; **24**(17): 2899-2908.

306. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997 May 15; **387**(6630): 296-299.

307. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. *Nature* 1997 May 15; **387**(6630): 299-303.

308. Hainaut P, Hernandez T, Robinson A, Rodriguez-Tome P, Flores T, Hollstein M, *et al.* IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. *Nucleic Acids Res* 1998 Jan 1; **26**(1): 205-213.

309. Jiang YH, Armstrong D, Albrecht U, Atkins CM, Noebels JL, Eichele G, *et al.* Mutation of the Angelman ubiquitin ligase in mice causes increased cytoplasmic p53 and deficits of contextual learning and long-term potentiation. *Neuron* 1998 Oct; **21**(4): 799-811.

310. Kishino T, Lalande M, Wagstaff J. UBE3A/E6-AP mutations cause Angelman syndrome. *Nat Genet* 1997 Jan; **15**(1): 70-73.

311. Smith CL, DeVera DG, Lamb DJ, Nawaz Z, Jiang YH, Beaudet AL, *et al.* Genetic ablation of the steroid receptor coactivator-ubiquitin ligase, E6-AP, results in tissue-selective steroid hormone resistance and defects in reproduction. *Mol Cell Biol* 2002 Jan; **22**(2): 525-535.

312. Rotin D. Regulation of the epithelial sodium channel (ENaC) by accessory proteins. *Curr Opin Nephrol Hypertens* 2000 Sep; **9**(5): 529-534.

313. Lecker SH, Solomon V, Mitch WE, Goldberg AL. Muscle protein breakdown and the critical role of the ubiquitin-proteasome pathway in normal and disease states. *J Nutr* 1999 Jan; **129**(1S Suppl): 227S-237S.

314. Shamu CE, Story CM, Rapoport TA, Ploegh HL. The pathway of US11-dependent degradation of MHC class I heavy chains involves a ubiquitin-conjugated intermediate. *J Cell Biol* 1999 Oct 4; **147**(1): 45-58.

315. Johnston JA, Dalton MJ, Gurney ME, Kopito RR. Formation of high molecular weight complexes of mutant Cu, Zn-superoxide dismutase in a mouse model for familial amyotrophic lateral sclerosis. *Proc Natl Acad Sci U S A* 2000 Nov 7; **97**(23): 12571-12576.

316. Gao X, Mohsin SK, Gatalica Z, Fu G, Sharma P, Nawaz Z. Decreased expression of e6-associated protein in breast and prostate carcinomas. *Endocrinology* 2005 Apr; **146**(4): 1707-1712.

317. Khan S, Barhoumi R, Burghardt R, Liu S, Kim K, Safe S. Molecular mechanism of inhibitory aryl hydrocarbon receptor-estrogen receptor/Sp1 cross talk in breast cancer cells. *Mol Endocrinol* 2006 Sep; **20**(9): 2199-2214.

318. Sivaraman L, Nawaz Z, Medina D, Conneely OM, O'Malley BW. The dual function steroid receptor coactivator/ubiquitin protein-ligase integrator E6-AP is overexpressed in mouse mammary tumorigenesis. *Breast Cancer Res Treat* 2000 Aug; **62**(3): 185-195.

319. Deng S, Zhou H, Xiong R, Lu Y, Yan D, Xing T, *et al.* Over-expression of genes and proteins of ubiquitin specific peptidases (USPs) and proteasome subunits (PSs) in breast cancer tissue observed by the methods of RFDD-PCR and proteomics. *Breast Cancer Res Treat* 2007 Jul; **104**(1): 21-30.

320. Coulombe P, Rodier G, Bonneil E, Thibault P, Meloche S. N-Terminal ubiquitination of extracellular signal-regulated kinase 3 and p21 directs their degradation by the proteasome. *Mol Cell Biol* 2004 Jul; **24**(14): 6140-6150.

321. Wang J, Sampath A, Raychaudhuri P, Bagchi S. Both Rb and E7 are regulated by the ubiquitin proteasome pathway in HPV-containing cervical tumor cells. *Oncogene* 2001 Aug 2; **20**(34): 4740-4749.

322. Bernassola F, Karin M, Ciechanover A, Melino G. The HECT family of E3 ubiquitin ligases: multiple players in cancer development. *Cancer Cell* 2008 Jul 8; **14**(1): 10-21.

323. Ceol CJ, Pellman D, Zon LI. APC and colon cancer: two hits for one. *Nat Med* 2007 Nov; **13**(11): 1286-1287.

324. Dai Z, Quackenbush RC, Courtney KD, Grove M, Cortez D, Reuther GW, *et al.* Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway. *Genes Dev* 1998 May 15; **12**(10): 1415-1424.

325. Wu L, Pu Z, Feng J, Li G, Zheng Z, Shen W. The ubiquitin-proteasome pathway and enhanced activity of NF-kappaB in gastric carcinoma. *J Surg Oncol* 2008 Apr 1; **97**(5): 439-444.

326. Trivedi AK, Pal P, Behre G, Singh SM. Multiple ways of C/EBPalpha inhibition in myeloid leukaemia. *Eur J Cancer* 2008 Jul; **44**(11): 1516-1523.

327. Subramanian L, Benson MD, Iniguez-Lluhi JA. A synergy control motif within the attenuator domain of CCAAT/enhancer-binding protein alpha inhibits transcriptional synergy through its PIASy-enhanced modification by SUMO-1 or SUMO-3. *J Biol Chem* 2003 Mar 14; **278**(11): 9134-9141.

328. Bengoechea-Alonso MT, Ericsson J. The ubiquitin ligase Fbxw7 controls adipocyte differentiation by targeting C/EBPalpha for degradation. *Proc Natl Acad Sci U S A* Jun 29; **107**(26): 11817-11822.

329. Talis AL, Huibregtse JM, Howley PM. The role of E6AP in the regulation of p53 protein levels in human papillomavirus (HPV)-positive and HPV-negative cells. *J Biol Chem* 1998 Mar 13; **273**(11): 6439-6445.

330. Louria-Hayon I, Alsheich-Bartok O, Levav-Cohen Y, Silberman I, Berger M, Grossman T, *et al.* E6AP promotes the degradation of the PML tumor suppressor. *Cell Death Differ* 2009 Aug; **16**(8): 1156-1166.

331. Lochab S, Pal,P.,kanaujiya,J., Bhatt,M.L.B.,Sanyal,S.,Behre,G.,Trivedi,A.K. Proteomic identification of E6AP as a molecular target of Tamoxifen in MCF7 cells. *Proteomics (accepted manuscript)* 2012.

332. Cheng J, Yu DV, Zhou JH, Shapiro DJ. Tamoxifen induction of CCAAT enhancer-binding protein alpha is required for tamoxifen-induced apoptosis. *J Biol Chem* 2007 Oct 19; **282**(42): 30535-30543.

333. Kuhne C, Banks L. E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box. *J Biol Chem* 1998 Dec 18; **273**(51): 34302-34309.

334. Kumar S, Talis AL, Howley PM. Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. *J Biol Chem* 1999 Jun 25; **274**(26): 18785-18792.

335. Oda H, Kumar S, Howley PM. Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination. *Proc Natl Acad Sci U S A* 1999 Aug 17; **96**(17): 9557-9562.

336. Thomas M, Banks L. Inhibition of Bak-induced apoptosis by HPV-18 E6. *Oncogene* 1998 Dec 10; **17**(23): 2943-2954.

337. Mani A, Oh AS, Bowden ET, Lahusen T, Lorick KL, Weissman AM, *et al.* E6AP mediates regulated proteasomal degradation of the nuclear receptor coactivator amplified in breast cancer 1 in immortalized cells. *Cancer Res* 2006 Sep 1; **66**(17): 8680-8686.

338. Ross SE, Radomska HS, Wu B, Zhang P, Winnay JN, Bajnok L, *et al.* Phosphorylation of C/EBPalpha inhibits granulopoiesis. *Mol Cell Biol* 2004 Jan; **24**(2): 675-686.

Publications

1. **Pal P**, Kanaujiya JK, Lochab S, Tripathi SB, Bhatt ML, Singh PK, Sanyal S, Trivedi AK. "2-D gel electrophoresis-based proteomic analysis reveals that ormeloxifen induces G0-G1 growth arrest and ERK-mediated apoptosis in chronic myeloid leukemia cells K562." **Proteomics.** 2011, Feb.doi: 10.1002/pmic.201000720. PMID: 21360677

2. Lochab S, **Pal P,** Kanaujiya JK, Tripathi SB, Kapoor I, Bhatt ML, Sanyal S, Behre G, Trivedi AK. "Proteomic identification of E6AP as a molecular target of tamoxifen in MCF7 cells." **Proteomics,** 2012, 12, 1-17. DOI 10.1002/pmic.201100572. (Accepted manuscript)

3. Kumar R, Gupta L, **Pal P**, Khan S, Singh N, Katiyar SB, Meena S, Sarkar J, Sinha S, Kanaujiya JK, Lochab S, Trivedi AK, Chauhan PM. Synthesis and cytotoxicity evaluation of (tetrahydro-beta-carboline)-1,3,5-triazine hybrids as anticancer agents." **Eur J Med Chem.** 2010 Jun;45(6):2265-76. Epub 2010 Feb 10.PMID: 20207053

4. **Pal P**, Lochab S, Kanaujiya J, Sanyal S, Trivedi AK."Ectopic expression of hC/EBPs in breast tumor cells induces apoptosis." **Mol Cell Biochem**. 2010 Apr; 337(1-2):111-8. Epub 2009 Oct 23.PMID: 19851833

Review Articles

1. Trivedi AK, **Pal P**, Behre G, Singh SM. "Multiple ways of C/EBPalpha inhibition in myeloid leukaemia." **Eur J Cancer**. 2008 Jul; 44(11):1516-23. Review.PMID: 18515086

2. Kanaujiya JK, Lochab S, **Pal P**, Christopeit M, Singh SM, Sanyal S, Behre G, Trivedi AK. "Proteomic approaches in myeloid leukemia." **Electrophoresis.** 2011 Feb;32(3-4):357-67. doi: 10.1002/elps.201000428. Epub 2011 Jan 20.PMID: 21254132