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1 Abstract

Although the survival rates of 80% in acute lymphocytic leukemia (ALL) is a remarkable achievement, the 20% of affected children are facing the risk of death and toxicity of the treatment is significant. The drugs used in ALL are anthracyclines and steroids. Glucocorticoids exert their therapeutic effects in leukemia by inducing apoptosis through intracellular glucocorticoid receptor (GR). Anthracyclines have the ability to inhibit the DNA topoisomerase II causing DNA double strands breaks and the end result is activation of p53 protein. The exact mechanisms of action of those drugs are not well understood, and significant proportion of children develops drug resistance. Moreover, it has recently been described that microenvironment can also contribute to leukemia cell survival and protect leukemia cells from the cytotoxic effect of drugs. Increased understanding of the mechanisms that lead to 80% curable rate will help bring curable rate to this level in other cancer types as well.

The aim of this study was to better understand of the role played by GR in improving the treatment and the role of bone marrow environment in inducing resistance. We have used ALL cell lines that are sensitive (CEM-C7-14) and resistant (CEM-C1-15) to glucocorticoid induced apoptosis and treated them with anthracyclines (etoposide-Etop) and glucocorticoids (dexamethasone-Dex) in the absence and presence of conditioned media (CM) obtained from bone marrow cells thus mimicking clinical settings. Our results demonstrate Bim down-regulation and Bax up-regulation highlighting the possibility of alternative pathways being utilized for apoptosis when both drugs are used. Also, analysis of the post translatonal modifications of GR indicated that a complex crosstalk may be involved, and the GR phosphorylation status differs when cells were treated with either Dex or ETOP alone or in combination. Additionally, CM has significant effects on GR phosphorylation profile in both cell lines. Furthermore, our preliminary data indicates that dexamethasone induces autophagy as well as apoptosis in leukemia cells. The activation of autophagy appears to be through beclin which is activated by glucocorticoids and activation of beclin is considered as a reason for efficient killing of the leukemic cells by dexamethasone. That may highlight beclin as a new target of glucocorticoids and open new therapeutic possibilities.

To have wider view, microarray technique was adapted. The interesting preliminary data revealed the repressive effect of CM on particular genes linked to cell death or

survival including NFKBIB (an inhibitor of NK-κB), RUNX3, Bad and RIP-1.Repressive effect of bone marrow microenvironment on those genes leads to cell survival, which is one way for the bone marrow microenvironment to develop resistance.

RIP-1 is significantly repressed in cells grown in CM media and it has been linked to not only apoptosis, but also with other forms of cells death including autophagy and necroptosis In addition RIP-1 is also involved in the regulation of MAPK and NF-κB, which are pathways that play an important role in regulating GR. Therefore, we initiated analysis and validation of RIP-1 to evaluate RIP-1 as a key switch between apoptosis and autophagy, and their significance with respect to cancer development and treatment. The data showed that inhibition of RIP-1 resulted in increased autophagy and not apoptosis. On the other hand, Dex treatment alone or combined tends to exert apoptotic stress on the cells rather than autophagy.

In conclusion, our findings can increase the understands of the biological and molecular aspects of the differences between the sensitive and resistant cells in acute lymphoblastic leukemia and the effect of the stem cell niche on the therapy which can contribute to more efficient, less toxic therapy.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

Copyright Statement

for my father soul
Who was supporter and encourager for education
and a victim of cancer

I wouldn't have come this far if it wasn't for you

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As she always being there for me, listening and helping

3 Abbreviations

β-GP	β-Glycerol phosphate
µl	Microliter
ACL	Aclarubicin
ACTH	Adrenocorticotropic hormone
AD1	Activation domain 1
AF-1	Activation function-1
AF-2	Activation function-2
AKT	Protein kinase B (PKB)
Ang-1	Angiopoeitin-1
AP-1	Activator protein 1
APAF- 1	Apoptosis protease activating factor
APS	Ammonium per sulphate
ATF2	Activating transcription factor 2
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3 related
Bad	Bcl-2 antagonist of cell death
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma-2
BH-3	Bcl-2-homology
Bik	Bcl-2 interacting killer
Bim	Bcl-2-interacting mediator of cell death
BIR	Baculovirus inhibitor repeat
BM	Bone marrow
BMSC	Bone marrow stromal cells
BOX1	Bone living cells
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer-binding protein
CDKN1A	p21 is a cyclin-dependent kinase inhibitor 1A
CDKs	Cyclin dependent kinases
ChIP	Chromatin immunoprecipitation
Chk1	Serine/threonine-protein kinase 1
Chk2	Serine/threonine-protein kinase 2
CKI	Cyclin dependent kinase inhibitor
CM	Conditioned media
CNS	Central nervous system
CRH	corticotropin-releasing hormone
CXCL12	CXC-chemokine ligand 12
Cyt c	Cytochrome c
DAU	Daunorubicin
DBD	DNA-binding domain
DCC	Dextran coated charcoal
DD	Death domain
Dex	Dexamethasone
DMEM	Dulbecco's mModified Eagle's mMedium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DTT	Dithiothreitol

EDTA	Ethylenediaminetetraacetic acid
EPI	Epirubicin
ERK	Extracellular signal-regulated kinase
ETOP	Etoposide
FAB	French-American-British
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death domain protein
FCS	Foetal calf serum
Fdr	False discovery rate
FITC	Fluorescein isothiocyanate
Foxo3a	Forkhead box O3
GCs	Glucocorticoids
G-CSF	Granulocyte colony-stimulating factor
Gilz	Glucocorticoid-induced leucine zipper
GR	Glucocorticoids receptor
GRE	Glucocorticoid response elements
GSK-3b	Glycogen synthase kinase 3 beta
HPA	Hypothalamic-pituitary-adrenal
HPC	Hematopoietic progenitor cell
HSCs	Hematopoietic regenerator cell
HSLB	High Salt Lysis buffer
Hsp	Heat-shock proteins
IAP	Inhibitor of apoptosis protein
IL1	Interleukin 1
IL10	Interleukin 10
IR	Infra-red
IRF3	Interferon regulatory factor 3
I-κB,	Inhibitor of NK-κB
JAK/STAT	The Janus kinase/signal transducers and activators of transcription
JNK	Jun N-terminal kinase
KOH	Potassium hydroxid
LBD	Ligand-binding domain
LC3	Microtubule-associated protein 1A/1B-light chain 3
LFA-1	Leukocyte function antigen-1
MAC	Mitochondrial outer membrane
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MAPKs	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukaemia-1
MDC	Monodansylcadaverine
MDM2	Mouse double minute 2 homolog
MEF2C	Myocyte enhancing factor-2c
MIT	Mitoxantrone
MMP-2	Matrix metalloproteinase-2
MMP-9	Osteoclasts express matrix metalloproteinase-9
MM	Multiple myeloma
MSCs	Mesenchymal stem cells
NaCl	Sodium chloride
NaOV	Sodium orthophosphate
NaPPi	Sodium pyrophosphate

N-CoR	Nuclear receptor co-repressor
Nec-1	Necrostatin-1
NES	Nuclear export signals
NF-κB	Nuclear factor-kB
NFKBIB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta
ng	Nanogram
nGREs	Negative glucocorticoid response elements
NK	Natural killer cell
NL1	Nuclear localisation 1
NL2	Nuclear localisation 2
NLS	Nuclear localization signal
OD	Homo-oligomerisation domain
p38 MAPK	p38 mitogen-activated protein kinases
PBS	Phosphate buffer saline
PCA	Principal components analysis
PCNA	Proliferating cell nuclear antigen
PE	Phosphatidylethanolamine
PH	Pleckstrin homology
PI	Propidium iodide
PI	Protease inhibitor
PI3K	Phosphatidyl inositol 3-kinase
PIP2	Phosphatidylinositol 4, 5-biphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PIR	Pirarubicin
PKB,	Protein Kinase B
PMSF	Phenylmethylsulphonylfluoride
PS	Phosphotidylserine
PTEN	Phosphatase and tensin homolog
PTMs	Post translational modifications
qRT-PCR	Quantitative real time polymerase chain reaction
RIP-1	Receptor interacting protein 1
RMA	Robust multiarray average
ROS	Reactive oxygen species
Rpl19	Ribosomal protein L19
Rpm	Round per minute
RPMI-1640	Roswell park memorial institute-1640
RT	Room temperature
RUNX3	Runt-related transcription factor 3
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SMAC	Second mitochondria derived activator of caspase
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SNS	Sympathetic nervous system
SUMO	Small biquitin-related modifier1
TAD	Transcription activation domain
T-ALL	T-Acute Lymphoblastic Leukaemia
TBS	Tris buffered saline
TEMED	Tetra methyl ethylene diamine

Th-1	T-helper cell
TNF	Tumour necrosis factor
TNF-R	Tumour necrosis factor receptor
TRADD	TNF receptor-associated death domain
UBCs	E2 ubiquitin-conjugating enzymes
UV	Ultraviolet
VDAC	Voltage-dependent anion channel
VLA-4	Very late antigen-4
WHO	World health organization

4 Introduction

4.1 Cancer

Cancer is a condition caused by uncontrolled growth that results from changes in normal cell division. It has the ability to invade surrounding healthy tissues and nearby organs. In advanced cases, it can spread to other organs and areas in the body through the lymphatic system or bloodstream. This type of spreading is known as metastasis (Ruddon, 2007).

There are over 200 different types of cancer. According to a 2008 study by Cancer Research UK, an estimated 12.7 million people are diagnosed with cancer worldwide every year. It is predicted that the number will increase due to increases in population. In the UK, approximately 324,579 people are diagnosed with cancer every year and 8,257 out of this number are diagnosed with leukemia. Among the most commonly diagnosed cancer worldwide, leukemia occupies the 11th place out of 200 types of cancer (Figure 4.1.1 A) and the 10th place as the most common cause of death from Cancer Worldwide (Figure 4.1.1 B) (Cancer Research UK, 2013). Additionally, leukemia occupies the first diagnosed cancer in children (Figure 4.1.1 C) and the second major cause of death among children, after brain tumours (Cancer Research UK, 2013).

4.1.1 Leukaemia

Leukaemia refers to blood cancer and results from overproduction of leukocytes (white blood cells) in the bone marrow. Depending on the affected types of white blood cells, Leukaemia may be classified as chronic or acute. Acute leukaemia develops within days or weeks. It progresses quickly and as such requires immediate treatment. Compared to acute leukaemia, chronic leukaemia develops slowly, taking several months or even years. Leukaemia is further classified depending on the type of affected blood cells. Leukaemia may be classified as lymphocytic leukaemia when it affects lymphocytes or myeloid leukaemia when it affects myelocytes.

4.1.1.1 Acute Lymphoblastic Leukaemia (ALL)

Acute lymphoblastic leukaemia (ALL) is a malignant disorder of lymphoid progenitor cells (Yeoh *et al.*, 2002). The immature lymphocytes, commonly known as lymphoblasts or blast cells, undergo proliferation inside the bone marrow and prevent it from producing blood cells effectively. Leukaemia cells lack maturity and per se,

they do not perform the normal functions of the white blood cells. As a result, leukaemia leads to an increased risk of infection. Besides, the bone marrow gets filled up with immature white cells leading to reduction in red cells and platelets.

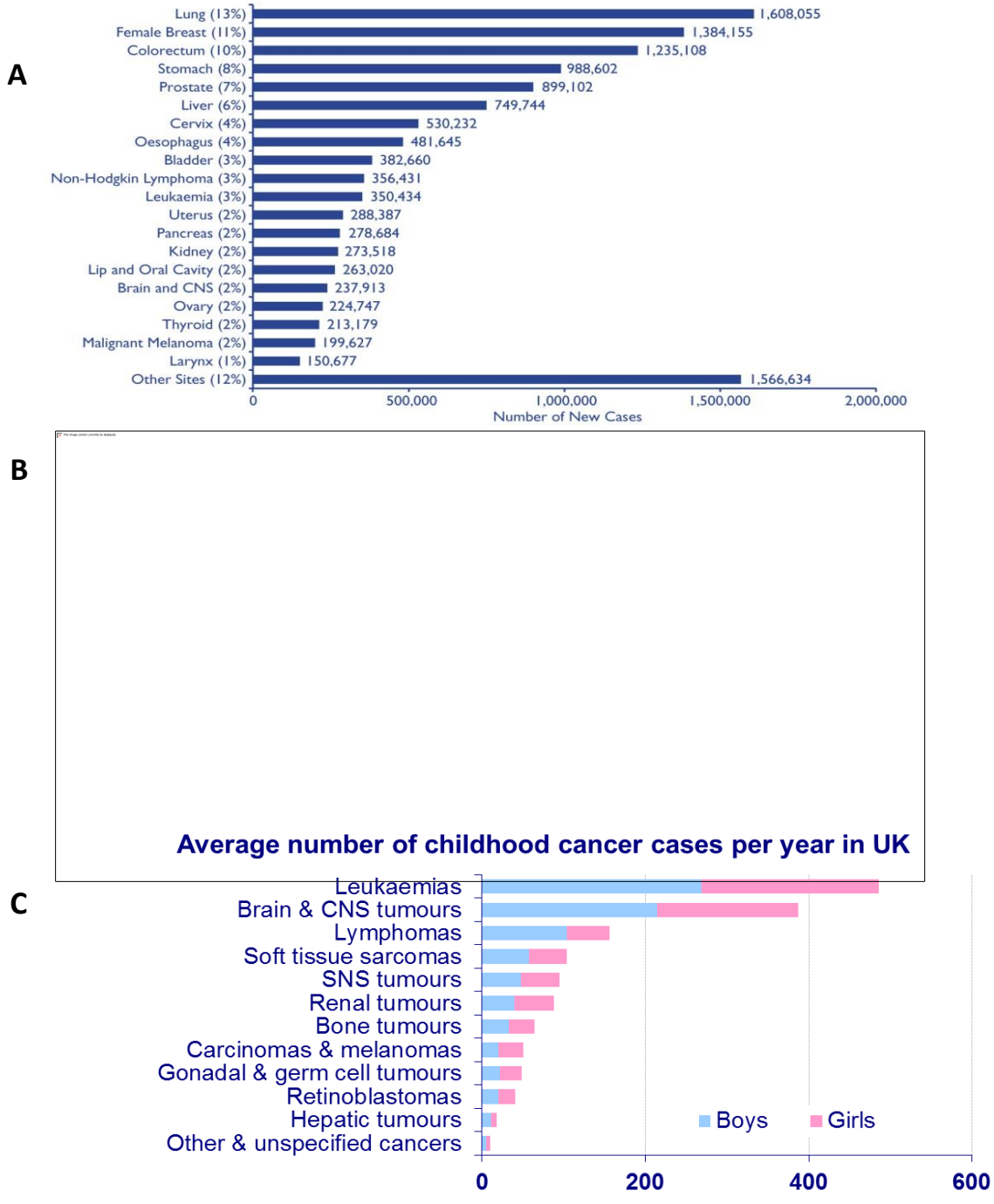


Figure 4.1.1: **(A) The 20 Most Commonly Diagnosed Cancers Worldwide for year 2008 according to cancer research UK, (B) Most Common Causes of Death from Cancer Worldwide, 2008 according to cancer research UK and (C) The Most Commonly Diagnosed Cancers in UK, average number of childhood cancer cases.** Taken from Cancer Research UK, UK Cancer Incidence (2010) by Country Summary, April 2013. CNS: central nervous system. SNS: sympathetic nervous system.

Acute lymphoblastic leukemia (T-ALL) is derived from T lymphoid progenitors. T-ALL forms approximately 15% of ALL (Cardoso *et al.*, 2008). ALL occurs frequently in children under 15 years of age with peak prevalence occurring in the age group, 2-5 years (Pui *et al* , 2008). ALL can also affect those between 15 and 25 years old, as well as elderly people. In 2010, 8.257 people in the UK were diagnosed with leukaemia (Cancer Research UK, 2013). Leukaemia is more common in males than in females (Pui *et al* , 2008).

There are two main types of ALL, namely, B-cell ALL and T-cell ALL. These types of ALL are only differentiated through immunophenotyping. The B-cell ALL is more common than the T-cell ALL. Most cases of B-cell ALL begin in the precursor B-cell. According to the World Health Organization (WHO), ALL may be classified using the cell line as B-cell and T-cell and also using the cell maturity as precursor or mature.

Based on WHO's classification, ALL has 4 further subtypes which are Precursor B-cell ALL, Transitional pre-B ALL, B-cell ALL and T-cell ALL. Precursor B-Type cell ALL has a higher prevalence as it accounts for 80–85% of all acute lymphoblastic leukaemia cases. Transitional pre-B ALL is characterized by unclear clinical picture and rare occurrence. It accounts for only 3% of all acute lymphoblastic leukaemia cases. B-cell ALL accounts for only 2-3% while T-cell ALL affects up to 13% of all acute lymphoblastic leukaemia cases. Another common classification method is the French-American-British (FAB) classification. This system classifies ALL as L1, L2 and L3 depending on the appearance of cells under the microscope. In L1 ALL, the cells are characterized by small size and regular shape blasts. L1 ALL is the most common. On the other hand, in L2 ALL, cells are characterized by large size and irregular nucleus. L3 ALL is rare and has large, round shaped cells (Bennett *et al*, 1976).

4.1.1.2 Etiology of ALL

The causes of childhood leukemia are not clear (Wiemels, 2012), however, environmental causes (risk factors) increase the chance of leukemia. Some of the known environmental factors include ionizing radiation and exposure to conditions such as parental smoking, pesticides, traffic fumes, paint and household chemicals (Wiemels, 2012). Furthermore, the congenital genetic syndromes such as Down's, neurofibromatosis, and Bloom's Syndrome, collectively account for less than 10% of

cases. Moreover, the occurrence of specific genetic features like translocations, the presence of extra chromosomes, and above all, most of the common genetic mutations in leukemia have been assessed for their occurrence during the fetal period. Genetic mutations such as the formation of Philadelphia chromosome due to a reciprocal translocation between chromosome 9 and 22, are specifically designated (9; 22) (q34; q11). Philadelphia chromosome is found in 25–30% ALL adults and 2–10% in pediatric cases (Talpaz *et al.*, 2006).

4.1.1.3 Treatment of T-ALL

The aim of treatments for ALL is to destroy the leukaemia cells and allow the bone marrow to function as required. The outcome of childhood acute lymphoblastic leukemia (ALL) has improved significantly over the past 50 years with modern frontline intensive regimens (Thomas *et al.*, 2010). In pediatric, ALL cure rates are currently exceeding 80%. Using similar therapeutic strategies in adult ALL, the overall cure rates remain in the range of 30% to 50% (Thomas *et al.*, 2010). Chemotherapy is the main type of treatment for ALL. Chemotherapy treatment is divided into three different phases: induction, intensification (consolidation) and continuing therapy (maintenance) (Apostolidou *et al.*, 2007).

Induction is the initial intensive phase of treatment. Induction normally lasts for 3-8 weeks, and works towards destroying as many leukaemia cells as possible. It usually achieves a remission (0% of lymphoblast in the peripheral blood) of the disease. The most widespread chemotherapy drugs used in this phase include: daunorubicin, doxorubicin, idarubicin, mitoxantrone, methotrexate, crisantaspase (asparaginase), mercaptopurine, cyclophosphamide and vincristine.

In addition, glucocorticoids (GCs) are used in combination therapy for treating acute lymphoblastic leukemia (ALL) (Medh *et al.*, 1998; Mcneer *et al.*, 2010; Schwartz *et al.*, 2010). The synthetic GC forms that can be used to treat leukaemia are predinsone, prednisolone and dexamethasone (Dex) (Bostrom *et al.*, 2003; Mitchell *et al.*, 2005). ALL treatment through GC synthetic compounds, Dex, appeared to be the most effective. This is due to its longer half-life and its effectiveness on the central nervous system (CNS) penetration (Pui *et al.*, 2006).

Intensification (consolidation) after the induction phase and increased chemotherapy is used to destroy any remaining leukaemia cells, most of which may not be visible in the blood or bone marrow. The treatment lasts for several months. The drug cocktail is likely to be the same as that used during the induction phase, but with the inclusion of cytarabine, etoposide and thioguanine.

Continuing therapy (maintenance) is one of the treatment phases and reduces the risk of relapse after treatment. It may last for a couple of years. Continuing therapy is a less intensive course of chemotherapy. Common drugs used are mercaptopurine, methotrexate, and vincristine with steroids, which is continued in short courses. However, the current treatment reflects a remarkable increase in the number of survivals but the upcoming drug resistance still can delay the treatments. Therefore, there is need for investigation in treatment methods that can overcome drug resistance.

4.1.1.4 Anthracyclines

Anthracyclines refers to a large family of chemotherapeutic agents such as aclarubicin (ACL), daunorubicin (DAU), doxorubicin (DOX), epirubicin (EPI), pirarubicin (PIR), mitoxantrone (MIT) and etoposide (ETOP). Anthracyclines have been used to treat cancer for the past 5 decades (Hortobagyi, 1997).

4.1.1.4.1 Doxorubicin

Doxorubicin (DOX) (the trade name is Adriamycin) is a member of chemotherapy drug of the anthracycline family. Chemical structure of DOX is illustrated in Figure 4.2.1. Adriamycin is administered intravenously to acute lymphocytic leukemia patients. However, serious side effects such as alopecia (hair loss), neutropenia (decreased level of neutrophils), congestive heart failure and dilated cardiomyopathy have been observed.

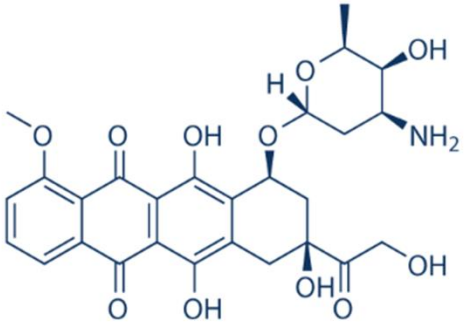


Figure 4.2.1: **Chemical structure of Doxorubicin.** Doxorubicin is a topoisomerase II inhibitor that leads to double DNA strands breakage by deregulate of the enzyme that stabilize the covalent complex between DNA and enzyme. Figure was drawn using TouchMol software http://www.scilligence.com/web/touchmol.aspx.

4.1.1.4.2 Etoposide

Etoposide (ETOP) (the brand name is VePesid) is another chemotherapy drug. It is a DNA topoisomerase II inhibitor, as well as, a member of the anthracyclines family. Chemical structure of ETOP is illustrated in figure 4.3.1. It is administered either intravenously or orally to ALL patients, in combination with other drugs. Etoposide has a number of side effects, but the most serious ones are inhibition of immune system response, bladder and kidney damage.

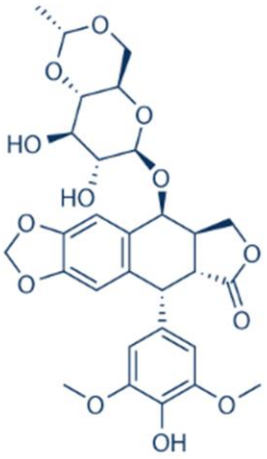


Figure 4.3.1: **Chemical structure of Etoposide.** Etoposide is topoisomerase II inhibitor and leads to cytotoxic DNA breaks in the cells. Figure was drawn using TouchMol software http://www.scilligence.com/web/touchmol.aspx

4.1.1.4.3 Mitroxantrone

Is also known as MIT and its trade name is Novantrone. The chemical structure of MIT is illustrated in Figure 4.4.1. Although its exact molecular mechanism is not clear, mitoxantrone is known to be toxic to cell division. It is administered to patients by intravenous drip. The dose varies according to the patient`s weight. Mitroxantrone's side effects are similar to those of doxorubicin and include heart damage, immunosuppression and alopecia.

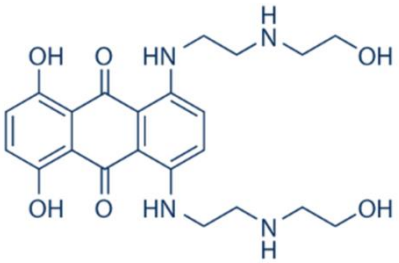


Figure 4.4.1: **Chemical structure of Mitroxantrone.** Mitoxantrone is an anticancer treatment that is toxic to dividing cells, but its molecular mechanism is not yet clear. Figure was drawn using TouchMol software http://www.scilligence.com/web/touchmol.aspx.

4.2 p53 mode of action

p53 is a tumour suppressor protein mutated in over half of human cancers (Cuadrado *et al*., 2006). In a normal cell, p53 is inactivated by MDM2 (negative regulator). p53 is activated in response to stress types such as UV, IR, or chemical agents such as hydrogen peroxide.The ataxia-telangiectasia mutated (ATM) protein kinase is the crucial trigger for DNA damage checkpoints pathway, DNA repair and apoptosis(Sarkaria *et al.*, 2001). ATM can be activated by ionizing radiation (IR) (Cuadrado *et al.*, 2006), whereas ATM Rad 3-related (ATR) is activated by ultraviolet radiation (Figure 4.5.1). DNA damage activates ATM and ATR and subsequently cell signaling pathway, which regulate cell cycle checkpoints pathway (Sarkaria *et al.*, 2001). ATM and ATR are activated by auto-phosphorylation and subsequently activate downstream kinases, Chk2 and Chk1, respectively (Cuadrado *et al*., 2006). p53 functions through three major mechanisms to prevent cancer. It may activate DNA repair proteins in case of DNA damage. It arrests cell cycle for a period long enough to allow the DNA repair proteins to fix the damage by activating p21, p57 and p27. In case of high levels of DNA damage, p53 can initiate apoptosis through the activation of Bax (Nicholas *et al.*, 1999).

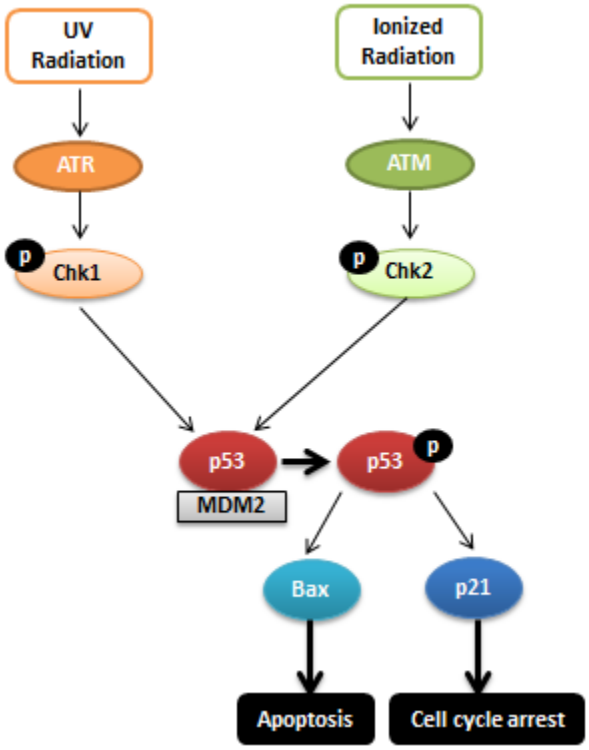


Figure 4.5.1: **Scheme of simplified p53 signaling pathway**. DNA damage results in activation of ATM and ATR cascade. ATM and ATR phosphorylate regulates Chk2 and ChK1. ATM is activated in response to DNA damage, caused by ionized radiation. Ionized radiation causes DNA single strand break. ATR is activated in response to DNA damage, caused by UV radiation. UV radiation causes DNA double strands break.

4.3 Tumour suppressor protein p53

p53 is considered the guardian of the human genome due to its role in protecting cells from cancer, through the regulation of different checkpoints in response to stress (Sarig R. *et al.*, 2008). Inactivation of p53 leads to genetic alterations that induce malignant transformation of cells. The cells that express mutant p53 proteins are more resistant to drug induced apoptosis. Therefore, p53 inactivation results from mutations in the p53 gene itself or the factors regulating the pathway (Lozano *et al.*, 2000). Tumor suppressor protein p53 is a transcription factor that is activated in response to cellular stress such as DNA damage, oncogenes, hypoxia and nutrient deprivation (Fridman *and* Lowe, 2003). p53 is activated by regulating various downstream target genes. Activated p53 gene triggers a series of cellular events like cell cycle arrest, DNA repair, senescence and apoptosis. Thus, activated p53 is responsible for promoting cellular homeostasis (Toledo *and* Wahl, 2007). The regulatory role of p53

is to allow mild damaged cell to undergo cell cycle arrest and DNA repair. Irreversibly damaged cells are excluded by apoptosis. Moreover, recent studies have demonstrated that p53 is involved in the regulation of autophagy (Maiuri *et al.*, 2010).

4.3.1 Tumour suppressor protein p53 structure

The p53 protein is encoded by the TP53 gene located on the short arm of chromosome 17 (Tawaki *et al.*, 2009). The wild type p53 protein consists of 393 amino acids and is composed of several domains (Figure 4.6.1).

Figure 4.6.1: **Functional domains of p53**. p53 protein comprises of several domains: the trans-activation domain; A proline-rich domain; The DNA-binding domain which consists of a variety of structural motifs; the C-terminus consists of three putative nuclear localization signals (NLS); within the oligomerization domain nuclear export signals (NES) has been identified.

The first domain is N-terminal transcription activation domain (TAD). TAD is also known as activation domain 1 (AD1), and is responsible for transcription of p53 target genes. TAD also facilitates the recruitment of transcription factors such as TBP, TAFs and TFIID (Lu *and* Levine, 1995). Moreover, this domain negatively regulates p53 by binding to MDM2 (Kubbutat *et al.*, 1997).

The next domain is the Proline rich domain, located in the N-terminus. It is involved in the apoptotic activity of p53 (Sakamuro *et al.*, 1997).

DNA-binding core domain (DBD) is the central domain and contains one zinc atom. The importance of this domain is to bind to the consensus sequences within the promoter of its target gene. Interestingly, 90% of p53 mutations occur in this domain (Bensaad *et al.*, 2006).

The domain required for p53 dependent transactivation and suppression is known as "Homo-oligomerisation domain" (OD). It allows tetramer formation and stabilises the

union of 4-subunits to form a single molecule of p53 tetramer, which is crucial for p53 gene functionality and activity (Pietenpol *et al.*, 1994).

The last domain is the C-terminal domain. This domain is involved in down regulation of DNA binding of the central domain by locking the domain in a latent conformation (Levine , 1997). It contains three nuclear localization signal sequences and one nuclear export signal sequence.

4.3.2 Tumour suppressor protein p53 target genes

There are numerous target genes related to acute lymphocytic leukaemia, but we will focus on two genes because of their ability to trigger apoptosis (Bax) and cell cycle arrest (p21) after p53 activation.

4.3.2.1 Bcl-2-associated X protein (Bax).

After irreversible cell damage, Bax is expressed as a result of high p53 protein level. Bax is a pro-apoptotic protein of the Bcl-2 gene family and it contains BH1, BH2 and BH3 domains (Wolter *et al.*, 1997). Bax is found in the cytoplasm, but when the cell undergoes apoptosis, Bax enters into mitochondrial membranes and induces opening of mitochondrial voltage-dependent anion channel (VDAC). Moreover, activated Bax and Bak form an oligomeric pore in the mitochondrial outer membrane, resulting in the release of cytochrome c and the other pro-apoptotic factors from mitochondria. This leads to activation of caspases (Gavathiotis *et al.*, 2008).

Tumour suppressor protein p53 up-regulates the expression of Bax, which is involved in p53-mediated apoptosis. In particular, p53 interacts with the regulatory region in the gene to activate Bax. This promotes its activation and insertion into the mitochondria to induce apoptosis when over expressed (Wolter *et al.*, 1997).

4.3.2.2 p21 WAF-1/CIP-1

Under mild stress conditions, p53 induces the expression of genes involved in cell cycle arrest $p21^{WAF\text{-}1/CIP\text{-}1}$. $p21^{WAF\text{-}1/CIP\text{-}1}$ is a cyclin-dependent kinase inhibitor 1A (CDKN1A) which is encoded by the CDKN1A gene that is located on chromosome 6 (Ouellet *et al.*, 2006). $p21^{WAF\text{-}1/CIP\text{-}1}$ protein functions as a regulator of cell cycle at G1 phase by binding and inhibiting the activity of cyclin- CDK2 or CDK4 complex (Gartel and Tyner, 2002). Tumour suppressor protein p53 controls the expression of

p21 gene and subsequently mediates the p53 dependent cell cycle G1 phase arrest in response to stress.

p21 protein also interacts with proliferating cell nuclear antigen (PCNA), which is an accessory factor of DNA polymerase. It also acts as a regulator of S phase DNA replication and DNA damage repair (Gartel and Tyner, 2002).

4.3.3 Post-translational modifications of p53

The post-translational alteration of p53 influences the signal transduction in stress conditions. Mechanisms involved in regulating p53 like phosphorylation, acetylation, sumoylation, ubiquitination and others are key players in maintaining the constancy and transcriptional performance of p53. The human p53 gene gets phosphorylated at N and C terminal by various kinases (Helton and Chen, 2007). Phosphorylation events mostly occur after DNA damage. These events are usually carried out by phosphatidylinositol-3-kinase (PI3K) related kinase such as ataxia telangiectasia mutated (ATM), and Rad3-related (ATR). These enzymes phosphorylate p53 at Ser15 and Ser20 by activating Chk1 and Chk2 which inhibits the interactions between gene p53 and MDM2, ultimately stabilizing p53 protein (Bai and Zhu, 2006). Phosphorylation is responsible for not only regulating p53's DNA binding activity but also for the recruitment of cofactors such as PCAF (Xenaki *et al.*, 2008). During DNA damage p53 transcriptionally activates $p21^{WAF-1/CIP-1}$, causes cell cycle arrest and activates Bax to initiate apoptosis (Helton and Chen, 2007). On the other hand, ubiquitination is involved mainly in the p53 degradation (Dai and Gu, 2010).

4.4 Glucocorticoid hormone

The glucocorticoids (GC) are a subclass of steroid hormones, used to treat children with ALL (Beesley *et al.*, 2009) due to their ability to induce apoptosis in lymphoblastic cells (Ploner and Kofler . 2009). Dexamethasone and prednisolone are the synthetic forms of glucocorticoids and are used to treat paediatric ALL (Tegethoff and Pryce , 2009).

Since glucocorticoids have potent anti-inflammatory and immunosuppressive properties, they are widely used as a treatment for inflammation and autoimmune disease. Glucocorticoids play an important role in a variety of biological processes,

such as metabolism, development, differentiation, immunity, reproduction and neural activity. The chemical structure of Dexamethasone is illustrated in Figure 4.7.1.

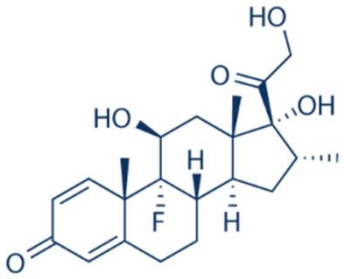


Figure 4.7.1: **Chemical structure of Dexamethasone.** Dexamethasone is the synthetic form of cortisol. It is widely used to treat acute lymphoblastic leukaemia, inflammation and autoimmune diseases as it exerts its effect after binding to glucocorticoid receptor and transcriptionally activating a variety of proteins. Figure was drawn using TouchMol software http://www.scilligence.com/web/touchmol.aspx.

4.4.1 The hypothalamic-pituitary-adrenal axis

This stress-hormone response engages the hypothalamic-pituitary-adrenal (HPA) axis (Figure 4.8.1). Hormonal response begins in the hypothalamus (a small gland at the base of the brain) wherein corticotrophin-releasing hormone (CRH) is excreted into the bloodstream. Then, CRH travels through the blood vessels to the pituitary gland (a pea-sized structure attached by a short stalk below the hypothalamus gland) and stimulates the release of adrenocorticotropic hormone (ACTH). ACTH, in turn, travels in the bloodstream from the pituitary to the adrenal glands (one perched atop each kidney), which release cortisol. Cortisol (the most potent glucocorticoid in humans) travels through the body as an adaptation to external stress (Bateman *et al.*, 1989).

Moreover, cortisol provides a "negative feedback system." When cortisol arrives in the hypothalamus region, it inhibits further excess release of CRH, thus, restoring ACTH and cortisol to normal levels (Flier *et al*, 1995).

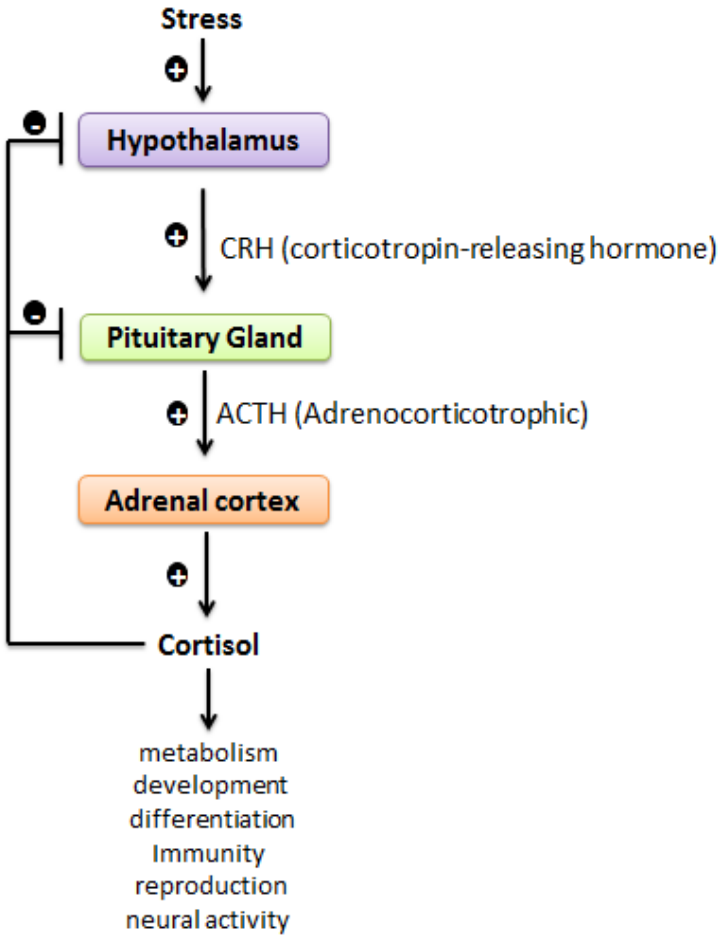


Figure 4.8.1: **An overview of the HPA axis**. In stress, the hypothalamus releases a hormone known as corticotrophin-releasing hormone (CRH). CRH is then transported to the pituitary gland which then secretes another hormone, adrenocorticotropic hormone (ACTH). ACTH then stimulates the adrenal cortex and the outcome is the secretion of cortisol as an adaptation to stress.

4.4.2 Glucocorticoid and related disorders

Cushing's syndrome, also known as hypercortisolism, is a disorder caused by the chronic intake of cortisol. This results from a tumour that can produce cortisol or intake of synthetic glucocorticoids. The symptoms of Cushing's syndrome include weight gain, thinning skin, excessive sweating, fat deposits in the face (moon face), depression and anxiety. Cushing's syndrome is treated by either reducing the prescribed glucocorticoids or surgical removal of the tumour (Nieman *et al.*, 2008).

Addison's disease is the opposite of Cushing's syndrome, whereby the level of cortisol present in the blood is insufficient. This occurs when the adrenal glands do not produce sufficient steroid hormones as "primary adrenal insufficiency", or can be caused by damage from the body's own immune system. Symptoms include fatigue,

muscle weakness and weight loss. The condition can be treated with the administration of synthetic glucocorticoids (Nerup, 1974).

4.4.3 The Glucocorticoid Receptor

The glucocorticoid receptor (GR) is a transcription factor that is activated by the binding of the GC hormone. GR is known as NR3C1 since NR3C1 is a GR gene located on chromosome 5. NR3C1 belongs to the steroid nuclear receptor family. The steroid nuclear receptor family belongs to a superfamily called the nuclear receptor superfamily. This superfamily shares a common structure which is a DNA binding domain (DBD) and Ligand binding domain (LBD). Moreover, GR is a 94kD protein member of the steroid-hormone-receptor family and is expressed abundantly (Japiassú *et al.*, 2009). GR contains two zinc fingers in their DNA binding domain which is controlled by ligand binding, cofactor binding and posttranslational modifications (Japiassú *et al.*, 2009). Adequate expression of GR is vital for GC induced apoptosis.

4.4.4 Structure of GR

GR consists of three functional domains. The first one is the N-terminal domain which contains transactivation functions (AF-1). The second one is the DNA-binding domain (DBD) with two zinc-finger motifs namely, a common DNA binding motif and a ligand-binding domain (LBD). The zinc-finger motifs consist of 12 alpha-helices, which are normally involved in the formation of the hydrophobic ligand-binding pocket (Figure 4.9.1). The N-terminal domain contains a ligand independent domain known as activation functions (AF-1) which is spanning from 77-262 amino acids (Figure 4.9.1) (Stade *et al.*, 1997). AF-1 region in the GR can pre-initiate some factors as TFIID and TBP and can interacts with the GR cofactors (Duma *et al.*, 2006). Moreover, AF-1 region contains target residues for post translational modifications (PTM) such as phosphorylation, sumoylation or ubiquitination (Ismaili and Garabedian, 2004; Rajkumar *et al.*, 2005). In terms of phosphorylation, the AF-1 region in the human GR contains phosphorylation residues such as Ser 203, Ser 211 and Ser226 which are targeted by cyclin-dependent kinase (CDK) and mitogen activated protein kinase (MAPK) families. Phosphorylation plays an important role in resistance toward treatment.

DNA-binding domain (DBD) is spanning from 421-488 amino acids. It contains highly conserved amino acid sequences with two zinc-finger motifs that is targeted and binds to specific DNA (Figure 4.9.1), (Schoneveld *et al.*, 2004; Mcmaster and Ray, 2008*).* GR dimerization, nuclear translocation and activation of GR through binding the GRE in the target gene can also occur in the DBD (Schaaf and Cidlowski, 2002). Nuclear translocation is based on two nuclear localisation sequences, namely, nuclear localization 1 (NL1) and nuclear localization 2 (NL2) (Picard and Yamamoto, 1987 ; Freedman and Yamamoto , 2004).The NL1 and NL2 are located next to DBD and LBD (Figure 4.8). NL1 is spanning from 479-506 amino acids and it has a major role of binding to importin proteins family in the GR translocation inside and outside the nucleus (Freedman and Yamamoto , 2004). NL2 is poorly defined (Freedman and Yamamoto, 2004).

Ligand-binding domain (LBD) is spanning from 527-777 amino acids. It is involved in the formation of the hydrophobic ligand-binding pocket because it is composed of α-helix and β sheet. It plays a role in GR dimerization together with the DBD region. Also, it controls GR selectivity binding ability (Figure 4.9.1) (Mcewan *et al.*, 1993). LBD, joined by a hinge region with DBD at the C-terminus, consists of a dimerization surface accompanied with Activation Function-2 (AF-2). AF-2 is similar to AF-1 but has a minor trans-activation domain across amino acids 727-763 (Bledsoe *et al.*, 2002). However, AF-2 is ligand dependent and allows binding to co-regulators.

The selectivity of the co activators or the co repressor is normally controlled through the C-terminal helix as it determines the proteins that bind with AF-2 region (Figure 4.9.1) (Mcmaster and Ray, 2008). While GR binds with the agonists, C-terminal alters from the open position to closed, and subsequently presents a platform for binding with activators. Characteristically, the antagonists prevent the C-terminal helix from closing appropriately over the bound ligand due to the presence of the side chains, which are too long to be controlled within the binding cavities. As a result, altered receptor surfaces are produced to act together with co-repressors and prevent co-activators communication (Bledsoe *et al.*, 2002).

The GR is encoded by nine exons. Alternative splicing at exon 9 produces many isoforms, but the most dominant in humans are; GRα and GRβ which differ at their carboxyl terminus. The two isoforms are identical through amino acid 727, but then

differ at GRα which has an additional 50 amino acids and GRβ which has an additional 15 amino acids (Sionov *et al.*, 2009).

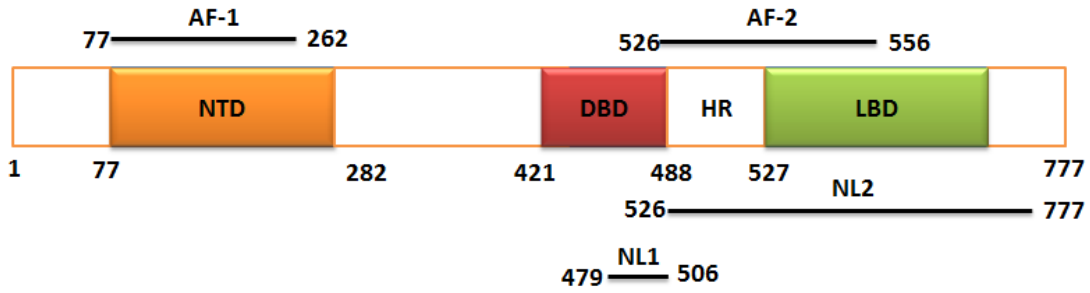


Figure 4.9.1: **Functional GR domains**. Functional domains are indicated as; DNA-binding domain with two zinc-finger motifs; LBD: Ligand-binding domain which has the NL1 and 2: Nuclear translocation signal 1 and 2; and N-terminal domain containing transactivation functions (AF-1).

4.4.5 Overview of transcription

Transcription is the major regulatory mechanism of gene expression, in which RNA polymerase enzyme carry out the process of copying particular genetic information from the DNA to complementary antiparallel RNA strand.

For initiation of transcription, the process requires RNA polymerase and DNA core promoter (Figure 4.10.1). The promoter can be located 30, 75 or 90 base pairs upstream from the transcription start site (TSS). Then, RNA polymerase binds to core promoter in the presence of various general basal transcription factors including TFIID (Orphanides *et al.*, 1996).

The most recognized DNA promoter sequence is TATA box. TATA box is a core DNA sequence and it is an A and T rich sequence. TFIID is the first of the general transcription factors that binds to the TATA box via the TATA binding protein (TBP) complex. TFIID is followed by the other general basal transcription factors essential for transcription initiation. These factors include TFIIA, TFIIB, TFIIE, TFIIF and TFIIH (Thomas and Chiang, 2006). The transcription factors and RNA polymerase enzyme bind to the TATA box, forming a transcription initiation complex. After that, RNA polymerase is released from the promoter, and transcription continues downstream from TSS, through elongation and eventually terminates (Orphanides *et al.*, 1996) Elongation is when RNA polymerase reverses the template strand and uses base pairing complementarity with the DNA template to create an RNA copy.

Eventually, the RNA transcription should terminate. This happens when the newly synthesized RNA molecule forms a G-C-rich pair hairpin loop.

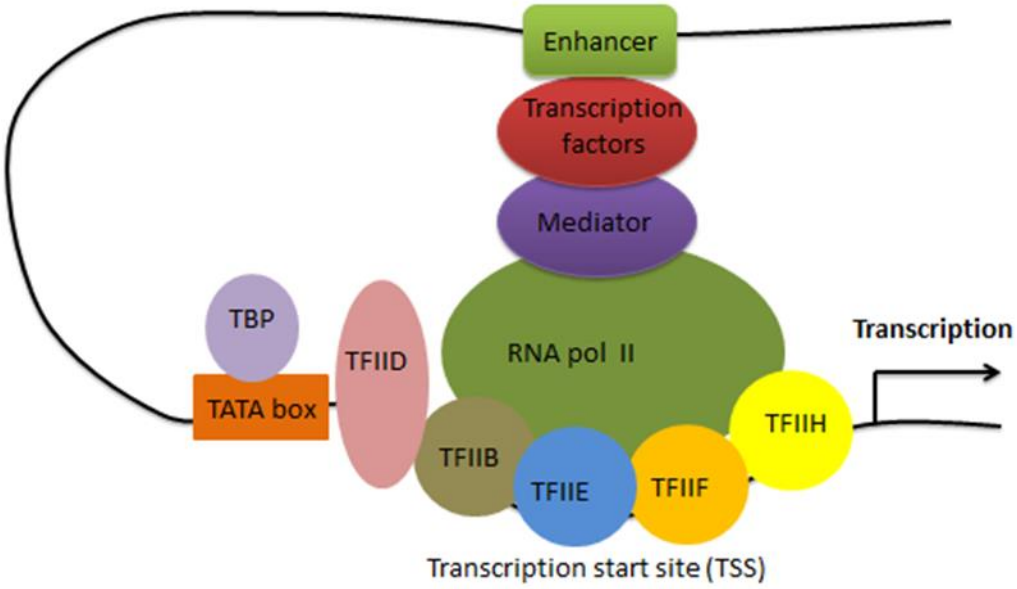


Figure 4.10.1: **Overview of the general transcription**. To initiate transcription, RNA polymerase II requires the general transcription factors which are TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH. TFIID is bound to TATA box, which is located 30 nucleotides away from the site where transcription is initiated, allowing transcription to begin.

4.4.6 The GR activation and mode of action

Glucocorticoid receptor (GR) is located in the cytoplasm and it translocates into the nucleus after binding with GC to activate or repress the transcription of the target genes (Freedman and Yamamato, 2004).

The GC hormone is able to enter the cell by passive diffusion and once it reaches cytoplasm, it can bind to the GR (Figure 4.11.1). During the absence of the GC, GR residues present in the cytoplasm undergo a stabilizing reaction by forming a heteroligomeric complex along with the heat shock proteins (hsp) 90, 70, 50 and immunophilins and other various chaperone complexes (Schmidt *et al.*, 2004). After binding of GC to the GR, the receptor complex undergoes conformational change and the hsp's dissociate. This exposes the nuclear localization signal (NLS) domains of the receptor (Sionov *et al.*, 2009). Once GR dissociate from the chaperone complex, the homo-dimer GC–GR complex translocates to the nucleus where it interacts with GREs (consensus sequence GGT ACANNN TGT TCT), located in the promoter region of GC genes, hence, leading to either gene activation or repression (Geng Y. *et*

al., 2005). Both transactivation (via GRE-binding) and trans-repression (via interaction with AP-1 or NF-κB) processes can induce apoptosis of GC-sensitive cells, but it is yet unknown if one or both are important in ALL (Tissing *et al.*, 2003). The accurate system for the glucocorticoid response termination is not completely known. It could be that GR termination occurs through the protein degradation via the ubiquitin-proteasome pathway (Wallace and Cidlowski, 2001).

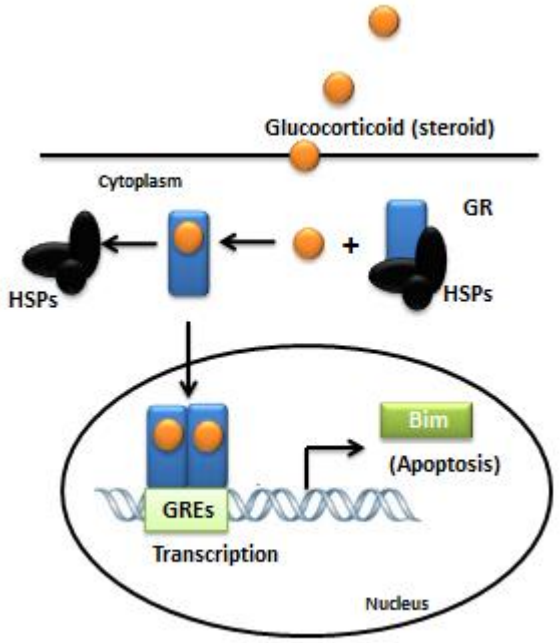


Figure 4.11.1: **Model of GC induced apoptosis in leukemic cell.**The extracellular glucocorticoid is transported into the cytoplasm of the target cell by either passive diffusion or active transport. When GR is not active it binds to the Hsp90 dimer in the cytoplasm which acts as its chaperone. The GC binds to GR and translocate to the nucleus as a dimer. In the nucleus, it binds to its DNA binding site and activates a variety of genes that play role in apoptosis.

4.4.6.1 Transactivation

The positive regulation of gene expression by the GR is mediated by the direct binding of receptor homo-dimers to specific sequences which are glucocorticoid responsive elements (GREs) in the regulatory regions (promoter) of target genes. GREs are present as two half sites sequences of AGAACA arranged in a 9, in the middle, there is a 3 base pair and the consensus sequence is GGT ACANNN TGT TCT. During transactivation, one active GR monomer binds to the 3`half site and after that the second active GR binds, the 5`half site in order to form dimer via the zinc fingers in DBD region on GR (Japiassú *et al.*, 2009).

GC can bind the transcription factor, glucocorticoid receptor, to either enhance or repress transcription of glucocorticoid-responsive genes (Martens *et al.*, 2005). The enhancer region is a very short region of DNA that binds to protein and is located upstream or downstream from TSS. When GR binds to enhancer, another protein complex called mediator can send signals and make contact with general transcription factors (Thomas and Chiang, 2006) (Figure 4.12.1).

Moreover, there are five types of binding sites for GR, namely, glucocorticoid response elements (GREs), glucocorticoid response units (GRUs), glucocorticoid response element half-sites (GRE1/2s), negative glucocorticoid response elements (nGREs) and tethering glucocorticoid response elements (Schoneveld *et al*., 2004). These are explained below.

Glucocorticoid response elements (GREs): Here, GR can activate gene transcription by direct binding to glucocorticoid-responsive elements. Transactivation by the GR through simple and composite GREs depends on the binding of the activated GR homo-dimer to DNA. Simple GR binding occurs during positive regulation of the genes (Van Tilborg *et al.*, 2000)(Figure 4.12.1 a).

Glucocorticoid response units (GRUs): This interaction site does not depend on GR binding to a GRE, but rather requires the binding of other transcription factors closely to the binding sites (Figure 4.12.1 b) (Schoneveld *et al.*, 2004).

GRE half-sites (GRE1/2): Here, the GR can bind the DNA as a monomer and known GRE half-sites (GRE1/2). GRE1/2s requires additional elements to mediate a glucocorticoid response (Segard-Maurel *et al.*, 1996) (Figure 4.12.1 c).

Negative GREs (nGREs): In this case, GRE negatively influence gene expression of responsive genes. This is referred to as negative GREs (nGREs). In this type of regulation, direct binding of GR to the nGRE is required (Figure 4.12.1 e).

In addition, there is another negative GRE model characterized by competition between the transcription factors and the GR. In this competition model, GR binding to the nGRE interferes with other transcription factors for binding to their response elements, leading to a negative response (Figure 4.12.1 f).

Tethering glucocorticoid response elements: In tethering GREs, GR does not bind directly to the DNA, but it binds to other DNA-bound transcription factors such as AP1, Stat3 and NF-κB. Under certain circumstances, these results in increased transcriptional activity and the effects can be both positive and negative. This is

because the GR behaves as a ligand-inducible co-regulator that employs protein–protein interactions to exert its effect (Schmidt *et al.*, 2004) (Figure 4.12.1 d and g).

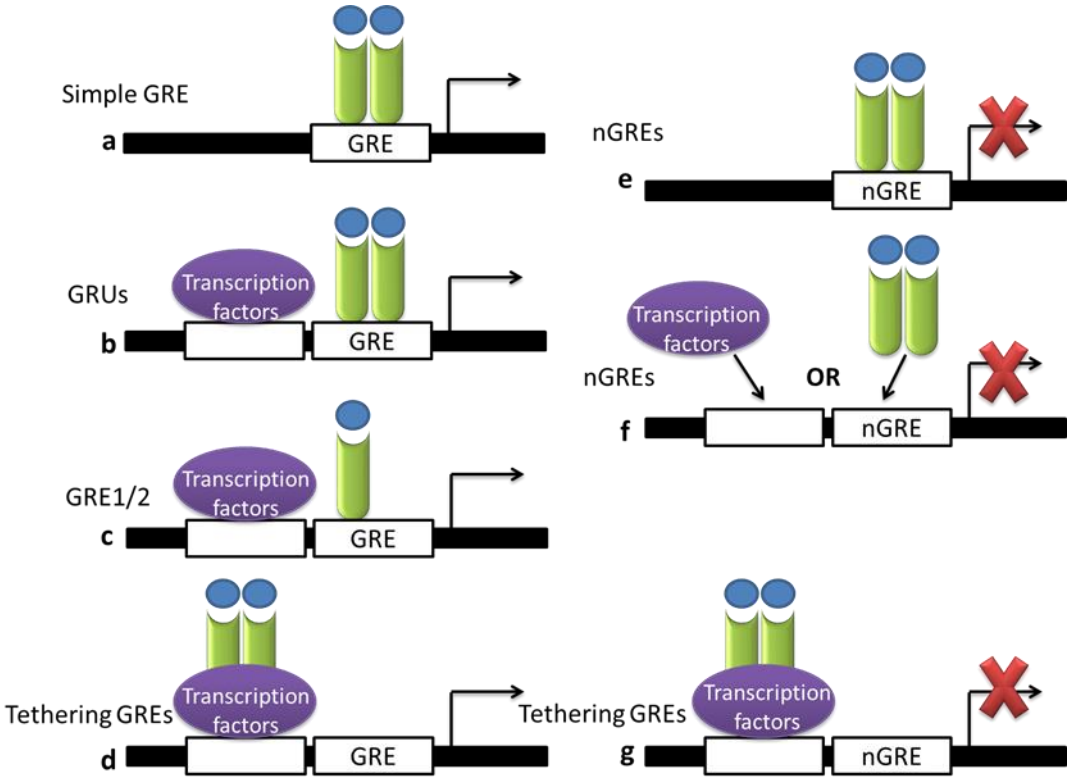


Figure 4.12.1: **Mechanism of GR interaction with regulatory elements**

4.4.6.2 Trans-repression

GR negatively regulates gene expression by binding to negative glucocorticoid response elements (nGREs). The negative glucocorticoid response elements have the sequence ATYACnnTnTGATCn. In trans-repression, the GR monomer binds to the negative glucocorticoid response elements and suppresses transcription of the gene (Japiassú *et al.*, 2009). The transcription factors to which the GR binds include activator protein 1 (AP-1), nuclear factor-kB (NF-κ*B*) and interferon regulatory factor 3 (IRF3). These transcription factors are involved in the regulation and expression of the pro-inflammatory genes. Negative regulations of these genes by GR are understood to be a feature of the anti-inflammatory and immuno-suppressive activity of corticosteroids. There are various other mechanisms that provide explanation for the trans-repressive activity of GR. In such mechanisms, GR binding to Jun N-terminal kinase (JNK) leads to the suppression of JNK activity and, subsequently, to the inhibition of AP-1(Bruna *et al.*, 2003).

4.4.7 Cofactors

When GR binds to the promoter of its target gene, it needs to recruit cofactors to complete the transcriptional process. Those cofactors can be co-activators or co-repressors. Co-activators are molecules that interact with the AF-1 or AF-2 of the GR and form several protein complexes. Co-activators like p160, p300/CBP and p/CAF build a bridge between GR bound to DNA and the transcription initiation complex, in which glucocorticoid signals activate RNA polymerase II (Kino *et al*., 2003). Co-repressors repress the transcription process of specific target genes. The most studied co-repressors of the nuclear hormone receptors are nuclear receptor co-repressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) Both N-CoR and SMRT share 45% of amino acids of the molecular structure containing N-terminal transcription domain and C terminal nuclear receptor interaction domain (Privalsky , 2004).

4.6.8 Post-translational Modifications of GR

The nuclear hormone receptors are regulated through post-translational modifications (PTMs). PTMs includes processes such as phosphorylation, acetylation and sumoylation (Krstic-Demonacos and Demonacos, 2001). The most studied area is the phosphorylation in GR function. In the human GR, Ser113, Ser 141, Ser 203, Ser203, Ser211 and Ser226 are the five phosphorylated residues in the N terminal domain (AF-1), (Wang *et al*., 2002). Particularly, the phosphorylation events at Ser 211 and Ser 226 are of high significance in transcriptional regulation (the above mentioned sites are discussed in details in human GR phosphorylation at Ser211 and Ser226). Post-translational modifications can affect transcription of a gene in several ways. Ubiquitin activating enzyme (UBAs), E2 ubiquitin-conjugating enzymes (UBCs) and E2 ubiquitin-ligase enzymes are considered to be the key players in regulating the ubiquitin proteasome pathway. GR is also considered the target for ubiquitination (Kinyamu *et al.* , 2005). Moreover, the small ubiquitin-related modifier1 (SUMO) is responsible for targeting transcription factors, co-regulators and chromatin re-modellers that are involved in gene regulation (Le Drean *et al.*, 2002). SUMO triggers sumoylation, which is responsible for maintaining protein stability, localisation and the transcriptional regulation (Davies *et al.*, 2008). On the other hand, it is considered that JNK dependent GR phosphorylation is linked to sumoylation, thereby, affecting

its target gene transcription (Duma *et al*., 2006). Acetylation and methylation of the co-regulators alters gene transcription through chromatin remodelling. The diversity of post-translational modifications is reversible and directly linked to GR functions. Collectively, they indicate that GR signaling is a combination of various processes rather than a single role (Kinyamu *et al*., 2005).

4.6.9 Phosphorylation and GR function

GCs are targeted as treatment sites in many pathologic conditions. Therefore, GC is a key player in gaining therapeutic benefit where sensitivity of tissues is involved. Compounding factors influencing the hormonal actions of GCs and modifications in those factors can cause GC resistance or hypersensitivity. The phosphorylation of the various residues of GR influences the transcriptional activity (Ismaili and Garabedian, 2004), indicating that GR phosphorylation type significantly affects the efficiency of existing GC therapies. GC resistance results from the activation of cell-signaling components within the GR pathway rather than from mutation of the GR (Miller *et al.*, 2007). The pathways participating are MAPKs, CDKs, and GSK-3b which is also involved in phosphorylation of GR. Hence, cellular environment that can modify GR phosphorylation status may alter GC signaling and decrease or improve GC resistance. Phosphorylation of a range of serine and threonine residues at Ser113, Ser141, Ser203, Ser211, Ser226, and Ser 404 can modify the transcriptional activity of human GR (Galliher-Beckley and Cidlowski, 2009)(Ismaili and Garabedian, 2004). These phosphorylation sites are mainly situated inside the transactivation domain-1 (AF- 1) (Kfir-Erenfeld *et al.*, 2010).

4.6.9.1 Human GR phosphorylation at Ser211

GR becomes hyper-phosphorylated after binding with a ligand. Ser203 and Ser211 are the main residues that are hyper-phosphorylated following hormone binding (Kfir-Erenfeld *et al.*, 2010). Phosphorylation of Ser211 residue (Figure 4.13.1) is ligand-dependent and regulates the transcriptional activity of GR. The amount of phosphorylation of Ser211 residue is highly correlated to the transcriptional activity of GR, hence, indicating that its phosphorylation is a biomarker for activated GR (Wang *et al.*, 2002; Blind and Garabedian, 2008; Kfir-Erenfeld *et al.*, 2010). On the contrary, phosphorylation of Ser211 residue (Figure 4.13.1) is ligand-dependent and regulates the transcriptional activity of GR.

Moreover, GR has the maximum transcriptional activity whilst phosphorylation of Ser211 residue is higher than that of the phosphorylation of Ser226 residue. Reports suggest that the phosphorylation of human GR by p38 MAPK at Ser211residue is observed both *in vitro* and within intact cells (Miller *et al.*, 2005). In addition Ser211 phosphorylation has been shown to be mediated by cyclin dependent kinases, particularly by the cyclin A/CDK2 complex, in addition to the p38 MAPK.

p38MAPK has an impact on GC-induced apoptosis because it is involved in the apoptotic signaling cascade. It has been demonstrated that p38 activation is essential for GC-induced apoptosis in S49 cells, which are partially resistant cells, and in CCRF-CEM T-ALL cells. However, in the thymocytes and thymic lymphoma cells which are GC-sensitive cells, p38 inhibition do not affect GC- induced apoptosis (Saffar *et al.*, 2008). Likewise, in eosinophils or MM cells, p38 inhibition do not affect GC-induced apoptosis (Spokoini *et al.*, 2010). This indicates the cell-type specific requirement for p38, the GC response in S49, and CCRF-CEM cells. This suggests that p38 is necessary for overcoming their GC-resistant phenotype. Indeed, increased p38 phosphorylation has been observed in CCRF-CEM cells, 24 hrs after Dex treatment (Kfir-Erenfeld *et al.*, 2010), suggesting a GC-mediated activation of p38. Importantly, it has been shown that p38 is required for FoxO activation and Bim up-regulation in CCRF-CEM cells. On the other hand, S49 cells already express elevated levels of Bim, suggesting that p38 may have additional effects in apoptosis through Bim activation in CCRF-CEM cells. Indeed, p38 can directly interact with Bim and increase its pro-apoptotic activity through phosphorylation of Bim at Ser 65 (Kfir-Erenfeld *et al.*, 2010).

Previous works suggested that lack of Ser211 phosphorylation is associated with GC resistance of human lymphoid cells (Miller *et al.*, 2007). Ser211-phosphorylated GR was considered as a transcriptionally active form of the receptor, as it was observed by ChIP assays to be recruited at numerous GRE-containing promoters (Blind and Garabedian, 2008).

4.6.9.2 Human GR phosphorylation at Ser226

The transcriptional reaction of GR can be enhanced by blocking the phosphorylation of Ser226 residue (Chen *et al.*, 2008). Therefore, these studies are stressing the importance of Ser211 phosphorylation in the transcriptional activity of GR (Galliher-Beckley and Cidlowski, 2009) (Figure 4.13.1). Moreover, phosphorylation of human

GR at Ser226 by JNK is also involved in blunting the hormone signaling by improving the nuclear export of the GR (Kfir-Erenfeld *et al.*, 2010). These evidences prove that activated MAPK signaling pathways are responsible for altering the GR signaling. Generally, GR phosphosylation on Ser226 is not hormone dependent and often has negative effect on GR function. However, it is possible that this depends on both cell type and target gene.

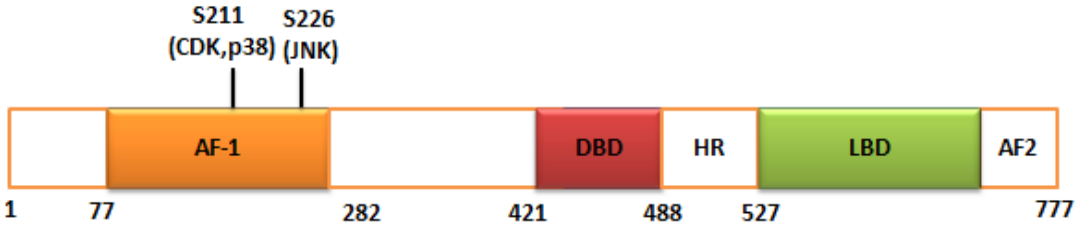


Figure 4.13.1: **The Ser211 and Ser226 phosphorylation sites of glucocorticoid receptor.** Human GR phosphorylation sites at Ser211 targeted by p38 MAPK and CDK and Ser 226 targeted by JNK.

4.4.10 GR target genes

GR is expressed in almost all body cells, but it exerts cell specific effects. In addition, there are numerous target genes of GR involved in the regulation of a wide spectrum of processes such as inflammation, immune response, metabolism, etc. Depending on the cell type, experimental conditions and stringency of the approach, GR target genes range from 50 to 2000, revealing the complexity of the GR action. As such, researchers have chosen to investigate recently identified GR target genes such as Mcl-1 and Bim, which have shown to be crucial in GR-induced apoptosis in leukaemia cells (Kfir-Erenfeld *et al.*, 2010).

4.4.10.1 Myeloid cell leukaemia-1 (Mcl-1)

Mcl-1 is a protein encoded by the MCL-1 gene, which belongs to the Bcl-2 family. Mcl-1 is a highly expressed protein in malignancies (Akgul C. *et al.*, 2000; Pepper *et al.*, 2008). Mcl-1 protein is involved in the control of apoptosis and promotes cell survival by interfering with the apoptosis process during the early stages, hence, enabling the mitochondria to release cytochrome c. Mcl-1 is frequently over expressed in different types of leukemia, especially those of myeloid origin (Sionov *et al.*, 2006; Akgul, 2009), and it is over expressed in GC resistance T-ALL cells (Wei *et al.*, 2006; Saffar *et al.*, 2008). However, knockdown of Mcl-1 is sufficient for sensitizing T-ALL cell lines to GC apoptosis (Wei *et al.*, 2006).

4.4.10.2 Bim (Bcl-2-interacting mediator of cell death)

Bim is a BH3 only, pro-apoptotic member of the Bcl-2 family. Bim has three isoforms that are produced by alternative splicing. They include the short Bim(S), long Bim(L) and extra-long protein variants Bim (EL)(Ewings *et al*., 2007). Bim(S) is the most potent pro-apoptotic member in GC sensitive cells CEM C7-14 (Thompson and Johnson, 2003; Strasser, 2005). Bim is an important mediator of cell death and as such, it is more likely to play a role in promoting death, though indirectly, by interacting with other proteins (Gillings *et al.*, 2009). Bim can induce apoptosis either by binding to Bcl-2 members to inactivate their anti-apoptotic activity or by forming oligomerization bonds with Bax and activating caspases mediated apoptosis (Chan and Yu, 2004; Ploner *et al.*, 2007). Bim is an indirect target of GR and is necessary for GR induced apoptosis because Bim knockdown by siRNA inhibits GC mediated apoptosis in CEM cells (Kfir-Erenfeld *et al.*, 2010).

4.5 Cell death

It has been demonstrated that the phenomenon of cell death is of considerable importance in the cell's life cycle (Chaabane *et al.*, 2013). Cell death can be categorized as autophagic, necrotic and apoptotic, depending on the cell morphology (Kroemer *et al.*, 2010).

Identification of dead cells can be done on the basis of morphological or molecular features which include: a) loss of cell membrane integrity. The loss can be detected using dyes such as propidium iodide (PI); b) fragmentation of cell organelles for instance nucleus; and/or c) phagocytosis of cell fragments (Table 1). Kroemer *et al.* (2010) proposes that the cells that are arrested in some cell cycle phases are considered to be live cells. These forms of cell death, together with their defining morphological traits are summarized in Table 1 and they are also represented by Figure 4.14.1.

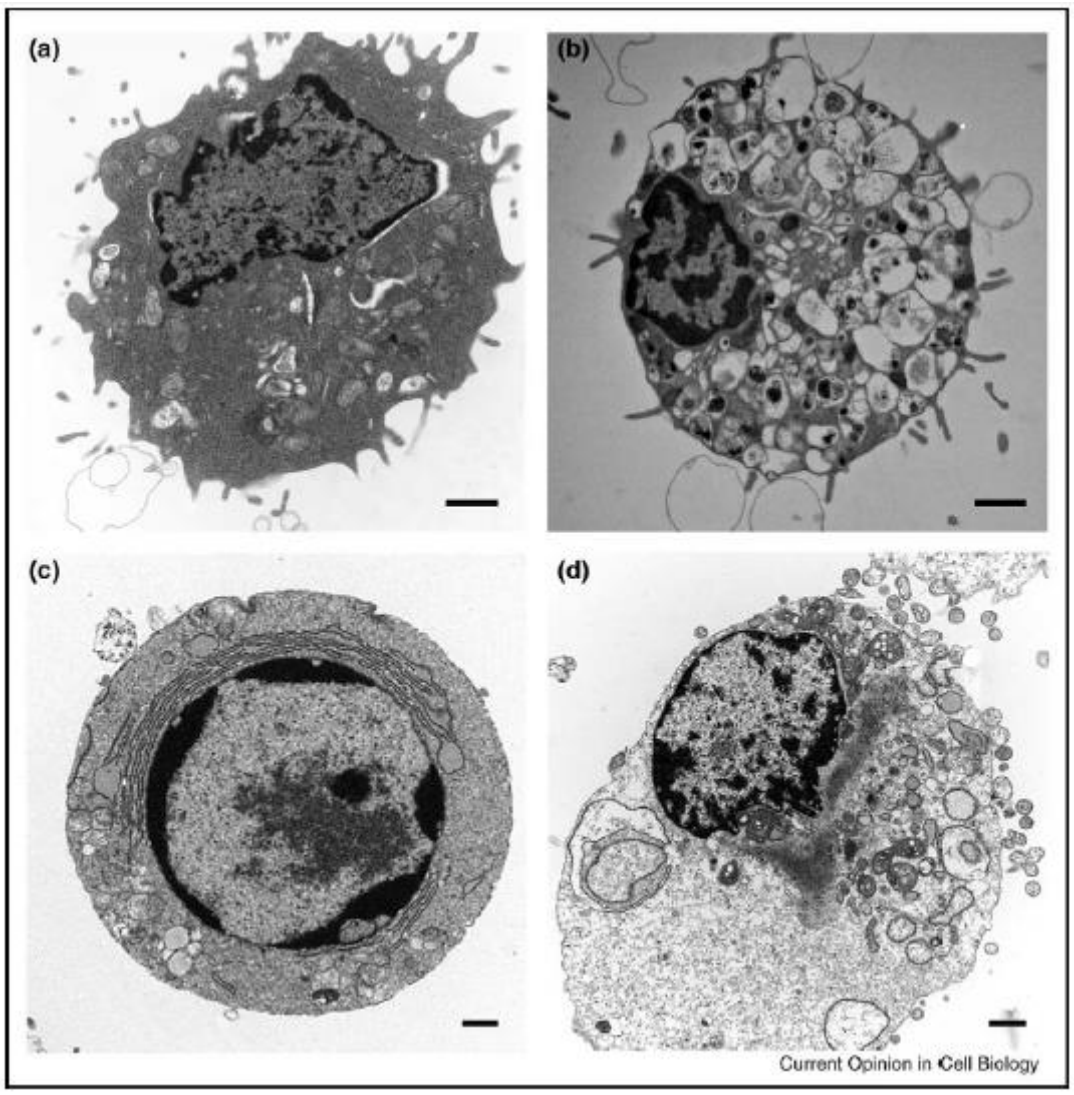


Figure 4.14.1: **Electron microscope figure to show the morphological features and differences between apoptosis, autophagy and necrosis versus normal cell**. (a) Normal cell, (b) autophagic showing the massive formation of (double-membraned) autophagic vacuoles, (c) apoptotic cell showing the typical round shape and chromatin condensation and (d) necrotic cell showing increased cell size, rupture of cell membrane and vacuoles formation. Morphological features are defined in detail in table (1). Figure is taken from (Edinger and Thompson, 2004).

Type of cell death	Morphological features and differences	Figure
Apoptosis	Cell becomes typical round in shape Cell reduces in size Nuclear chromatin condensation (pyknosis) Nuclear fragmentation (karyorrhexis) Formation of blebs in the plasma membrane (blebbing) Apoptotic cell engulfed by phagocytes No involvement of immune system	Figure 4.14.1 Compare a and c
Autophagy	No chromatin condensation Massive vacuolization of the cytoplasm Formation of (double-membrane) autophagic vacuoles Autophagy cell does not get engulfed by phagocytes	Figure 4.14.1 Compare a and b
Necrosis	Gain in cell volume Swelling in cytoplasm Swelling of cytoplasmic organelles Moderate chromatin condensation Rupture of plasma membrane loss of intracellular contents Massive involvement of immune system	Figure 4.14.1 Compare a and d

Table 1: **Comparative morphological features between apoptosis, autophagy and necrosis.**

4.5.1 Apoptosis

In a healthy adult human being, around 10 million cells die via apoptosis every day. Apoptosis is mostly described as programmed cell death since it is a phenomenon during which a cell sets off its own death i.e. suicide (Curtin and Cotter, 2003). The above mentioned degree of apoptotic deaths is important in maintaining homeostasis, as well as, in controlling shape and size of tissues during various developmental processes. This programmed cell death is also essentially required to reduce the number of immune effector cells once the pathogen has been eliminated (Los *et al.*, 1999).

During apoptosis, a cell becomes round, and its volume is reduced. This process is called pyknosis. In addition, chromatin material becomes condensed and the nucleus becomes fragmented. This phenomenon is called karyorrhexis. Apoptosis is also

accompanied by plasma membrane blebbing. However, there occurs insignificant ultra-structural modifications of cellular organelles. Blebs serve as the progenitors of apoptotic bodies having small, almost round cytoplasmic fragments surrounded by cell membrane. Moreover, it is possible that apoptotic bodies contain functional organelles enclosed in unbroken cell membrane (Elmore, 2007; Ghavami *et al.*, 2009). Apoptotic bodies express phosphatidylserine on their surface. It is a phospholipid attached to the cell membrane and it functions as an "eat me" signal in order to attract phagocytic cells such as macrophages, which phagocytose these bodies (Elmore, 2007; Ghavami *et al.*, 2009; Chaabane *et al.*, 2013) (Figure 4.15.1).

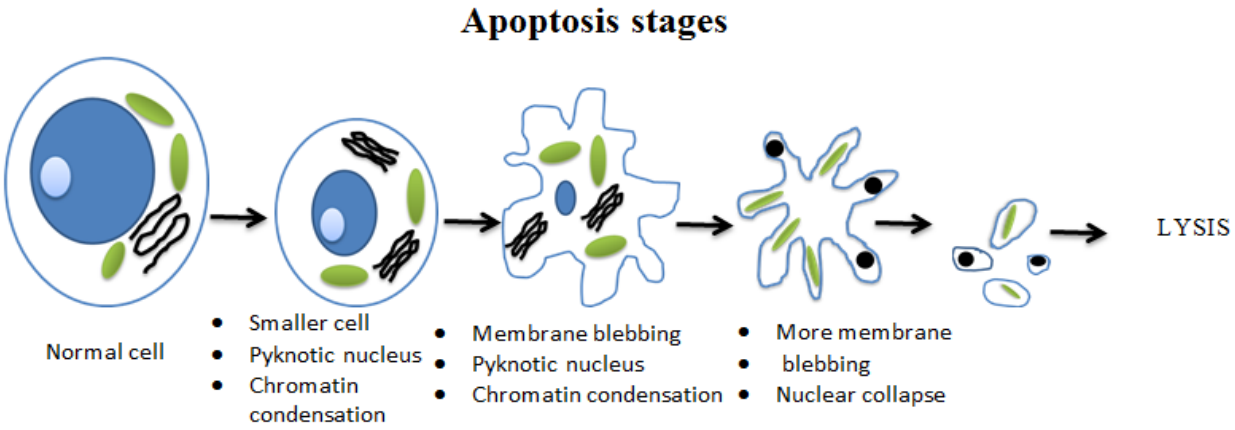


Figure 4.15.1: **Apoptosis stages.** Apoptosis stages can be described when the chromatin condenses, the cell detaches from the surrounding, starts to shrink and the cell membrane starts blebbing.

There are two main pathways to apoptosis, namely, the death receptor pathway (extrinsic pathway) and the intracellular stress signals pathway (intrinsic pathway) (Suen *et al.*, 2008).

The extrinsic pathway is activated by signals such as toxins, hormone, growth factors, nitric oxide or cytokines. The signals come from outside the cell and transduce through the plasma membrane for apoptosis to occur. The intrinsic pathway initiates in response to glucocorticoid and signals such as heat, radiation, hypoxia, viral infection, or raised calcium level inside the cell (Chalah and Khosravi-Far, 2008).

4.5.1.1 Extrinsic Pathway (death receptor pathway)

Tumour necrosis factor (TNF) is the cytokine generated from activated macrophages and initiates the apoptosis through the extrinsic pathway (Chalah and Khosravi-Far, 2008). TNF combines with tumour necrosis factor receptor (TNF-R) and subsequently activates caspase, a fundamental apoptotic protein (Figure 4.16.1). The intermediate membrane protein called TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD) signals to caspase for apoptosis (Suen *et al*., 2008).

4.5.1.2 Intrinsic pathway

The transduction of intrinsic pathway inside the cell can increase permeability of mitochondrial outer membrane, thus, allowing mitochondrial proteins to escape and disperse into the cell cytosol (Figure 4.16.1) (Cory and Adams, 2005).

Mitochondria are the most crucial organelles in the regulation of apoptosis. Cytochrome c (Cyt c) is the first protein released from mitochondria for apoptosis, through configurative channels on mitochondrial outer membrane (MAC). Cyt c binds to apoptosis protease activating factor (APAF-1) to form Cyt c/ APAF-1 complex termed as apoptosome (Rong and Distelhorst, 2008). Cyt c/ APAF-1 complex consequently binds to pro-caspase-9.; then apoptosome cleaves pro-caspase into active form with the purpose of activating caspase 3 (Cory and Adams, 2005).

Voltage dependent anion channel (VDAC) is a mitochondrial outer membrane protein that can control apoptosis by regulating mitochondrial outer membrane pores permeability. It interacts with BAK to keep the apoptotic process under control (Chalah and Khosravi-Far, 2008).

The second mitochondria derived activator of caspase (SMAC) is a regulatory protein released in the cell, as a result of an increase in mitochondrial permeability. It plays an important role in apoptosis, as it deactivates inhibitor of apoptosis protein (IAP) which represses caspase activity (Chalah and Khosravi-Far, 2008; Suen *et al.*, 2008). Smac/Diablo is the second apoptotic factor after Cyt c but with a different function. Cyt c is the direct trigger of APAF-1 and caspase-9 whereas Smac/Diablo antagonize baculovirus inhibitor repeat (BIR - a domain of IAP) and facilitates activation of caspase indirectly (Chalah and Khosravi-Far, 2008).

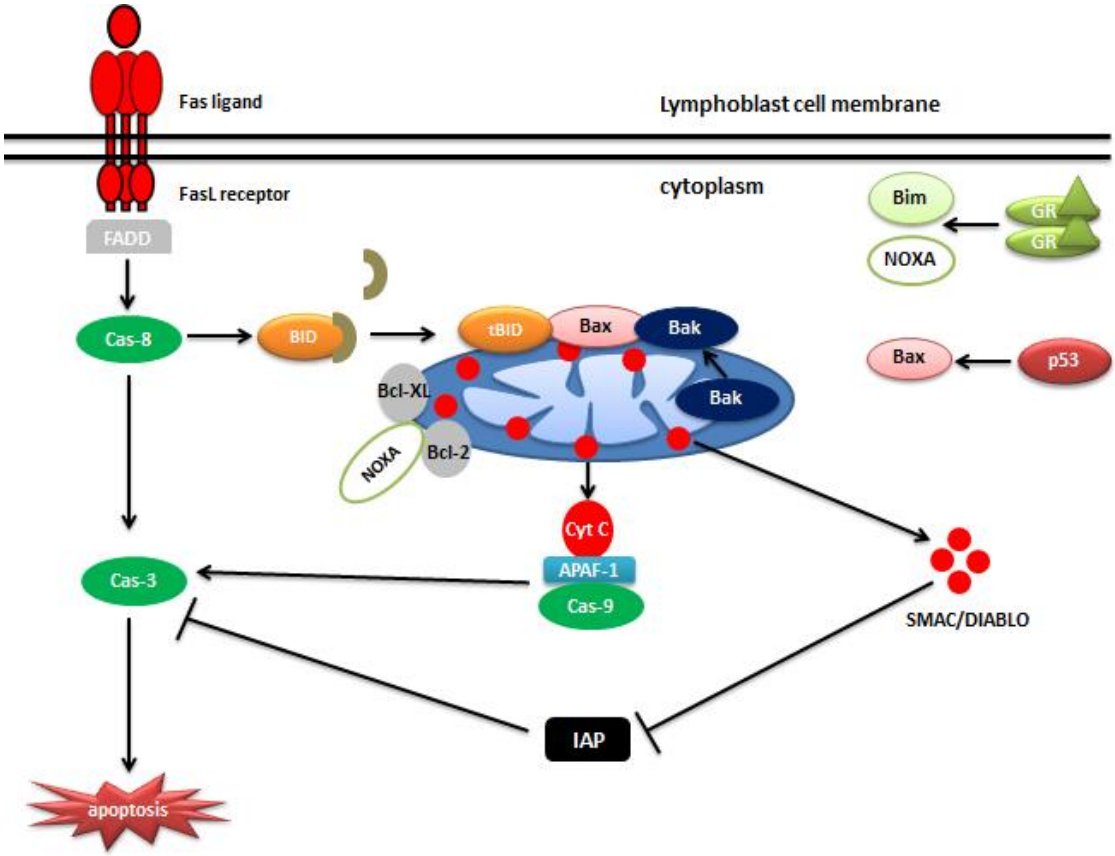


Figure 4.16.1: **Intrinsic and extrinsic apoptosis in conjunction with GR and P53.** Apoptosis can be activated by two pathways, the extrinsic and intrinsic pathway. The extrinsic pathway is activated by extracellular signals such as TNF that bind to cell surface death receptors, resulting in caspase cleavage and an activation cascade, that transforms pro-caspases into active caspases. The intrinsic pathway is activated by intracellular signals such as DNA damage, chemotherapy and UV irradiation. This regulates the Bcl-2 family of proteins that induce mitochondrial outer membrane potential (MOMP), and the release of pro-apoptotic factors such as cytochrome C and second mitochondrial-derived activator of caspases /direct IAP-bind protein with (SMAC/DIABLO). Cytochrome C binds to an adaptor protein APAF1, forming the apoptosome that cleaves and activates caspase proteins. SMAC/DIABLO inhibits the inhibitor of apoptosis (IAP) family of proteins, which prevent apoptosome formation. Caspase proteins can cleave and stimulate other caspase family members, thus, inducing apoptosis.

4.5.2 Bcl-2 family and regulation of apoptosis

B cell lymphoma-2 (Bcl-2) family members are important regulators of apoptosis. According to structural homology and function, the Bcl-2 family is divided into three groups (Cory and Adams, 2005).The first group includes Bcl-2, Bcl-XL and induced myeloid leukaemia cell differentiation protein 1 (Mcl-1), wherein, all are anti-apoptotic factors. The second group includes Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak), all of which are pro-apoptotic proteins. The third group includes the BH3 domain only (Rong and Distelhorst, 2008). BH3 domain only is a pro-apoptotic protein that includes Bcl-2 interacting mediator of cell death

(Bim), Bcl-2 antagonist of cell death (Bad), and Bcl-2 interacting killer (Bik) (Ploner *et al.*,2009). Bcl-2 family action is regulating the mitochondrial permeability of intracellular membrane to the ions and proteins. Bax and Bak (proapoptotic members) enhance cytochrome c released from mitochondria in order to activate caspase during cell apoptosis (Rong and Distelhorst, 2008).

4.5.3 Autophagy

Around fifty years ago, researchers discovered the phenomenon of autophagy. However, substantial molecular information about this process has been obtained during the past 5-7 years. This information has given insight into the form and functions of this phenomenon (Mizushima, 2007; Mizushima *et al.*, 2008). It has now been established that autophagy is the major cellular catabolic mechanism that safeguard the cell from different stresses such as deficiency of growth factors, nutrients or hypoxia instigated stress. It plays a crucial role in recycling the cellular organelles, proteins and other components. Therefore, it ensures cleanup of the cell, as well as, the provision of new constituents to build up various parts of the cell (Gump and Thorburn, 2011). Autophagy is an ubiquitous phenomenon that take place in all types of cells. However, deficiency of nutrients or other stresses can result in upregulation of this process (Gump and Thorburn, 2011). Researchers have also determined an association between autophagy and etiology of a number of human diseases such as cancer, metabolic disorders and neurodegenerative disorders (Meijer and Codogno, 2009).

Autophagy is of substantial importance for humans, especially in protecting the human body from a number of disorders. Its role in causation and prevention of different disorders is now well established. Numerous myodegenerative and neurodegenerative disorders are described by an elevated, yet inadequate autophagic activity. This shows how important autophagy is for the quality control of proteins. Likewise, if protein damage, mitochondrial damage or DNA damage caused by cellular stress is not followed by appropriate autophagic repair pathways, the affected cells become susceptible to becoming cancerous cells (Isabel Colombo and Simon, 2009).

As mentioned above, induction of autophagy occurs in response to different cellular stress conditions like nutrient deficiency. Autophagosomes which are intracellular

organelles and proteins impounded by double membrane vesicles are delivered to lysosomes during this process, so that they can be degraded. A growing number of evidence indicates that autophagy acts as a membrane trafficking system which supplies the parts of cytoplasm to the lysosomes for degrading protein in bulk, thereby, ensuring not only the survival of the cells but also quality control of the protein content of the cell through the elimination of abnormal proteins. Even though autophagy is viewed as a pathway for ensuring survival of the cell, unnecessary autophagy can induce necroptosis i.e. the so called type II programmed cell death. Necroptosis is a kind of programmed cell death which is different from apoptosis in many ways as mentioned in section 4.5.4. During the course of autophagy, an autophagosome is formed when phagophore closes after engulfing cytoplasmic constituents and cellular organelles. Phagophore is a pre-autophagosomal structure having the shape like cup and is also known as isolation membrane (Mizushima *et al.*, 2002). The next step is normally the fusion of autophagosome with the lysosomal vesicles, resulting in proteolytic breakdown of the engulfed substances through the action of lytic enzymes contained in the lysosomes as shown in Figure 4.17.1 (Eskelinen, 2005).

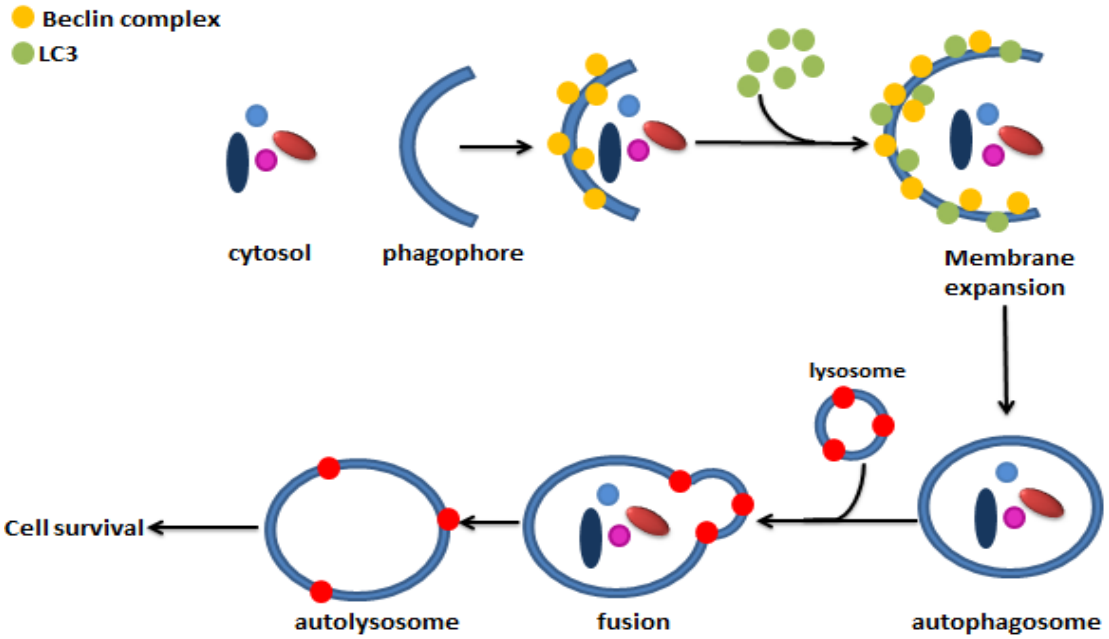


Figure 4.17.1: **Autophagy steps.** Autophagy begins with the formation of the phagophore (vesicle nucleation step) which sequester the cytoplasmic cytosol. In the presence of Beclin complex and LC3, the phagophore membrane expands into an autophagosome (vesicle elongation). The autophagosome fuses with a lysosome to form an autolysosome, in which the enclosed cargo is degraded by acid hydrolases (fusion steps). Finally, the sequestered material is degraded inside the autophagolyosome (vesicle breakdown and degradation) and recycled.

A pair of ubiquitin-like modifications is needed for the preliminary development of the sequestering membrane. These include the formation of Atg12-Atg5 conjugation system and the Atg8-phosphatidylethanolamine (PE) complex (Mizushima *et al.*, 1998). In Atg12-Atg5 conjugation system, a covalent bond binds the smaller Atg12 protein with the Atg5 (Ichimura *et al.*, 2000). Atg7 acts as E1 enzyme and activates the Atg12 first, followed by the development of thioester bond between the two proteins. Then, Atg12 gets linked to Atg10 which is E2-like enzyme, resulting in the formation of a new thioester bond. Finally, through its glycine at carboxyl end, the Atg12 protein gets attached to amino group in the lysine residue of Atg5 (Ichimura *et al.*, 2000)(Shintani *et al.*, 1999); however, this bonding is irreversible. Through its coiled coil domain at carboxyl end, the Atg16 gets attached to the Atg12-Atg5 complex and this, bigger complex, forms temporary association with the isolation membrane or phagophore (Mizushima *et al.*, 1998; Shintani *et al.*, 1999; Ichimura *et al.*, 2000). The second conjugation system needs this complex for its formation. However, the Atg12-Atg5 conjugate splits after the formation of autogphagosome.

The Atg8/LC3-PE complex is the 2nd conjugation system and is the best-defined marker for autophagosomes. Cysteine proteinase Atg4 processes Atg8 by cleaving the arginine residues at carboxyl end, thereby, exposing a glycine residue. Atg7 activates this glycine followed by transference of Atg8, an E2-lke enzyme, to Atg3(Ichimura *et al.*, 2000). Eventually, the conjugation of Atg8 with phospholipids PE occurs, thus, permitting the binding of protein with the autophagosomal membrane. The Atg8–PE complex is dissociated by the same Atg4 enzyme, leading to the recycling of Atg8 back to the cytoplasm. The cytosolic LC3-I form is produced when an Atg4 cysteine proteinase i.e. autophagin cleaves the newly formed LC3. The LC3-I form, having molecular weight of 18kDa, undergoes few ubiquitination-like reactions, leading to the generation of the membrane bound protein LC3-II. This LC3-II protein (16kDa) binds to both the outer and the inner sides of the developing autophagosome (Kabeya *et al.*, 2000).

4.5.3.1 Two kinases in autophagy cascade

Phosphatidyl inositol 3-kinase (PI3K) was one of the first kinases to be verified as involved in autophagy. There are three groups of PI3K in mammal's cells (Petiot *et al.*, 2000). Among these groups, class III PI3K has an important role in the

authophagy mechanism. Class III PI3K activates autophagy. On the other hand, autophagy is negatively regulated by class I PI3K. It has also been established that class III PI3K demonstrates interaction with Beclin 1 and p150. Beclin 1 was actually isolated as Bcl-2 interacting protein. Moreover, it serves as the first autophagy related tumour suppressor gene (Liang *et al.*, 1999).

Serine/threonine kinase Atg1 is the 2nd major kinase involved in the pathway of autophagy. It develops a complex through interaction with different regulatory proteins and under different nutrient conditions, leading to the formation of complexes with different compositions. Partial dephosphorylation of Atg13 occurs in starvation conditions, followed by development of autophagosome, through an interaction between dephosphorylated Atg13 and Atg1. However, in nutrient-rich environment, phosphorylation of Atg13 occurs and its interaction with Atg1 is inhibited, thereby, leading to inactivation of autophagy. Moreover, under nutrient-deficient conditions, association between Atg17 i.e. another Atg protein, and the Atg1-Atg13 complex is augmented (Kabeya *et al.*, 2005).

4.5.3.2 Autophagy proteins

4.5.3.2.1 Beclin 1

Beclin 1 was the first mammalian gene to be identified as having a role in mediating autophagy. Beclin 1 is a novel Bcl-2-homology (BH)-3 domain only protein. Anti-apoptotic Bcl-2 family members interact with the BH3 domain of Beclin 1(Kang *et al.*, 2011). Beclin 1 is localized within the plasma-membrane, the cytoplasm and the nucleus in human and murine (Li *et al.*, 2009). Transcriptionally, nuclear factor (NF)-*κ*B is involved in the regulation of Beclin 1 expression. NF-*κ*B directly binds the Beclin 1 promoter and upregulates its mRNA and protein levels, leading to positive modulation of autophagy in T cells (Copetti *et al.*, 2009).

4.5.3.2.2 Microtubule-associated protein 1A/1B-light chain 3 (LC3)

All mammalian tissues and cultured cells possess Microtubule-associated protein 1A/1B-light chain 3 (LC3), which is a soluble protein with a molecular weight of around 17 kDa. Various components of cytoplasm such as cellular organelles and proteins found in cytosol are engulfed by autophagosomes during the course of autophagy. At the same time, LC3-I i.e. a cytosolic form of LC3, conjugates with phosphatidylethanolamine, resulting in the formation of LC3-phosphatidylethanolamine conjugate (LC3-II). This conjugate is then recruited to

autophagosomal membranes. Autolysosomes are formed by the fusion of autophagosomes and lysosomes. Hydrolase enzymes contained in the lysosomes degrade the intra-autophagosomal components. The degradation of LC3-II in the lumen of autolysosomes occurs simultaneously (Tanida *et al*., 2008).

4.5.4 Linking autophagy and apoptosis

There are two possible ways through which one can link the phenomena of autophagy and apoptosis. First, the autophagy could demonstrate a regulatory role for apoptosis by increasing or decreasing its chances to occur. Second, apoptosis could demonstrate a regulatory role for autophagy by enhancing or reducing it (Gump and Thorburn, 2011). Beclin 1 is an autophagic substance which is overexpressed under the influence of nutrient depletion, hypoxia, angiogenesis inhibitors, immunotherapy, irradiation, or chemotherapy (Kang *et al.*, 2011). Cancerous cells are protected from apoptosis through Bid knockdown which also stimulates autophagy and this is manifested by enhanced expression of Beclin 1. However, the current literature is still deficient in elaborating the exact mechanism involved in inhibition of apoptosis by Beclin 1.

During apoptosis, Beclin 1 can be cleaved by caspases, resulting in the confiscation of its pro-autophagic action. Caspases are cysteine aspartyl proteases which play a crucial role in apoptosis and are stimulated by a death receptor ligand i.e. TRAIL (Kroemer and Martin, 2005; Reef *et al.*, 2007). Beclin 1 cleavage, mediated by capase-3, -7 and -8, results in the formation of C- and N- terminal fragments, leading to loss of the ability to induce autophagy. The C-terminal fragments move to the mitochondria and this make the cells sensitive to apoptotic signals (Djavaheri-Mergny *et al.* , 2010). Autophagy is decreased by apoptosis triggered by the proapoptotic protein Bax via increasing Beclin 1 cleavage at D149 mediated by caspase (Luo and Rubinsztein, 2009). Nevertheless, it has been reported by some latest research studies that autophagy can result in degradation of a death receptor effector i.e. caspase-8 (Hou *et al.*, 2010), which implies that living systems have a feedback mechanism for cross-regulation of apoptosis and autophagy. Even though autophagy is inactivated by the apoptosis-related cleavage of Beclin 1 and Atg5, caspase-3-mediated Atg4D cleavage produces a fragment, capable of demonstrating enhanced autophagic activity (Kang *et al.*, 2011).

4.5.4 Necroptosis

It has been established that members of the death receptor family are essentially involved in the regulation of cell population and removal of unwanted cells or cells infected with viruses. Researchers had previously reported that the stimulation of death receptors can either lead to the promotion of survival of cell through the activation of NF-κB or the induction of apoptosis, leading to cell death. However, a third mechanism known as necroptosis or programmed necrosis has now been discovered (Bell and Walsh, 2009) .

Kinnally *et al.*, 2011; Smith and Yellon (2011) have proposed that there are two apoptotic pathways which are partially interlinked. These include the classical apoptosis which is dependent on caspase, and caspase-independent programmed cell death. Both of these mechanisms can be associated with each other, since caspases may cause the activation of non-caspase proteases and vice versa.

Apoptosis is promoted by death receptors through an intracellular motif termed death domain (DD), causing the activation of caspases-8 and thereby, inducing apoptosis. Once the caspase-8 is inhibited, activity of the Jun amino-terminal kinase (JNK) and DD-containing kinase RIP-1 results in the induction of a type of cell death which leads to the production of autophagosome, and which is dependent on Atg6/Beclin 1 and Atg7 (Kang *et al.*, 2011).

It has been recently determined that T lymphocytes, stimulated by mitogens, induce nonapoptotic active capases-8. Moreover, T cells devoid of this caspases-8 activity can enter the cell cycle. Nevertheless, they are unable to accumulate and undergo caspase-independent cell death. It was determined that this cell death results from chemical inhibition of RIP-1 and the necroptotic inhibitor Nec-1 caused restoration of T cells increasing in number. A parallel study conducted by Chen and colleagues has reported that knockdown of RIP-1, mediated by Nec-1, restores the ability of T cells lacking in caspase-8, to demonstrate clonal expansion. This implies that kinase activity of RIP-1 is important for this mechanism of cell death (Ch'en *et al.*, 2008).

4.5.4.1 Receptor interacting protein 1 (RIP-1)

The receptor interacting protein 1 (RIP-1) is essentially required for the induction of necrosis. It is a serine/threonine kinase which is regulated by ubiquitination and caspases. Three different domains can be identified in RIP-1. These are the C-terminal

death domain, the intermediary RIP-1 homotypic interaction motif (RHIM)-domain and the N-terminal kinase domain (Holler *et al.*, 2000; Vandenabeele *et al.*, 2010). Upon stimulation of TRAIL or TNF, necrosome is formed, and it in turn, activates RIP-3. Upon activation, RIP-3 interacts with enzymes regulating glycolytic flux, glutaminolysis, leading to the formation of reactive oxygen species in the mitochondria (Holler *et al.*, 2000; Vandenabeele *et al.*, 2010). It has now been established that the activity of RIP-1 is linked specifically with necrosis, instead of apoptosis. Moreover, necrostatin-1 (Nec-1), a small molecule which inhibits necroptosis, impedes the RIP-1 kinase activity (Degterev *et al.*, 2008). So far, researchers are unable to identify any substrate of RIP-1 that could have a role in necroptosis (Chaabane *et al.*, 2013).`

It is interesting to note that the ligands that stimulate apoptosis such as TRAIL, FASL and TNFα can stimulate necroptosis, as well. This implies that cell death caused by the activation of death receptor can occur through alternative mechanisms i.e. necroptosis or apoptosis. Researchers are busy exploring the mechanism involved in the decisions made by the cell when undergoing either necroptosis or apoptosis, as well as, the mechanism responsible for necroptosis execution (Christofferson and Yuan, 2010).

4.6 Regulation of pathways crucial for the survival and proliferation of leukemia cells

4.6.1 The MAPK Pathways

A group of serine/threonine kinases, the MAPK family, plays a crucial role in generating different intracellular responses to extracellular stimuli as shown in Figure 4.18.1. These responses include regulation of cell death, proliferation and differentiation (Lewis *et al.*, 1998; Cardoso *et al.*, 2008). Biochemical characterization of three MAPKs belonging to mammalian cells has been done. These MAPKs include the p38 MAPK, the c-Jun N terminal kinase (JNK), also known as stress-activated protein kinase, and the extracellular signal-regulated kinase (ERK). This signaling pathway cascade comprises of a module of three kinases. The first one is MAPK, phosphorylated and activated by MAPK kinase (MAPKK). MAPKK is phosphorylated and activated by a MAPK kinase kinase (MAPKKK) (Roux and Blenis, 2004).

Mitogens such as growth factors mainly activate the ERK, while on the other hand, the p38 MAPK and the JNK are activated by a wide variety of stress conditions such as osmotic shock, heat, DNA damage, hydrogen peroxide exposure and ultraviolet irradiation. In addition, the p38 MAPK and the JNK have also been reported to be activated by a number of chemotherapeutic drugs (Cardoso *et al.*, 2008). Activation of these MAPKs has been found to be associated with apoptosis. Numerous transcription factors are phosphorylated by these three MAPKs (p38, JNK and ERK), once they are activated. These transcription factors include stress-activated protein-1, CCAAT/enhancer-binding protein [C/EBP] - homologous protein/growth arrest DNA damage-inducible 153 (CHOP/GADD153), myocyte enhancing factor-2c (MEF2C), activating transcription factor 2 (ATF2), c-Jun and c-Myc (Pearson *et al.*, 2001). Phosphorylation of different transcription factors has varying biological outcomes. Since an extensive range of factors activate MAPKs, these signaling mechanisms may act as a common pathway and may collaborate with other signaling mechanisms to regulate responses of the cell to various extracellular stimuli (Hu and Kong, 2004).

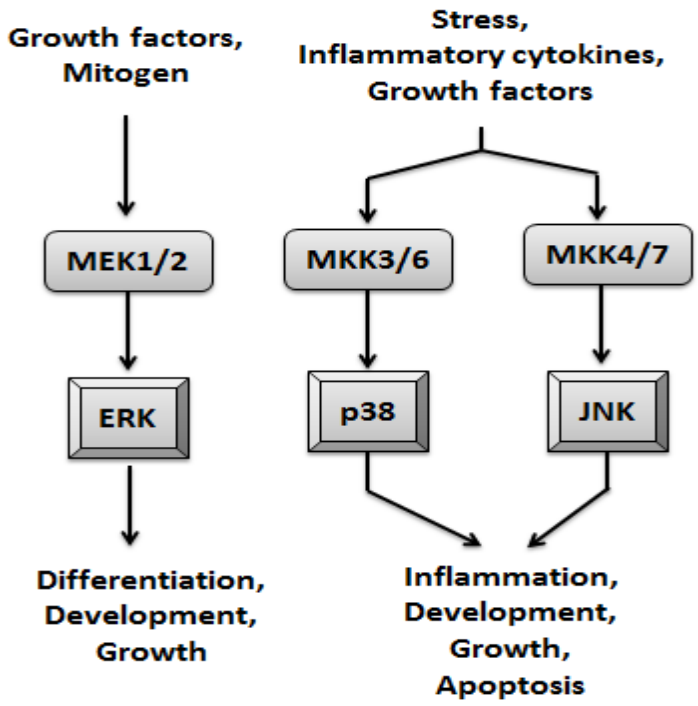


Figure 4.18.1: **Organization of the MAPK family of proteins.** Upon stimulation of receptors MAPK kinase family of proteins is activated. It is composed of a triple kinase module which consists of MAPKKK, MAPKK and MAPK that act up their substrate proteins. Various mitogenic and environmental signals activate the MAPKKK proteins, which phosphorylate their downstream MAPKK targets. The phosphorylation of the MAPKK proteins induces activation, which in turn phosphorylates their MAPK targets. Once activated MAPK proteins regulate the activity of numerous proteins, regulating their activity.

4.6.1.1 c-Jun N-terminal kinases (JNKs)

The JNKs control phosphorylation of numerous target proteins including c-Jun on Ser-63 and Ser-73. This occurs with the transcriptional activation domain of the c-Jun. These are the MAPKs that are activated by mitogens in response to stress conditions such as osmotic shock, heat shock, ultraviolet irradiation and cytokines. The JNKs are also involved in cellular apoptosis pathway and differentiation of T cells. A pair of MAPKs is involved in the activation. These are MKK4 and MKK7 (Pearson *et al.*, 2001).

4.6.1.2 p38 mitogen-activated protein kinases (p38 MAP kinase)

The p38 MAPK represents a group of MAPKs involved in cellular processes such as autophagy, apoptosis and cell differentiation and activated by mitogens. A number of different stress conditions activate p38 MAPKs. These conditions include ultraviolet light, inflammatory cytokines and osmotic shock (Pearson *et al.*, 2001)

4.6.1.3 MAPK and GR

The MAPK demonstrates dual roles in the lymphoid cells. Its activation status inside the lymphoid cells leads to variations in GR phosphorylation patterns. These variations can control cell responses to GCs. More specifically, Ser211 phosphorylation of GR induced by p38 MAPK leads to increased GC sensitivity (Cardoso *et al.*, 2008). On the other hand, JNK induces Ser226 phosphorylation, and reduces the level of Ser211 phosphorylation, thereby, leading to the amplification of GC resistance. Intriguingly, reduced phosphorylation of Ser211 within GR decreases its nuclear confinement and also reduces the transcriptional activity of the receptor on a number of target genes. Hence, activation status of GR seems to increase with an increase in the degree of Ser211 phosphorylation (Kfir-Erenfeld *et al.*, 2010).

4.6.2 The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway

The Janus kinase-signal transducer and activator of transcription (Jak/STAT) pathway, is normally conserved during the evolutionary process, and is involved in a number of biological phenomena including cellular immunity mediated by cytokines and growth-factors, cell differentiation and proliferation, and apoptosis (Dreesen and Brivanlou, 2007). The Jak family of tyrosine kinases is activated by binding of cytokines, which leads to oligomerization of the cytokine receptor present on cell

surface (Valentino and Pierre, 2006). Upon activation, Jaks cause phosphorylation of the cytoplasmic domain of the receptor, resulting in the formation of docking sites for STATs. Jaks phosphorylate STATs dimerizes and transfers to the nucleus where they regulate transcription of genes for cell death, proliferation and cell growth (Cardoso *et al.*, 2008).

4.6.2.1 Mechanism of the JAK/STAT signaling

The activation of JAKs requires that the regulatory molecule bind with the receptor molecules so that they form a dimer as shown in Figure 4.19.1. The two JAKs are brought close to each other through dimerization so that they may phosphorylate each other (Valentino and Pierre, 2006). JAK is further activated by the phosphorylation of the receptor. It is also known that the phosphotyrosine residues found on the receptor acts as target sites for the binding of STAT proteins, which are latent transcription factors. They are localized in the cytoplasm and are activated by JAK. Upon phosphorylation, two STATs join to give rise to a STAT dimer (Schindler *et al.*, 2007). During this dimerization, a bond is formed between a STAT molecule and phosphotyrosine of the other STAT molecule. The STAT dimer is formed and acts as an active transcription factor. It migrates to the nucleus where it executes its activities by binding with specific regions of DNA. Dephosphorylation results in the inactivation of these proteins (Ward *et al.*, 2000).

Remarkably, various STAT proteins are common in cells since they are formed from the activation of different cytokine receptors. In order to play its role as a transcription factor, each STAT dimer interacts with the particular DNA region present, in the promoter of the gene. Hence, it can be stated that certain genes are activated by certain cytokines, resulting in a specific cellular response (Ward *et al.* , 2000; Cardoso *et al.*, 2008).

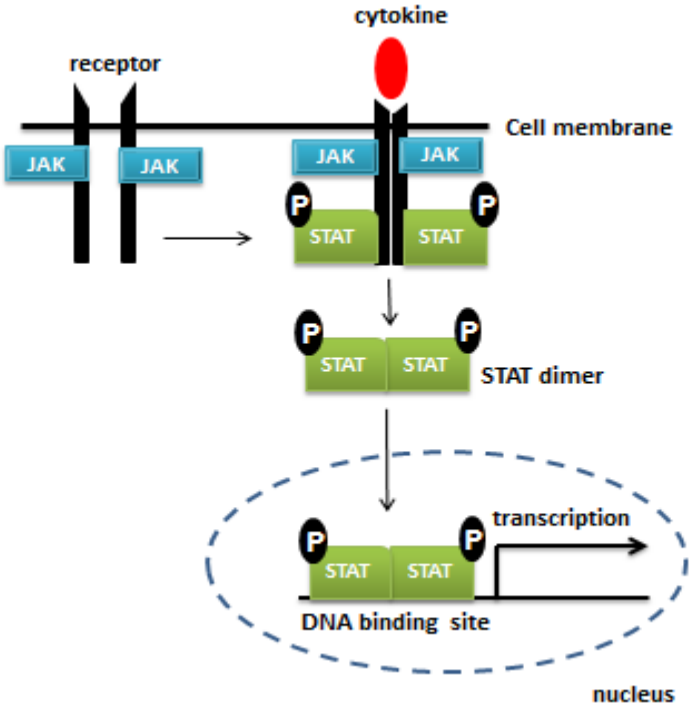


Figure 4.19.1: **Jak/STAT pathway.** Upon ligand binding of cytokines and growth factors to their corresponding receptors. JAK pathway is activated and phosphorylates the receptor and STAT proteins on specific tyrosine residues. STATs translocate to the nucleus and bind to a specific DNA binding site as a dimer and initiate the transcription of target genes of cellular functions, including proliferation, growth, haematopoiesis, and immune response.

4.6.2.2 JAK/STAT signaling pathway and GR

The GCs influence cytokine signaling at more than one level. The most researched issue in this regard is the direct transcriptional repression of the expression of cytokine genes by activated GR (Rogatsky and Ivashkiv, 2006). The transcriptional interference between NF-κB or AP-1 and GR at different cytokine promoters has turned into a common demonstration of the phenomenon of repression via protein-protein interactions. Yet, all cytokines are not repressed, for example, anti-inflammatory cytokines like IL-10, are upregulated by GCs (Darnell *et al.*, 1994). The scenario becomes even more complex because of the impact of GR on regulation of transcription by STAT, i.e. the finishing point of the cascade initiated by cytokine signaling. GCs seem to be in synergy with various cytokines instead of impeding their activities. Moreover, it appears to be specifically counterintuitive that GR strengthens IL-2 signaling to STAT5 and IFNγ signaling to STAT1 (Rogatsky and Ivashkiv, 2006). Differentiation of helper T cells is promoted by both of these signaling and this differentiation is radically inhibited by GCs (Rogatsky *et al.*, 2003). It has been

established, though from only a few research studies, that a wide variety of mechanisms are involved depending on the physiological state, cellular surrounding, the gene, as well as the promoter that influences the availability and actions of accessory proteins and transcriptional cofactors (Rogatsky and Ivashkiv, 2006).

4.6.3 Phosphatidylinositol-3OH-kinase (PI3K) pathway

A number of different cellular functions such as apoptosis and cell proliferation are regulated by Phosphatidylinositol-3OH-kinase (PI3K) pathway, through the activation of various downstream effectors (Cardoso *et al.*, 2008). Ser/Thr kinase Akt, also known as protein kinase B (PKB) is the most prominent one among these effectors. The phosphatidylinositol 4, 5-biphosphate (PIP2) present at the 3-position of the inositol ring is phosphorylated by PI3K, resulting in the formation of phosphatidylinositol 3, 4, and 5-triphosphate (PIP3). PIP3 function as second messengers. Moreover, for the proteins having a pleckstrin homology (PH) domain (e.g. PDK1 and Akt), the PIP3 behave as a membrane anchor. The PDK1 and Akt colonalization at cell membrane promotes phosphorylation of Akt leading to its activation (Cantley, 2002). Pro-proliferative and anti-apoptotic signals are generated by Akt via inhibitory phosphorylation of caspase-9, Forkhead (FOXO1), GSK3 and Bad, which in turn occurs via activating phosphorylation of mTOR and also through the discharge of transcriptional factors such as NF-κB (Barata *et al.*, 2005). PIP3 is dephosphorylated by tumour suppressors PTEN, meaning that they behave as negative regulators of PI3K/Akt pathway (Cantley, 2002). Apoptosis inhibition via FOXO3a inactivation by PI3K pathway is represented by Figure 4.20.1.

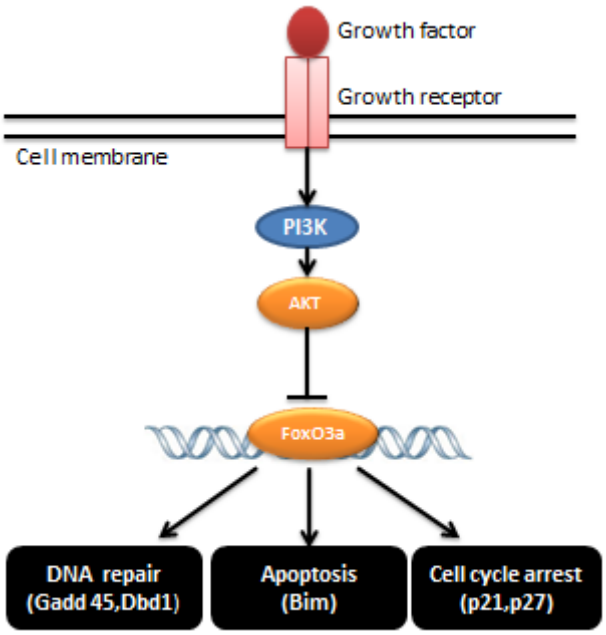


Figure 4.20.1: **Regulation of FOXO transcription factors by the PI3K-AKT pathway**. On stimulation of PI3K AKT signaling by growth factors, AKT phosphorylates FOXOs and inactivate it which leads to inhibit nucleus translocation and transcriptional activation of Foxo. Foxo3a transcriptionally controls different target genes responsible for a variety of cellular process such as apoptosis, DNA repair, and metabolism, ROS detoxification and cell proliferation.

4.6.3.1 PI3K pathway and T-ALL

Activation of the PI3K/Akt pathway in T-ALL is executed by the growth factors found in the leukemic setting. They signal through the cytokine receptors (Barata *et al.*, 2004). In addition, Akt molecules that are constitutively phosphorylated independent of the external growth factors are found in T-ALL cell lines (Palomero *et al.*, 2007). Moreover, in certain primary T-ALL samples, a downstream target of PI3K/Akt i.e. NF-κB is found to be constitutively activated (Kordes *et al.*, 2000). However, there is no activating mutation of Akt and/or PI3K in T-ALL. Some researchers propose that over-activation of PI3K/Akt pathway occurs as a consequence of downregulation of PTEN protein (Cardoso *et al.*, 2008). As discussed earlier, a number of downstream signal transduction pathways such as mTOR are activated by PI3K. Research studies report that rapamycin, an inhibitor of mTOR pathway, is capable of restoring the usual activity of hematopoietic stem cells from PTEN-deleted mice (Yilmaz *et al.*, 2006), thus, reverting the chemo-resistance of leukemic cells overexpressing Notch1(Mungamuri *et al.*, 2006) and stimulating cell death of pediatric T-ALL cells (Avellino *et al.*, 2005).

4.7 Bone marrow niches and mesenchymal stem cells

Bone marrow niches support leukemic cell proliferation, metastasis and drug resistance by secretion of numerous factors including cytokines. When leukemic cells grow, they disrupt normal hematopoietic progenitor cell (HPC) bone marrow niches and create abnormal microenvironments. Also, niches recently have been linked to the development of drug resistance (Saha and Kearns, 2011).

One type of stem cell that resides in the bone marrow niche is called Mesenchymal stem cells (MSCs) (Herzog *et al.* , 2003). MSCs are adherent, non haematopoietic stem cells and have the ability to self-renew and to differentiate into multiple lineages including osteoblasts, chondrocytes and adipocytes (Dwyer *et al.*, 2010). MSCs express markers such as CD29, CD44, CD51, CD73 (SH3/4), CD105 (SH2), CD166 (ALCAM) and Stro-1. MSCs express a wide range of molecules, including growth factors, chemokines, adhesion molecules and toll-like receptors, on their surface. In terms of cell migration, MSCs are known to functionally express chemokine receptors CCR1, CCR4, CCR7, CCR9, CCR10, CXCR4, CXCR5, CXCR6, CX3CR1 and c-me (Dwyer *et al.*, 2010) .

MSCs have potential successful uses in regenerative medicine, but we are focusing on their potential role in supporting resistance in leukemia. We focused on analyzing differences in gene expression of leukemia cells caused by secreted factor from bone marrow stromal cells. Understanding the factors involved in secreted factors' effect on leukemia is essential to ultimately develop novel clinical strategies aimed at using MSCs as vehicles to deliver antitumor factors or block actions of secreted factors to reduce tumour growth. The potential role of MSCs in tumour initiation or promotion is a significant concern that must be addressed fully to allow MSC-mediated therapy for cancer to realize its full potential (Spaeth *et al.*, 2008).

4.7.1 Haemopoeitic stems (progenitor) cells (HSCs)

HSCs are the precursors that give rise to all blood cell types including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lineages (T-cells, B-cells, Natural killer (NK)-cells) (Figure 4.21.1). The niche is the microenvironment of the bone marrow that regulates HSCs maintenance, production and differentiation (Colmone *et al.*, 2008). The stem cell niche concept was first introduced in 1970 by

Schofield (Arai *et al.*, 2005). In the bone marrow, there are two types of supportive niches for HSC, namely, the Endosteum niche (Osteoblastic) and the vascular niche (Colmone *et al.*, 2008).

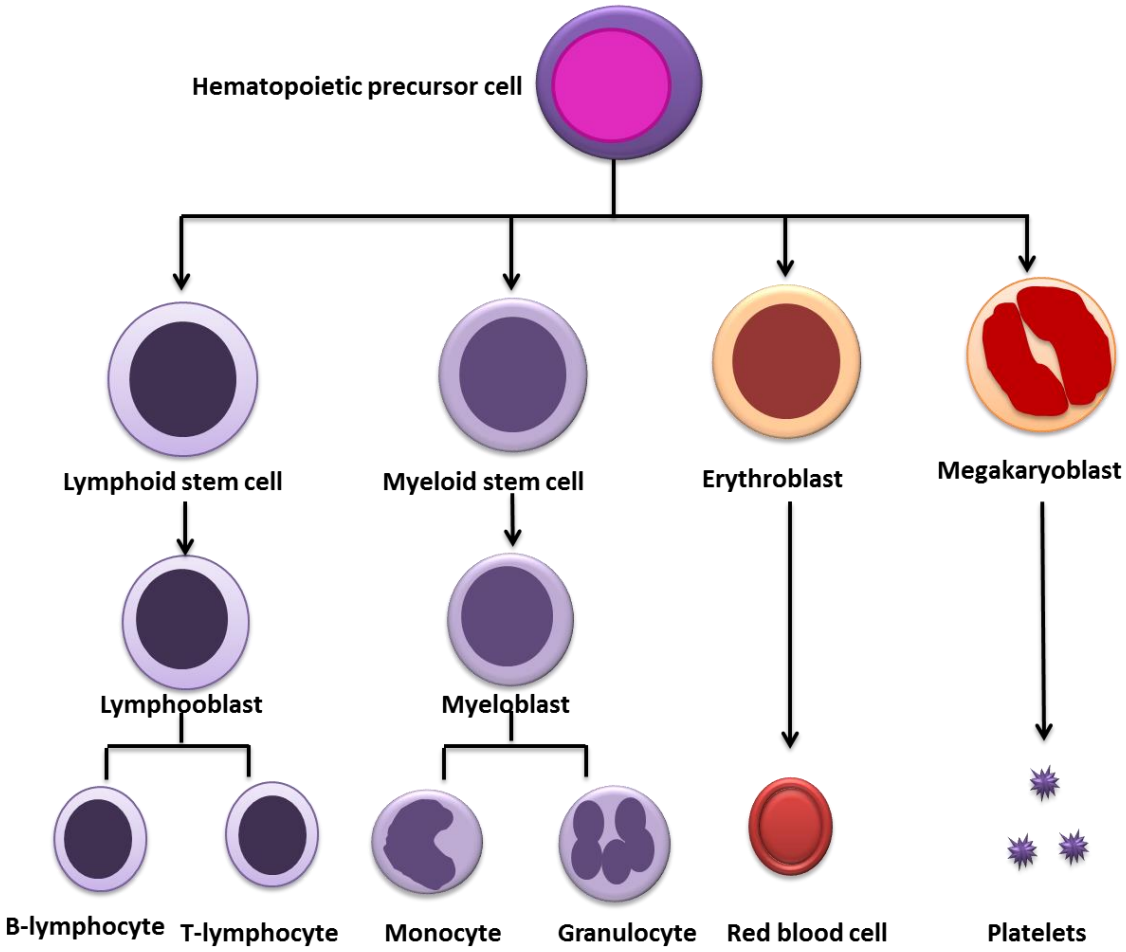


Figure 4.21.1: **Formation of blood cells (Haematopesis).** The formation of all kind of blood cells takes place in the bone marrow, soft inner part of the bones.

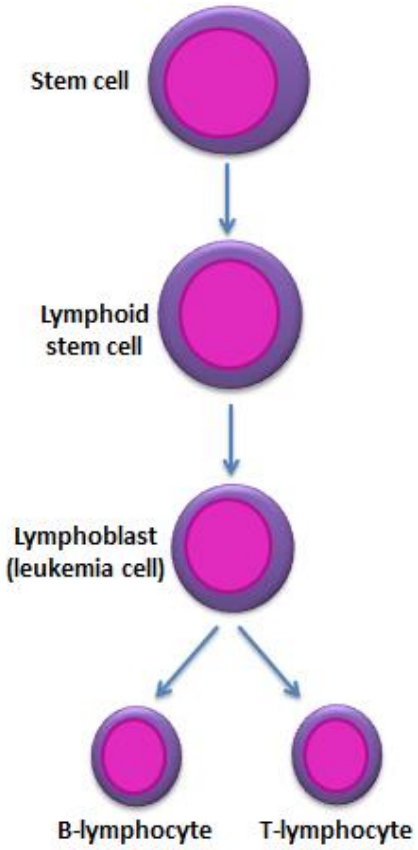


Figure 4.22.1: **Lymphoblast formation.** In acute lymphoblastic leukemia, the bone marrow filters with too many lymphoblasts. Lymphoblasts are not fully functional cells and do not protect against infection. Lymphoblasts can be T or B cells depending on the origin of formation. T-Lymphoblast arises from the bone marrow, while B lymphoblasts arise from the lymph node.

4.7.2 Endosteal niche and HSC maintenance

Endosteum is the highly vascularized interface between the bone and the bone marrow (Kiel and Morrison, 2008). Endosteum is covered by a layer of bone cells that give rise to bone forming cells called osteoblasts. Besides osteoblasts, endosteum contains osteoclasts which are bone resorbing cells. Osteoblasts and osteoclasts play a crucial role in bone formation and remodeling. Both cells are found in endosteum in the equilibrium state (Kiel and Morrison, 2008).

Osteoblasts secrete factors to streamline the HSC in the bone marrow so as to maintain bone marrow quiescence. These positive factors include angiopoeitin 1 (Ang 1), thrombopoietin and CXC-chemokine ligand 12 (CXCL12). Angiopoeitin 1 (Ang 1) and thrombopoietin are responsible for controlling HSC quiescence, whereas,

CXC-chemokine ligand 12 (CXCL12) is responsible for regulating HSC movement and stability in the bone marrow (Kiel and Morrison, 2008).

HSC obtain osteoblasts factors using different means such as direct adhesion to the osteoblasts, or by communication with neighboring cells and releasing of soluble factors when N-cadherin ligand is not vital (Kiel and Morrison, 2008). Osteoclasts express matrix metalloproteinase 9 (MMP9), cathepsin K and CXCL12 to contribute in HSC maintenance and localization inside the bone marrow.

4.7.3 Vascular niche and HSC maintenance

The sinusoidal niche is vascularized with a large number of sinusoids in haematopoeitic tissues. The sinusoids are small blood vessels in haematopoeitic tissue that allows cells trafficking in and outside the bone marrow (Kiel and Morrison, 2008). The interface between the bone cell, the HSC and the endothelial cells are crucial for haemopoeisis (formation of blood cells) and bone formation.

Sinusoids are lined by endothelial cells which allow hematopoietic cells to go in and out of the circulation. The presence of 60% of HSC around the sinusoids reflects vascular function in maintaining HSC. Twenty percent of the cells are found inside the endosteum and the rest are distributed in the bone marrow (Kiel and Morrison, 2008). Thus, the HSC microenvironment quiescence is maintained by osteoblastic niches, while both the proliferation and differentiation of the cells are supported by vascular niche (Arai *et al.*, 2005) (Figure 4.23.1).

Vascular niche plays three important roles in the bone marrow;

It supplies the nutrients, oxygen and growth factors for the HSC cells for proliferation and differentiation.

Sinusoidal endothelium along with chemokines encourages homing and mobilization of HSC. This occurs through endothelial mobilization when the HSC enters the vascular system, and homing when cell returns back to the bone marrow.

A vascular niche is important for cells outside the bone marrow, such as in the spleen which may replace BM niche in case of bone marrow suppression (Yin and Li, 2006a).

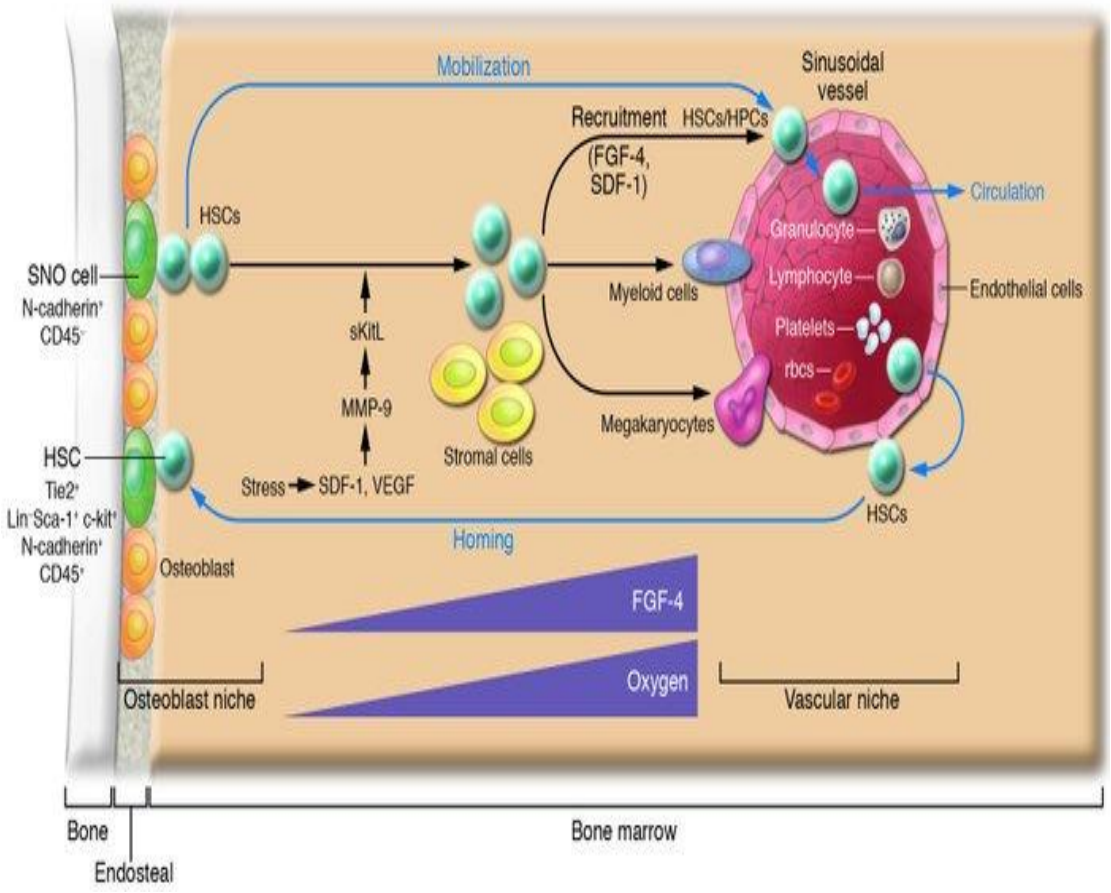


Figure 4.23.1: **The osteoblastic and vascular niches in bone** (Yi and Li., 2006b)

4.7.4 Chemokine involvement in HSC homing and mobilization

Chemokines are involved in HSC homing and mobilization. The ability of HSCs to mobilize and then return, or home, to the niche is regulated by specific molecules called chemokines. Chemokines are proteins secreted by the cell. The chemokine SDF-1 and its receptor CXCR4 play a crucial role in HSC mobilization and homing regulation. Endothelial cells, osteoblasts, and other stromal cells express SDF-1, while HSCs express CXCR4. Moreover, activation of adhesion molecules such as the very late antigen-4 (VLA-4) and the leukocyte function antigen-1 (LFA-1) is also required for this process and also for the subsequent migration of BM toward the osteoblast surface. Likewise, high levels of SDF-1 on the surface of osteoblasts attract HSCs to return home to the osteoblast niche. The Rho GTPases Rac1 and Rac2, in response to SDF-1 signals, are also involved in regulating HSC mobilization and homing (Kopp *et al.*, 2005).

Granulocyte colony-stimulating factor (G-CSF) inactivates SDF-1 in the bone marrow using proteolytic enzymes such as elastase, cathepsin G, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) (Yin and Li., 2006a). Homing of HSC to the osteoblast niche occurs when osteoblasts surface demonstrates raised level of SDF-1. HSC cells that express CXCR4, directly attach to SDF-1 on the surface of osteoblastic niches. Adhesion is strengthened by the N-cadherin and β-catenin complex (Kopp *et al.*, 2005).

4.7.5 Effects of leukaemia cells on bone marrow niches

Drug resistance, proliferation of leukemic cells and their metastasis is favoured in the bone marrow by cytokine secretion. The normal hematopoietic progenitor cell (HPC) bone marrow niches are disrupted by leukemic cell growth, which develops abnormal microenvironments (Konopleva *et al.*, 2009). It is still not clearly understood how leukemic cells bring about modifications in the microenvironment (Colmone *et al.*, 2008). During leukemia, number of CD34+ cells reduces with time and they become unable to travel to the peripheral circulation under the influence of cytokine stimulation. Neutralization of stem cell factor (SCF) released by leukemic cells blocks transport of CD34+ cells to malignant niches, normalizes the number of CD34+ cells and restores movement of CD34+ cells. HPC dysfunction is caused by tumour microenvironment through disrupting the normal HPC niches. However, the normal progenitor cell function can be sustained in malignant setting by the help of therapeutic blockage of HPC interaction with tumour niches (Colmone *et al.*, 2008). In addition, CXCR4 is expressed on the surface of leukemic cells in order to facilitate leukemic cells homing in the BM microenvironment, followed by targeting additional leukemic cells in the microenvironment of bone marrow (Yin and Li, 2006a).

5 Project aims

The aim of the project is to determine the role of the tumour microenvironment and steroid/DNA damage induced pathways in promoting drug resistance. The project also aims at identifying the key aberrant pathways that lead to leukemic cell survival. This will be achieved by following the effects on apoptosis and cell cycle progression of leukaemia ALL cells either exposed to mesenchymal stem cell based culture systems and/or exposed to steroids and anthracyclines.

1. First, we will assess the DNA damage response and response to GC of CEM C7-14 steroid sensitive and CEM-C1-15 steroid resistant ALL cells. We will also assess the alteration of these responses when cells are exposed to conditioned media secreted by stem cell niche from bone marrow derived cell line.

2. We will analyse glucocorticoid receptor pathway and p53 pathway components as markers of leukaemia cell response to dexamethasone and anthracyclines respectively.

3. We will analyze novel target genes by applying microarrays technology in lymphocytic cells treated with dexamethasone/anthracyclines as individual and combined therapy and also when the cells are exposed to stem cell niche. Microarray experiments will determine the dissimilarities in gene networks in ALL cells during individual and combined treatments with dexamethasone and topoisomerase II inhibitors. This will be essential for understanding the molecular and cellular basis of drug action and presumably result in therapy improvement.

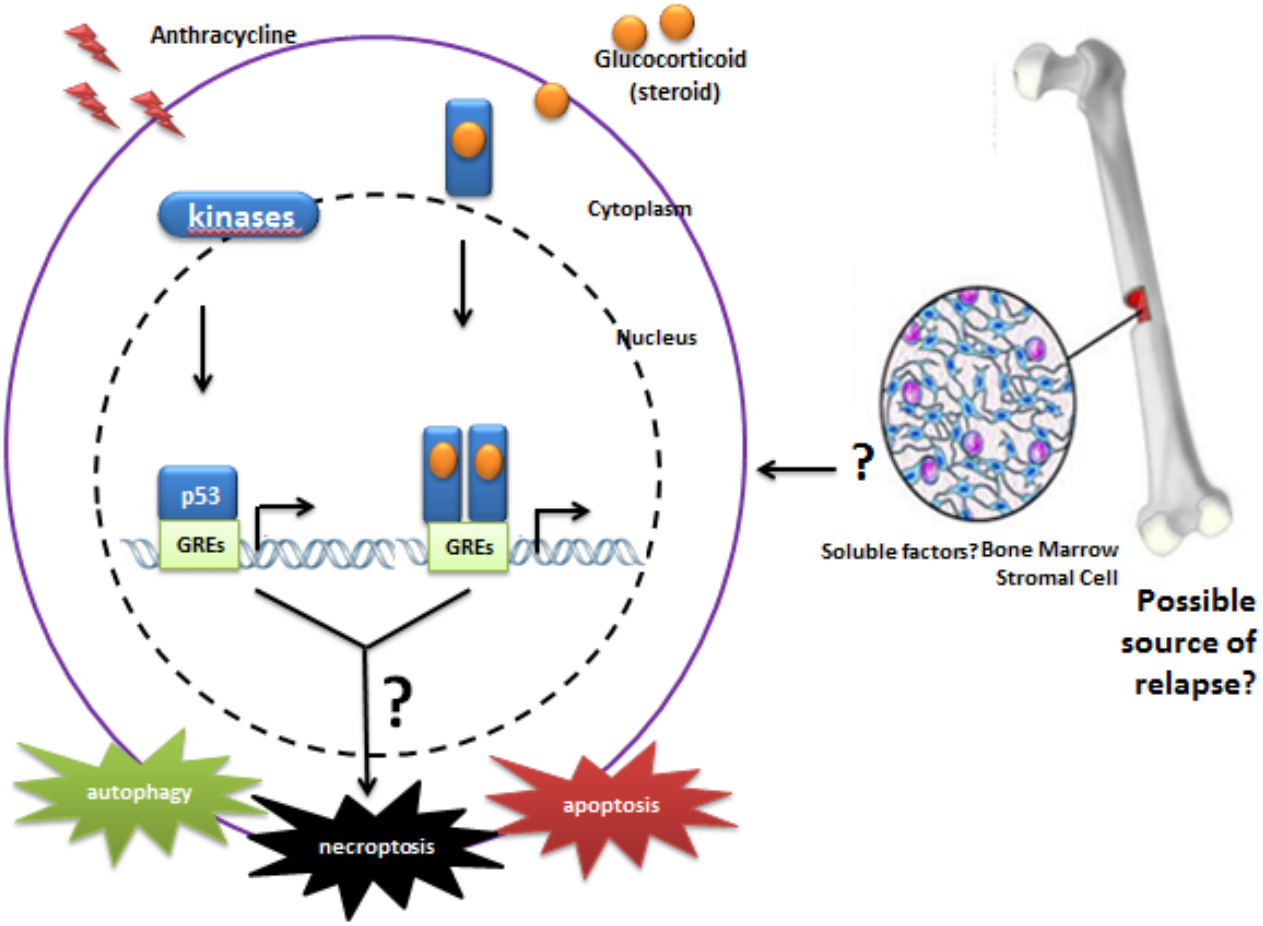


Figure 5.1.1: **Hypothesis of the project**. 25-30% of children affected with T-ALL are at risk of death due to resistance to prevailing treatment. We propose to study how combination of drugs improves therapy when compared to individual drug treatment. In addition, we have hypothesized that the bone marrow could be the source of resistance for the treatment.

6 Materials and Methods

6.1 Materials

6.1.1 Chemicals

All chemicals used in this study and their suppliers are listed in table 2.

No	Materials	Supplier	Product code
1	2-Mercaptoethanol	Sigma,UK	M3148
2	Acrylamide	Sigma,UK	A6050
3	Ammonium persulphate (APS)	Flowgen,UK	H17423
4	Calcium chloride	VWR Prolabo,UK	1.02379.1000
5	Chemiluminescent substrate	Chembio Ltd,UK	16031
6	Cryovails	Scientific laboratory supplies, UK	G122263
7	Dimethylsulphoxide (DMSO)	Sigma,UK	D8418
8	Di sodium hydrogen sulphate	Fisher,UK	S/P525/53
9	dNTPs	Bioline	Bio-39025
10	Dithithreitol (DTT)	Sigma,UK	D0632
11	Magnetic beads (Dynabeads)	Invitrogen,UK	100-01D
12	Ethanol absolute	Fisher,UK	E/0600/05
13	Etoposide	Sigma,UK	E1383
14	Formaldehyde 38%	Sigma,UK	F8775
15	Glycerol	VWR BDH Prolabo, UK	24387.361
16	Glysine	Fisher,UK	BPE381-1
17	HEPES	Sigma,UK	54457
18	Igepal	Sigma,UK	CA-630
19	Methanol	Fisher,UK	BPE1105-1
20	Page Ruler Prestained protein ladder	Fermentas,UK	SM0672
21	Phenylmethylsulphonyl fluoride (PMSF)	Sigma,UK	P7626
22	Propidium iodide (PI)	Sigma,UK	25535-16-4
23	Sodium butyrate	Sigma,UK	B5887

24	Sodium chloride	Fisher,UK	BPE358-1
25	Sodium dihydrogen phosphate	Fisher,UK	S/4480/60
26	Sodium dodecyl sulphate (SDS)	Fisher,UK	S/P530/48
27	Sodium hydrogen carbonate	VWR BDH Prolabo,UK	27778.293
28	Sodium orthovandate	Sigma,UK	S6508
29	Sodium pyrophosphate	Sigma,UK	221368
30	SYBR Green dye	Sigma,UK	S9430
31	Tetra methyl ethylene diamine (TEMED)	VWR BDH Prolabo,UK	443083G
32	Tris base	Fisher, UK	BPE152-1
33	Triton X100	Sigma,UK	T8787
34	Tween 20	Sigma,UK	P9416
35	X-Ray film	Fuji Films,UK	Super Rx

Table 2: **Suppliers of chemical and general laboratory reagents.**

6.1.2 Media and other biological materials

Cell culture media and other biological materials used for this study and their suppliers are listed in table 3

No	Materials	Suppliers	Product code
1	Roswell park memorial institute-1640 (RPMI-1640)	Sigma,UK	R1383
2	Dulbecco's Modified Eagle's Medium (DMEM)	Lonza,UK	BE12-604F
3	Foetal calf serum (FCS)	Lonza,UK	14-416F
4	Bovine serum albumin	Roche,UK	10711454001
5	Penicillin/Streptomycin	Lonza,UK	DE17-602E
6	Dextran Charcoal Coated medium (DCC)	(Hycloine)	SH30068.03

Table 3: **Suppliers of media and other biological materials.**

6.1.3 Enzymes, antibodies and kits

The enzymes, antibodies and ready-made kits used for this study and their suppliers are listed in table 4**.**

No	Products	Supplier	Catalog No
1	Actin	Abcam,UK	Ab8227
2	Annexin V kit	Ebioscience,UK	08. 12 29
3	Bax	Sigma,UK	SC-493
4	Beclin-1	Cell signalling,UK	AB15417
5	Bim	Abcam,UK	Ab15184
6	Bio-Rad protein assay kit	Bio-Rad,UK	500-0001
7	Cleaved caspase 3	Cell signalling,UK	Ab1871
8	Gilz	Santa Cruz Biotechnology,UK	SC-33780
9	H300 (total GR)	Santa Cruz Biotechnology,UK	SC-8992
10	LC3	Cell signalling,UK	ABC232
11	Mcl-1	Abcam,UK	Ab2910
12	p21	Santa Cruz Biotechnology,UK	SC-397
13	p53 (DO-1)	Santa Cruz Biotechnology,UK	SC-126
14	Proteinase K	Sigma,UK	P2308
15	Reverse transcriptase	Life technologies, UK	12328-019
16	ribonuclease A	Sigma,UK	R4875
17	RNeasy plus mini kit	Qiagen,UK	74134
18	Ser15 p53phosphorylation	Cell signalling,UK	7846
19	Ser20 p53phosphorylation	Cell signalling,UK	9287
20	Ser211GR phosphorylation	Cell signalling,UK	4161S
21	Ser226 GR phosphorylation	Abcam,UK	ab53692
22	Stabilized goat anti-Mouse HRP conjugated	GE Healthcare,UK	32430
23	Stabilized goat anti-Rabbit HRP conjugated	GE Healthcare,UK	32460
24	Taq DNA polymerase	Life technologies,UK	18038042

Table 4. **Suppliers of antibodies, enzymes and ready-made kits list.**

6.1.4 Buffers

All the buffers that used in this study are listed in table 5.

No	Buffers	Composition
1	0.1%PBS tween	PBS+0.1% Tween-20
2	3XSDS sample buffer	187mM Tris, 30% Glycerol, 6% SDS,15% 2-mercapto ethanol, 0.01% bromophenolblue
3	Chip blocking buffer	0.5% Bovine serum albumin (BSA) w/v phosphate buffer saline (PBS)
4	Chip buffer 1	50mM HEPES-KOH;pH7.5, 140 mM NaCl, 1mM EDTA,10% glycerol, 0.5% Igepal, 0.25% Triton X-100.
5	Chip buffer 2	10 mM Tris-Hcl; pH 8.0, 200 mM NaCl, 1mM EDTA, 0.5mM EGTA.
6	Chip buffer 3	10 mM Tris-Hcl; pH 8.0, 100 mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.1% Na- Deoxycholate, 0.5% N-lauroylsarcosine,
7	Chip elution buffer	50 mM Tris-Hcl; pH 8.0, 100 mM NaCl, 1% SDS w/v
8	Chip RIPA buffer	50 mM Tris-Hcl; pH 7.5, 500 mM LiCl, 1mM EDTA, 1% Igepal , 0.1% Na- Deoxycholate.
9	Formaldehyde solution	50mM HEPES-KOH, 100 mM NaCl, 1mM EDTA, 0.5mM EGTA, 11% formaldehyde.
10	High salt lysis buffer (HSLB)	45mM HEPES; pH 7.5, 400mM NaCl, 1mM EDTA, 10% Glycerol, 0.50% NP-40, 1mM DTT, 1mM PMSF, 1XT Protease inhibitor (PI), 2mM Sodium Orthophosphate (NaOV), 20mM β-Glycerol phosphate(β-GP), 5mM Sodium Pyrophosphate (NaPPi)
11	Phosphate buffer saline (PBS)	170mM NaCl,3.3mM KCL,1.8mM Na2HPO4, 10.6mM KH2PO4; pH7.4.
12	Stripping buffer	100mM 2-Mercaptoethanol, 2% SDS, 65.5mM Tris Hcl; pH 6.7.
13	Tris buffered saline (TBS)	20mM Tris-Hcl; pH 7.6, 150mM NaCl.
14	Western blocking solution	5% Milk in PBS, 0.1% Tween-20

15	Western transfer buffer	150mM Glycine, 25mM Tris-HCl; pH 8.3, 20% Methanol.

Table 5: **Buffers and their compositions**.

6.1.5 Cell signaling inhibitors list

All the inhibitors used in this study are listed in table 6

Pathway inhibitor	**Symbol**	**Supplier**
p38MAPK inhibitor	SB203580	Sigma,UK
JNK inhibitor	SP600125	Sigma,UK
PI3K inhibitor	LY294002	Cell signaling,UK
RIP-1 inhibitor	Necrostatin-1	Sigma,UK

Table 6: **Signalling pathway inhibitors list**.

6.2 Cell culture

6.2.1 Cell line and culture conditions.

The two cell lines were used through all the experiments of this research. They were CEM-C1-15 cells which is acute lymphocytic leukaemia cells that is resistant to glucocorticoid therapy and CEM-C7-14 which is glucocorticoid sensitive acute lymphocytic leukaemia cells line.The variations in the GC resistance of these cell lines provides a suitable system to study the differences between the two cell lines in terms of resistance mainly in gene expression. CEM cells were derived from the parental line CCRF-CEM, were chemically mutagenesis and then grown in Dex to select resistant clones (Medh *et al.*, 1998). Then CEM-C1-15 was recloned and isolated using semisolid agarose medium. CEM clones were expressed GR sites per cell. After recloned CEM-C1-15, both cell clones were treated continuously with Dex to develop affinity for Dex sensitive and Dex resistant, respectively (Medh *et al.*, 1998). In addition to that, CEM cell lines have two identical heterozygous mutations located in codon 175 (Arg-->His) (CGC-CGC/CAC) and in codon 248 (Arg-->Gln) (CGG- CGG/ CAG) which prolonged the half-lives of p53 in these cells (Cheng and Haas, 1990; Massumi, Ziaee and Sarbolouki, 2006).

K562 is an additional cell line which is chronic myeloid leukaemia cell line that is used to assess the effectiveness of glucocorticoid treatment. All cell lines cells were cultured in 10 ml Roswell park memorial institute-1640 (RPMI-1640, Sigma,UK)

medium supplemented with 10% foetal bovine serum (Sigma,UK) and 1% penicillin and streptomycin (Sigma,UK).The CM was generated from HS5 cell line which is human marrow stromal cell that sustain hematopoeisis (Torok-Storb *et al.*, 1999).

Cell line cultures were maintained in humidified incubators at 37^{o}C with a 5% CO2 atmosphere. Conditioned medium has been gifted from Prof. Vaskar Saha, from the Paterson Institute for Cancer Research. Also, Dextran Charcoal Coated medium (DCC) (Hycloine) was used before treatment with dexamethasone as small molecule in the serum were demonstrated to interfere with the dexamethasone and glucocorticoid receptor interaction and are absorbed by the charcoal (Chen, 1967).

6.2.2 Maintenance of cell culture

Cells were maintained in 75cm^2 vented tissue culture flasks and subcultured every 2-3 days when they were 70-80% confluent after centrifugation in RT as 2000 rpm for 3 minutes (Labofuge 400, Heraeus instrument).

6.2.3 Cell counts

The aim of counting the cell is to have appropriate number of cells for subculturing them in order to provide optimal cell density and consistency for future experiments.

Cells were counted by the haemocytometer (Bright-line, Hauser scientific, USA) covered by quartz coverslip (22 X 22 mm, scientific laboratory supplies). The total number of cells was counted in the four large corner squares. The cell concentration was calculated as:

Cell concentration per millilitre =

$$\text{Total cell count in 4 squares} / 4 \times \text{dilution factor} \times 10^{4}$$

6.2.4 Freezing and thawing of suspended cells.

The cells were pelleted by centrifugation at 2000rpm for 3min at room temperature. Then, the pellet was resuspended in 1 ml FBS and 1 ml of 20% FBS in DMSO as drop wise addition as drop wise to avoid osmotic shock (DMSO) will prevents the formation of intracellular ice crystals. Finally, cells suspension was aliquoted into cryovitals, transferred to -80 C^{o} for overnight before transfer to liquid nitrogen (-180).

For thawing the cells, 1ml of warm RPMI media was added to the frozen cells rapidly to avoid formation of ice crystals during the rehydration (Ryan, 2004). Then, 14 ml of warm RPMI medium (containing 10% FBS and 1% penicillin/streptomycin) was added to 25 cm^2 flask and the 1 ml cells suspension transferred to the flask. The flask incubated in the $37C^o$ incubator with 5% CO2 for 6 hours. The media changed to fresh media after 6 hours of incubation to avoid the cytotoxic effect of the DMSO on the cell. Then the cells maintained when they reach 70-80% confluence in $75cm^2$ flasks.

6.2.5 Cytotoxic stress condition

Cells were treated with different compounds, combinations and timepoints depending on the experiment. Most of the experiments were based on using dexamethasone and etoposide (Sigma–Aldrich). In viability test which was performed using trypan blue to determine the cytotoxic effect of the drug; mitoxantrone (MIT) and doxorubicin (DOX) were used as well beside etoposide. The reason of using 3 chemotherapy compounds is to determine the right compound for all the future experiment in this study. Mitroxantrone was a gift from Prof. Vaskar Saha from Paterson Institute for Cancer Research and Doxorubicin was a gift from Prof. Andy Sharocks, the University of Manchester.

Dexamethasone (GC) and etoposide (topoisomerase II inhibitor) were used then together to mimic the current treatment of the leukaemia. Dexamethasone induced apoptosis and etoposide induced DNA strands breaks and induce p53 (Zhou *et al.*, 1999).

To examine the microenvironment-mediated drug resistance in which tumour cells are transiently protected from apoptosis induced by chemotherapy, condition media (CM) was generated from Bone marrow stromal cells (BMSC). CM generation is clarified in section 6.8.1.

After fresh preparation of the drugs, they were added directly to the media to have a final concentration shown in table 7.

Compound	Purpose	Final concentration
Dexamethasone (Dex)	Induce apoptosis	1μM
Etoposide (Etop)	Induce p53	10μM
Mitoxantrone (Mit)	Induce p53	1μM
Doxorubicin (Dox)	Induce p53	10μM
CM generated from BMSC	Induce resistance	9 parts of CM against 1part of FBS DCC RPMI media

Table7: **The final concentration of the cytotoxic stress conditions.** Table shows the concentrations of the treatments that were used to induce cellular stress, concentration and the reason of using each regimen.

6.3 Cell death

6.3.1 Viability test using trypan blue

Haemocytometer-based trypan blue dye exclusion assay was used to measure the percentage of viable (live) cells in the cell suspension.

The percentage of viable cells was calculated as: Viable cells (%) =

Total number of viable cells per ml / total number of cells per ml X 100.

6.3.2 Flow cytometry

Fluorescence activated cell sorting (FACS) is a technique to sort cells according to their various properties depending on the individual dye or staining protocol used. Flow cytometer is an instrument that detects light-absorbing or fluorescing properties of cells passing through a narrow laser beam.

Cells were split into 3ml DCC-FBS RPMI media with cell count less than $1x10^6$. Cells were treated for different times before collection. At the end of treatment, Pellet was washed with cold PBS buffer. 1 ml of cold 50% Ethanol/PBS was added drop-wise whilst vortexing gently and then samples were incubated at -20°C for 30 minutes or alternatively placed at 4°C and then stored for up to a week. Cells were centrifuged and supernatant was removed completely. 200ul of 125U/ml ribonuclease A (Sigma,UK) and 800 ul of 50μg/ml Propidium Iodide (PI, Sigma,UK) staining solution was added and incubated at 37°C in the dark for at least 30 minutes before cell cycle analysis. Cell cycle analysis was performed using the Cyan flow cytometer

(Dako). The representations and percentages of cell cycle phases were analysed by Summit software.

6.3.3 Annexin V binding assay

Annexin V:FITC is used to measure changes in plasma membrane by binding to phospholipid phosphatidylserine (PS). PS is exposed at the cell surface during the early stages of apoptosis. PS is a very sensitive method for detecting cells entering apoptosis. This protocol is designed to measure apoptosis easily and quickly in a sample of suspended cells.

Cells were split into 3ml DCC-FBS RPMI media with cell count less than$1x10^6$ cells/well. Cells were treated for different times before collection. Then, cells were resuspended in binding buffer which was diluted 1:4 in distilled water (50ml binding buffer and 150 ml distilled water). 5ul of Annexin V:FITC was added to 195ul of the cell suspension and mixed well and was incubated 10 minutes in the dark, at room temperature. Cells were washed again and resuspended in 190ul prediluted binding buffer. 10ul of the Propidium Iodide solution was added to the suspended cells. Finally, cells were analysed by Cyan flow cytometer (Dako). The representations and percentages of cell cycle phases were analysed by Summit software.

6.3.4 Monodansylcadaverine (MDC) staining autophagy

After treatment of cells with specific compounds for specific time points, cells were counted, centrifuged for 3 min in 2000 rpm and washed with cold PBS. Then, the cells were labelled with 0.05 mM MDC in PBS at 37^oC for 10 minutes. After incubation, cells were washed 2-3 times with PBS and immediately analyzed for flow cytometry. Autophagy analysis was performed using the Cyan flow cytometer (Partec). The representations and percentages of cell cycle phases were analysed by Summit software.

6.4 Immunoblotting

6.4.1 Preparation of whole cell extract

Suspension cells were counted by haemocytometer and approximately $1x10^6$ cells were split into 6 wells containing 3 ml DCC-FBS RPMI media each, and treated with specific compounds for specific times. Suspended cells were harvested in 120µl high salt lysis buffer which is prepared according to (table 8 showed details of HSLB). Then, centrifuge tubes were placed on a rotator for 20 minutes at 4^oC, followed by

centrifugation at 13,000rpm for 15 minutes at 4°C. Supernatant was transferred carefully to a new centrifuge tube placed on ice. Protein concentration was determined by reading absorbance at 595 nm by spectrophotometer using the Bradford assay.

High salt lysis buffer	**Stock**	**Final**	**For 10ml**	
HEPES pH 7.5	1M	45mM	450µl	In 100 ml of distilled water
NaCl	5M	400mM	800µl	
EDTA	0.5M	1mM	20µl	
Glycerol	100%	10%	1ml	
NP-40	100%	0.50%	500µl	
DTT	1M	1mM	10µl	Take In 10 ml from 100ml
PMSF	100Mm	1mM	100µl	
1µg/ml of each Aprotinin, Leupeptin, Lepstatine (Protease inhibitor (PI))	N 1000x	1xT	10µ1	
Sodium Orthophosphate (NaOV)	1M	2mM	20µl	
β-Glycerol phosphate	500mM	20mM	400µl	
Sodium Pyrophosphate (NaPPi)	200mM	5mM	250µl	

Table 8. **High Salt Lysis Buffer**. High Salt Lysis Buffer contents which was used to harvest the cells for western blotting.

6.4.2 Bradford assay

Bradford assay is a colorimetric assay for measuring total protein concentration. It is based on using Bio-Rad protein assay Reagent. The Bio-Rad working solution was prepared as: 800µl of distilled water, 200µl of Bradford reagent (Bio-Rad) and 2µl of protein extract were added in a cuvette (Fisherbrand, UK) and mixed gently to avoid air bubbles. 2µl of distilled water was used as blank to calibrate the spectrophotometer. The Absorbance was measured at 595 nm using a light spectrophotometer (JENWAY, Genova). Protein concentration determination for

sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is calculated as:

Lowest absorbance reading = 40 µl of protein extract. Therefore

(Lowest absorbance reading = 40) = X

X/ Sample absorbance = amount of protein to be used.

Into clean centrifuge tubes, 3XSDS (table 9) loading buffer which is equal to half the volume of the protein extract was added to calculated protein extracts and either analysed immediately or frozen at -20^{o}C.

3XSDS sample buffer	**Stock**	**Final (1x)**	**Per 10ml (3X)**
Tris pH 6.95	1M	62.5mM	1.87ml
Glycerol	100%	10%	3ml
β-Mercapthoethanol	100%	5%	1.5ml
SDS	10%	2%	0.6g
H_20			3.03 ml (to bring up to 10 ml)
Bromophenol blue (sigma)			Small amount to give dark blue color.

Table 9: **3XSDS sample buffer contents.** The solution was prepared as shown in the table and stored at -20^{o}C.

6.4.3 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins according to their size in an electric field. SDS is an anionic detergent, it denatures the proteins by covering around the polypeptide backbone and which conferring the negative charge and allow proteins to move towards the positive electrode in an electrical field according to the polypeptide mass only. A 7.5%, 10 % and 15% of resolving gel were prepared according to the molecular weight of the protein in interest table 10. The gel casting apparatus (Mini-PROTEAN 3-Bio-Rad, UK) was set up according to manufacturer's instruction. Once resolving gel polymerised, 0.1% SDS was removed before pouring the stacking gel. Stacking gel was prepared according to table 10 and poured and 1.5 mm wide well combs was applied (Bio-Rad). After the stacking gel polymerised, the gels were fitted into

electrophoresis mini buffer tank (Bio-Rad) and then the tank filled with SDS-PAGE running buffer (SDS-PAGE running buffer was prepared according to table 11) and combs were removed to apply samples.

Protein samples were boiled for 3 minutes at 95°C, centrifuged 13,000 rpm for 1 minute at RT and loaded onto the gel using a Hamilton syringe. In the first well, 3 µl of the protein ladder was loaded as a molecular weight marker (Pageruler plus pre-stained protein ladder, Biolab, UK).

	7.5% gel		10% gel		15% gel	
Solutions	Resolving	Stacking	Resolving	Stacking	Resolving	Stacking
Water	13.3ml	6.73ml	10.94 ml	6.73ml	6.3ml	6.73ml
Acrylamide	7.0 ml	1.67ml	9.33ml	1.67ml	14ml	1.67ml
Tris pH 8.95 (1.5M)	7.0 ml		7.0ml		7.0ml	
Tris pH 6.95 (1M)		1.25ml		1.25ml		1.25ml
EDTA (0.2M)	280 µl	100µl	280 µl	100µl	280 µl	100µl
SDS (10%)	280 µl	100µl	280 µl	100µl	280 µl	100µl
APS (10%)	157µl	157µl	157µl	157µl	157µl	157µl
TEMED	17µl	17µl	17µl	17µl	17µl	17µl

Table 10: **Ingredients of 7.5%, 10% and 15% Polyacrylamide gels.**

7.5% gel which is used to detect proteins larger than 30kDa and 10% gel used to detect small molecular weight proteins and 15% to detect very small molecular weight.

1X SDS running buffer	Final	For 1Litre
Tris	25mM	3g
Glycine	190mM	14.4g
SDS	35mM	1g

Table 11: **1X SDS running buffer.** The buffer was prepared and stored at RT.

6.4.4 Western blotting and detection of protein

Western blot is used to detect one protein in a mixture according to the size of protein. This is done by using specific antibody against a desired protein.

A sandwich of filter paper, gel, nitrocellulose membrane (Millipore, UK) and one more layer of filter paper were prepared in a cassette, which was placed between platinum electrodes immersed in transfer buffer. 1x western transfer buffer was prepared according to table 12 and stored in room temperature.

1X Western transfer buffer	Final	For 1Litre
Tris	22mM	3.3g
Glycine	75mM	11.3g
Methanol	20%	200ml

Table12: **1x Western transfer buffer**. Ingredients of 1X Western transfer buffer to make 1 litre.

Transfer was performed at 0.4 amperes. Ice pack was used to avoid high temperature and changed half way through the run time which was 2 hours. Before adding primary antibody, membrane was blocked in 5% milk /PBS for 1 hour, to avoid any unspecific binding of antibodies to membrane. Nitrocellulose membrane was incubated with a primary antibody (1:1000) in 2.5% /PBS milk /Tween (0.1%) on a rocking platform overnight at 4^0C. Next day, membrane was washed 3 times with PBS/Tween (0.1%) for 10 minutes interval. Then, nitrocellulose membrane was incubated again with a secondary antibody, which was an antibody-enzyme conjugate. The membrane was incubated for 1 hour with 2µl anti-rabbit or anti-mouse IgG horseradish peroxidase conjugated secondary antibody dissolved in 10mls of 2.5% milk/PBS/Tween (0.1%)

and washed 3 times again with PBS/Tween (0.1%). Protein band were visualized by incubating the nitrocellulose membrane in chemiluminescent HRP substrate and visible bands can be captured on X-ray film (Fujifilm).

6.4.5 Densitometric analysis

Bands quantification was performed for some of the experiments using Image J 1.38e software. The phosphorylated proteins to total calculated as the intensity of the bands that correspond to the phosphorylated protein against total protein levels, normalized to the actin values.

6.4.6 Stripping the membrane

After detecting the first antibody in interest, the membrane is subjected to antibody stripping (table 5) .Membrane stripping is to allow unbind of the first primary antibody to allow the bind of the other primary antibody of close molecular weight. To remove the antibody, the membrane was incubated in the stripping solution which is prepared according to table 5 as 100mM 2-Mercaptoethanol, 2% SDS, 65.5mM Tris Hcl;pH 6.7 in 55^{o}C for 30min and washed three times with in PBS with 0.1% tween-20 . Then blocking buffer (table 5) was used for 1 hour in room temperature on rocking plate before incubating in primary antibody.

6.5 RNA extraction and qRT-PCR

6.5.1 RNA extraction

After treatment of cells with specific compounds for specific time points, cells were counted, centrifuged for 3 min in 2000 rpm and washed with cold PBS. Then, mRNA was extracted using the RNeasy plus mini kit (Qiagen, Catalogue number 74134) according to the manufacturer's guidelines. To remove any genomic DNA, gDNA eliminator was used and 70% ethanol was added to the flow through. RNA captured by RNAeasy column, which was washed with RW1 buffer and then washed again with RPE buffer (both buffers been included in the kit). The captured RNA in the RNAeasy column was eluted by 30µl of RNase free water. After RNA was extracted, a NanoDrop spectrophotometer (Thermo) was used to determine RNA concentration.

Eluted RNA concentration was determined by the NanoDrop spectrophotometer in duplicate.

6.5.2 Reverse Transcription

For reverse transcription, 1 ug of total RNA was primed with anchored oligo-dT primers (500ng/µl) (Thermo scientific) and incubated for 5 minutes at 70^{o}C, prior to adding remaining reagents (complete reaction listed in Table 13).

Component	Volume
RNA template	1µg
Anchored Oligo-dT (500ng/µl)	1µl
1st strand synthesis buffer (5x)	4µl
Reverse-iT Rtase blend	1µl
dNTP mix (10mM)	1µl
H_20	Up to 20µl

Table 13: **Reverse transcription reaction.**

6.5.3 qRT-PCR

Quantitative real time polymerase chain reaction (qRT-PCR) is a method for DNA amplification by thermocycles in which a fluorescent dye (SYBR Green) is used to detect the amount of PCR product in each well of the 96-well plate.

SYBR Green method (Sigma, UK) requires a double-stranded DNA dye in the PCR reaction which binds to newly synthesized double-stranded DNA and gives fluorescence (Figure 6.5.3).

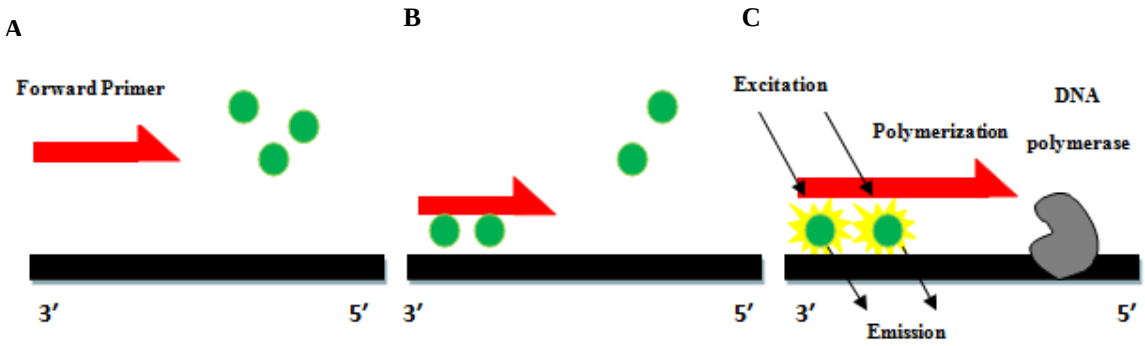


Figure 6.1.1: **SYBR Green principle**. (A) SYBR Green molecules are free. (B) SYBR Green binds to DNA and primers anneal. (C) Template elongated by DNA polymerase and the product forming resulting in fluorescence which is detected spectrophotometrically.

The cDNA of each sample was diluted 4 fold with RNease free water then serial dilution was made as 10%, 1% and 0%. Then, 4µl of the cDNA was added to 16µl of the mastermix, mixed and seeded as triplicates in 96 well plates (Bio-Rad). Components of mastermix are clarified in table 14. Reference gene Rpl19 (housekeeping) was used with each run.

Component	Company	Volume for 1x Reaction
Thermopol buffer (10x)	Biolabs	2µl
dNTP (10mM)	Bioline	0.4µl
MgCl2	Bioline	0.6µl
SYBR-green (1/2000)	Sigma	0.75µl
Taq DNA polymerase	Biolabs	0.2µl
Forward primer (50µM)	Eurogentec	0.06µl
Reverse primer (50µM)	Eurogentec	0.06µl
RNease free water	Sigma	11.93µl

Table 14: **qRT-PCR mastermix**. PCR set up. The contents are for 1 reaction

Finally, the PCR reaction was performed on a Chromo 4 machine (Bio-Rad) and the protocol is shown in table 15. Analysis was performed using Opticon monitor analysis software (version 3.1). All primers used were listed in table 15.

Parameters	Temperature	Time	Cycles
Initial denaturation	94^{o}C	10min	1
Denaturation	94^{o}C	30sec	35
Annealing	50^{o}C	30sec	35
Extension	72^{o}C	1min	35
Final Extention	72^{o}C	5min	1
Melting curves	60-90^{o}C, read every 1^{o}C	15sec	1

Table 15: **qRT-PCR protocol**.

Primers listed in table 16 were used to amplify cDNA.

qRT-PCR	F/R	Sequence	Supplier
Bax	F	TTTGCTTCAGGGTTTCATCC	Eurofins, UK
Bax	R	ATCCTCTGCAGCTCCATGTT	Eurofins, UK
Bim	F	GAGAAGGTAGACAATTGCAG	Eurofins, UK
Bim	R	GACAATGTAACGTAACAGTCG	Eurofins, UK
Mcl-1	F	TCAAAAACGAAGACGATGTGA	Eurofins, UK
Mcl-1	R	CAAAGGCACCAAAAGAAATGA	Eurofins, UK
p21	F	ATTAGCAGCGGAACAAGGAGTCAGACAT	Eurofins, UK
p21	R	CTGTGAAAGACACAGAACAGTACAGGGT	Eurofins, UK
RIP-1	F	GCACCAGCTGTCAGGGCCAG	Eurofins, UK
RIP-1	R	GCCCAGCTTTCGGGCACAGT	Eurofins, UK
Rpl19	F	ATGTATCACAGCCTGTACCTG	Eurofins, UK
Rpl19	R	TTCTTGGTCTCTTCCTCCTTG	Eurofins, UK

Table 16: **Primers list**.

6.6 Microarrays

Microarray technique is considered to be the most efficient technique for obtaining genome wide gene expression data in a short time with least number of experiments. First the cells are treated with certain compounds for a fixed time period and then they are centrifuged at 2000 rpm for three minutes followed by washing with cold PBS. Extraction of mRNA is the next step which is executed with RNeasy plus mini kit (Qiagen, Catalogue number 74134) and gDNA eliminator column. The extracted RNA is stored at -80^{o}C till it is supplied to the Core Facility in the Faculty of Life Sciences, Manchester University.

6.6.1 The Basic Steps of microarrays

Microarray technique works on the basis of hybridization probing. It is a method which employs nucleic molecules labelled with fluorescent substance as “mobile

probes" in order to detect complementary molecules and sequences capable of paring with each other. When two complementary sequences (like immobilized target DNA and mobile probe DNA, mRNA or cDNA) approach one another, their hybridization takes place.

Once the hybridization gets completed, the microarray is observed through a scanner or reader equipped with lasers, a specialized microscope and a camera. The laser excites the fluorescent tags, the microscope makes the phenomenon visible and the camera takes an image of the array. The collected data is stored in a computer system. Then specific program is employed for the computation of red-to-green fluorescence ratio or subtraction of background data for every spot on the microarray. Once these calculations are done, the program automatically generates a table comprising of ratios of the intensity of red-to-green fluorescence for each spot on the microarray.

6.6.2 The GeneChip Human Genome U133 plus 2.0 Array

The GeneChip Human Genome U133 plus 2.0 Array was chosen because of comprehensive analysis of genome-wide expression on a single array, complete coverage of the human genome U133 Set plus 6,500 additional genes for analysis of over 47,000 transcripts, great accuracy and reproducibility.

6.6.3 Microarray analysis

After treatment of cells with specific compounds, mRNA was extracted using the RNeasy plus mini kit and QIAshredder spin columns according to manufacturer's guidelines. GeneChip Human Genome U133 plus 2.0 Array was used to analyse the gene expression changes in CEM-C7-14 cells when treated with Dex, condition media and ETOP individually or in combination. Microarray background correction, quantile normalization, and gene expression analysis were performed using the robust multiarray average (RMA) in Bioconductor software package (Bolstad *et al*., 2003) Principal components analysis (PCA) was performed with Partek Genomics Solution (version 6.5, Copyright 2010, Partek Inc., St. Charles, MO, USA) to determine the significance of the array. Differential expression analysis was performed using the Limma package in the Bioconductor software to assess (Smyth et al, 2004) differential expression in microarray experiments. Tests were performed as two-way comparisons. Gene lists of differentially expressed genes were controlled for false discovery rate (fdr) errors using the method of QVALUE (Storey *et al*, 2003).

A genelist of differentially expressed genes (2632 unique genes) was created by filtering for probesets with a q-value less than 0.050 and fold change great than or equal to 2.0. This data set was segregated into 8 clusters based on similarity of expression profile across the dataset using a k-means clustering algorithm. Clustering was performed on the means of each sample group (log 2) that had been z-transformed (for each probeset the mean set to zero, standard deviation to 1). K-means clustering was done on the basis of similarity of profiles (Manhattan Distance) across the dataset using the "Super Grouper" plugin of maxdView software (available from http://bioinf.man.ac.uk/microarray/maxd/.

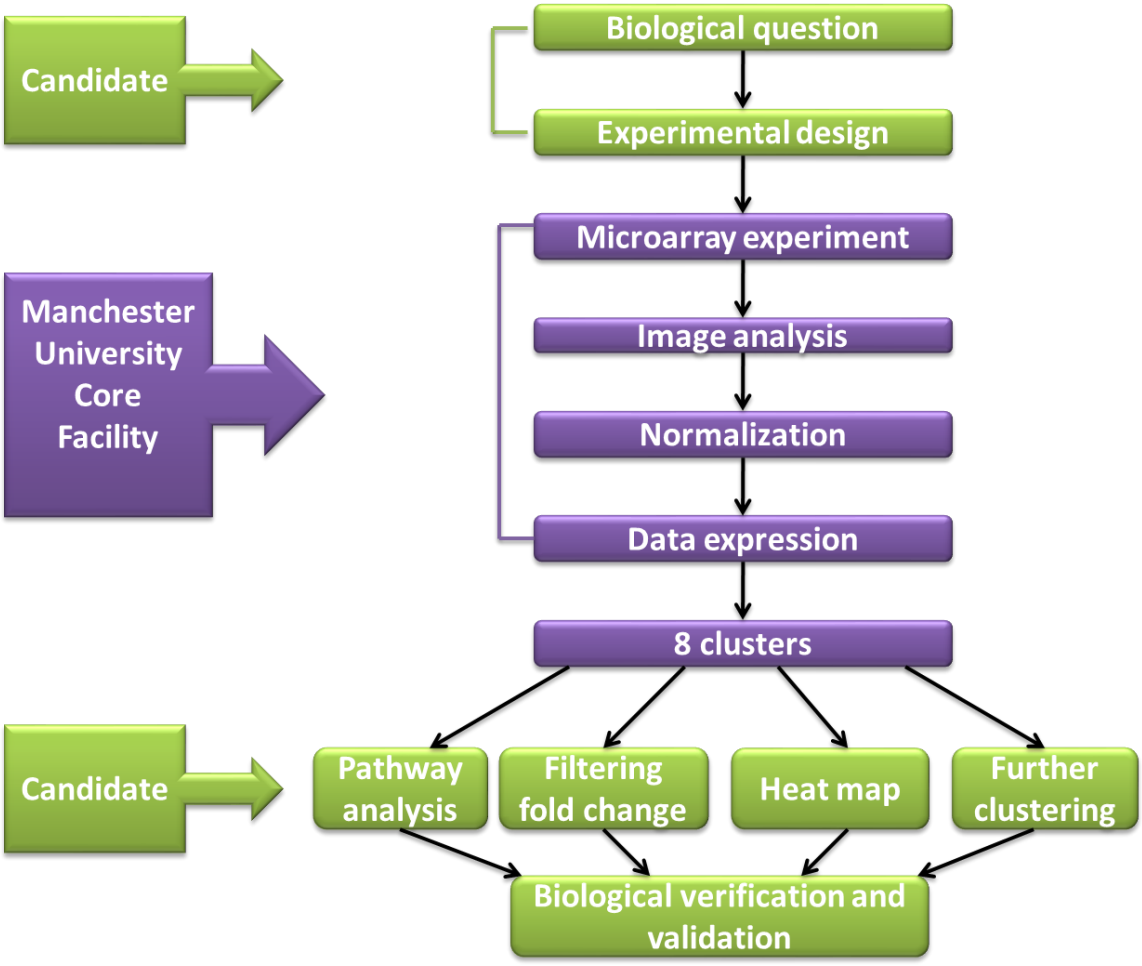


Figure 6.2.1: **Illustration about the analysis flow of microarray.** The purple work flow was done by University of Manchester core facility and the green work flow was done by the candidate.

6.7 Mesenchymal conditioned medium

Conditioned media is also called cell free culture supernatant. Conditioned media differ according to the cells it has been obtained from. A biological activity found in conditioned media is of unknown origin but most likely is from the growth factors and other soluble mediators that mimic in vivo conditions encountered by leukaemia cells

6.7.1 Bone marrow mononuclear cell isolation

HS5 cells which is Bone marrow stromal cell line (BMSC) was grown in RPMI-1640 medium supplemented with 10% foetal bovine serum (Sigma, UK) and 1% penicillin streptomycin (Sigma,UK) (Savasan S. *et al.,* 2005). The cells were grown to confluence and washed twice with cold PBS and then incubated with 15 ml of serum free RPMI 1640 at 37^{o}C from 24 to 48 hours with 5% CO_2. After 48 hours the cell-conditioned medium was collected, centrifuged at 1000 g for 10 minutes to remove cell debris and the supernatant stored at -80^{o}C as CM generated from BMSC.

7 Results

7.1 The cytotoxic effects of the clinically relevant compounds and stem cell microenvironment on leukaemia cells

7.1.1 Effect of clinically relevant compounds on leukaemia cell survival

Determination of the optimal dose and duration of glucocorticoid treatments of leukaemia cells

Glucocorticoids exert a wide range of physiological effects, including the induction of apoptosis in lymphocytes, which makes synthetic glucocorticoids frequently used therapeutics in the treatment of hematological malignancies (Smith and Cidlowski, 2010). The molecular mechanisms governing cancer cell death in response to chemotherapy in the tumor microenvironment have not been extensively studied. However, one of the major challenges in treating cancer is resistance to therapy (Saha and Kearns, 2011). In case of leukemia, resistance is mediated by several factors secreted from stromal cells of the bone marrow. We wanted to shed light on the factors secreted from the stromal cells from the bone marrow which might mediate resistance to treatment. The cells that used for the experiments are glucocorticoid sensitive and resistance in which the glucocorticoid sensitive cells are known as its capability to undergo apoptosis due to steroid treatment rather than the glucocorticoid resistant cells.

The purpose of this part of the study was to determine molecular basis of the DNA damage and GC response of steroid sensitive and resistant ALL cells and alteration of these responses when cells are exposed to microenvironmental factors. For this purpose the glucocorticoid-induced apoptosis-resistant CEM-C1-15 and -sensitive CEM-C7-14 ALL cells, as well as the glucocorticoid-resistant K562 acute myelocytic leukemia cell line were grown in conditioned media prepared as described in the Materials and Method (6.7.1) secreted by stromal cells from bone marrow. Since the treatment leads to cell death, it was important to measure the viability of the cells after the treatment and to assess the concentration, the duration of the treatments and the most effective dose of anthracyclin in order to establish the conditions for future experiments. Initially, the simple and rapid trypan blue viability test was employed. Trypan blue was used to measure the percentage of viable (live) cells in the cell suspension. Treated cells were mixed with an equal volume of 0.4% trypan blue dye and counted using a

hemocytometer. Viable or intact cells are not stained by trypan blue whereas dead cells are stained blue due to the fact that their damaged membrane permits the dye to enter and stain them.

The synthetic steroid compound dexamethasone (Dex), and the three anthracyclines etoposide (ETOP), doxorubicin (DOX) and mitoxantrone (MIT) were tested to determine the concentration and incubation time needed to induce apoptosis. The most effective anthracyclin was chosen to treat the cells.

Above mentioned leukemia cells were treated with different concentrations of Dex ranging from 1nM to 10µM for three different incubation periods (24hrs, 36hrs and 48hrs) to assess cell type dependent effects and distinguish them from glucocorticoid specific effects. The results showed that the onset of cell death in the sensitive and resistant cells occurs gradually 36 hours after addition of various concentrations of Dex (Figure 7.1.1 B, black and grey bars). CEM-C7-14 cells treated with different concentrations of Dex showed the highest sensitivity at 36 hours (Figure 7.1.1 B, grey bars) compared to 48 hours (Figure 7.1.1 C, grey bars) and 24 hours (Figure 7.1.1 A, grey bars) of Dex treatment.

CEM-C1-15 cells were less sensitive and K562 cells were the least responsive to glucocorticoids (Figure 7.1.1, compare black and white bars to grey bars in A, B and C). The 36 hour and 48 hour treatment yielded similar results at 1µM and 10µM dose and therefore 1µM Dex and 36 hour treatments were chosen as optimal and used in all future experiments.

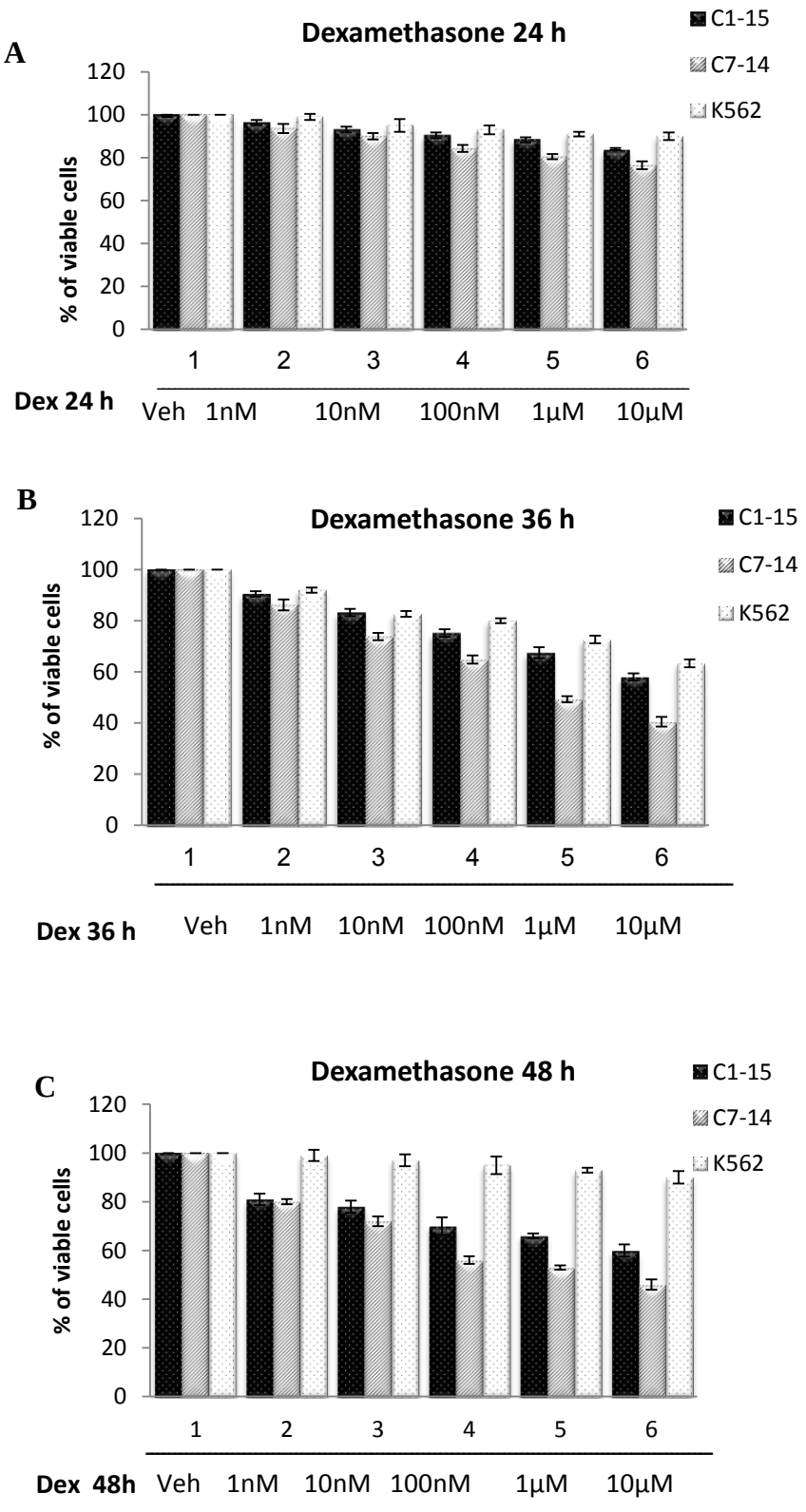


Figure 7.1.1: **Cytotoxicity curves of CEM and K562 cells.** Growth curves for CEM-C1-15 (black), CEM-C7-14 (grey) and K562 (white). Cells treated with different concentrations of Dex for (A) 24 hrs. (B) 36hrs and (C) 48 hrs were shown. Cell viability was determined using trypan blue exclusion and a hemocytometer and results shown are representative 3 independent experiments. Error bars represent standard deviation.

Determination of the optimal type, dose and duration of anthracyclines' effect on leukaemia cell death

CEM-C1-15 and CEM-C7-14 cells were also compared with regard to their relative sensitivities to etoposide (ETOP) (Figure 7.1.2 A), Doxorubicin (DOX) (Figure 7.1.2 B), and Mitroxantrone (MIT) (Figure 7.1.2 C). Cell viability gradually decreased in both cell lines in the presence of increasing concentrations of ETOP but CEM-C7-14 cells exhibited stronger response to ETOP after 24hrs treatment, ranging gradually from 94 % to 21 % viability (Figure.7.1.2 A, compare grey bars 2 to grey bars 6). High concentration of ETOP (10μM) caused a substantial reduction of CEM-C7-14 (21% viable) and CEM-C1-15 (34% viable) cell viability. Increased cell death with higher concentration and longer duration of the etoposide treatment was observed (Figure.7.1.2 A and B).Cells lines treated with MIT showed gradual decrease in cell viability in sensitive and resistant cell lines compared to untreated cells (Figure.7.1.2 B, compare black and grey bars to 1). Notably, CEM-C7-14 cells were more sensitive to MIT compared to CEM-C1-15 cells. Cell viability ranged from 90 to 62% in CEM-C7-14 and from 95 to 74% in CEM-C1-15 cells implying differential dose dependent cell viability in the GC sensitive compared to resistant cell lines (Figure.7.1.2 B).

Gradual decrease in leukemic cells viability with increasing concentration of DOX for 24 hrs was recorded (Figure.7.1.2 C). The number of viable glucocorticoid resistant CEM-C1-15 cells ranged from 90 to 54% (Figure 7.1.2 C, compare black bars 2-6 to black bar 1). The number of viable CEM C7-14 ranged between 84% to 40% following inversely the increase of DOX concentration (Figure.7.1.2 C, compare grey bars 2-6 with grey bar 1).

To further investigate the three of the anthracyclin agents, LC_{50} of each drug was measured for each cell line. LC_{50} is defined as the concentration of the drug that is lethal to 50% of leukemia cells (Pieters *et al.*, 1990). The aim of determining LC_{50} was to measure the short-term actual toxicity of the treatment. A dose response curve based on trypan blue viability assay was drawn and LC_{50} calculated using a best-fit line passing through most of the values. The point where the dose response curve crosses the 50% leukemia cell survival point was considered as LC_{50}. LC_{50} of ETOP, DOX and MIT were estimated as 1μM, 5μM and 3 μM, respectively (Figure.7.1.2. D. E. and F).

ETOP displayed a significant decrease in ALL cell viability, which was higher in CEM-C7-14 cells compared to CEM C1-15 (Figure 7.1.3 A, compare grey to black bars). MIT

and DOX exhibited weaker effect compared to that of ETOP (Figure 7.1.3, compare B and C to A). For this reason ETOP was chosen to work with throughout this study. In summary, 1 µM Dex for 36 hrs and 10 µM ETOP for 24 hrs treatment was chosen as optimal dose and duration and used in all subsequent experiments.

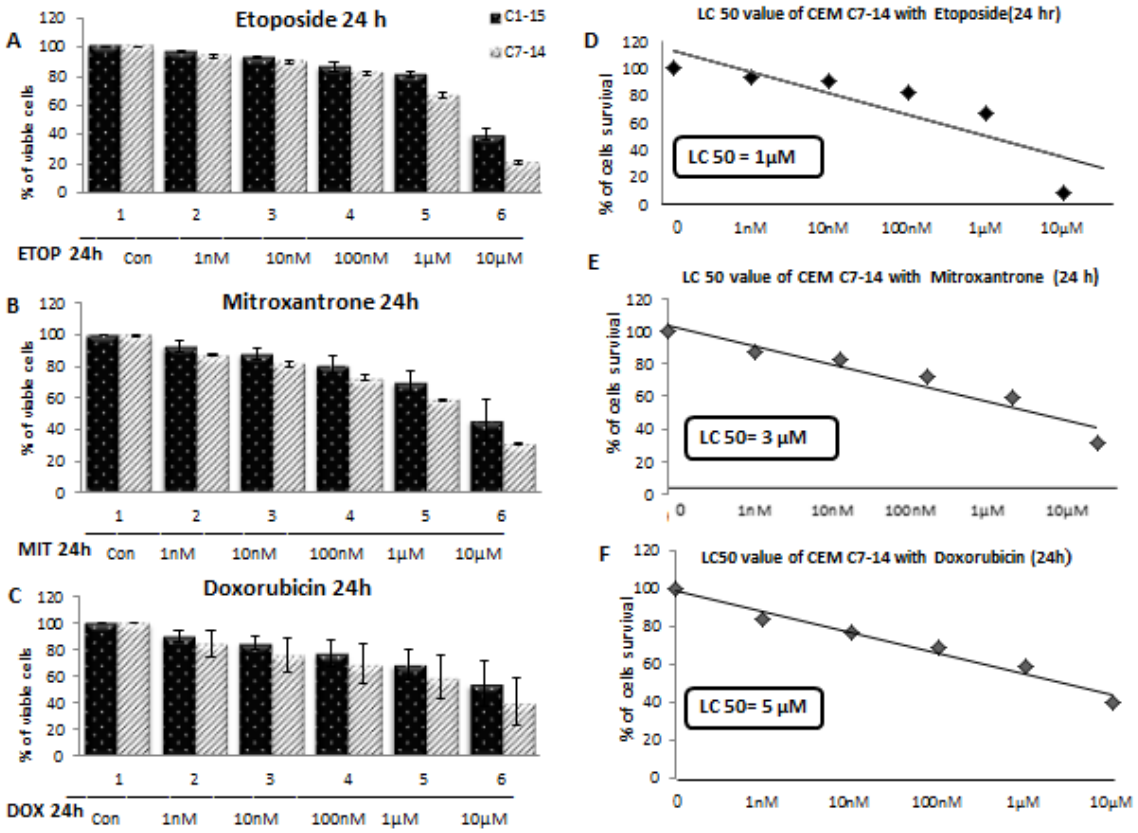


Figure.7.1.2: **Cytotoxicity curves of CEM cells.** Growth curves for CEM-C1-15 (black bars), CEM-C7-14 (grey bars) treated for 24hrs with different concentrations of etoposide (ETOP) (A). Mitroxantrone (MIT) (B) and Doxorubicin (DOX) (C). LC $_{50}$ values of CEM C7-14 cells for ETOP (D), DOX (E) and MIT (F) cells counted by trypan blue and hemocytometer as described in material and method. Results are representative of 3 independent experiments. Error bars represent standard deviation.

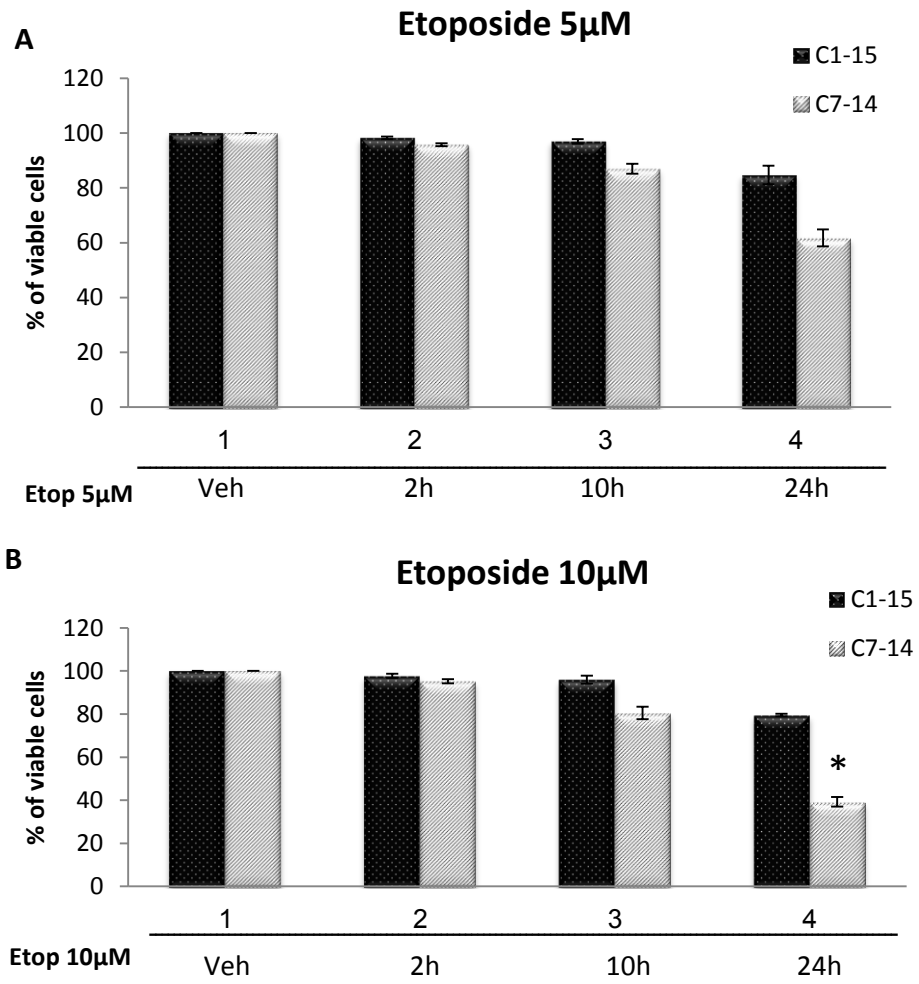


Figure 7.1.3: **Time course and dose dependency of Etoposide effect on ALL cells.** Growth curves for CEM C1-15 (black bars), CEM C7-14 (grey bars) treated for indicated times with 5µM (A) or 10µM (B) Etoposide. Cell viability was determined using Trypan blue assay and a hemocytometer. Results are representative of 3 independent experiments. Error bars represent standard deviation. Error bars indicate ± standard deviation. Asterisk indicates a significant difference at $P < 0.05$.

The effects of microenvironment and combined treatment on leukaemia cell viability

To determine the effects of dexamethasone and etoposide alone or in combination on leukemia cells viability CEM-C1-15 and CEM-C7-14 cells were left untreated or treated with dexamethasone (1µM) 36hrs and etoposide (10µM) 24hrs either alone or in combination. The role of the microenvironment in promoting drug resistance was tested using conditioned media (CM) generated from bone marrow stem cells (as described in the Materials and Methods 6.7.1) to mimic the conditions of growth environment in the niche and cell viability was assessed with the trypan blue assay (Figure 7.1.4).

Dex and ETOP combined treatment was more effective in inducing leukemia cell death

compared to individual treatment with Dex or ETOP alone (Figure 7.1.4 compare bars 7 with 3 and 5). Addition of CM increased slightly the number of viable cells (Figure 7.1.4 compare black and grey bars 3 ,5 and 7 to black and grey bars 4,6 and 8 respectively) indicating the potential role of CM in conferring resistance to leukemia cells. To test whether glucocorticoid or p53 signaling was preferentially affected by CM, leukemia cells were treated with Dex and ETOP in the presence or absence of CM or combination of Dex, ETOP and CM. The results shown in Figure 7.1.4 indicate that CM inhibited the glucocorticoid induced cell death (compare black and grey bars 3 to black and grey bars 4), whereas the effect of CM on ETOP was marginal (compare black and grey bars 5 to black and grey bars 6). The effect of CM on GR dependent signaling was more prominent in the resistant CEM-C1-15 compared to CEM-C7-14 (Figure 7.1.4 compare black bar 3 to black 4 and grey bar 3 to grey bar 4) while the effect of CM on the combination of Dex and ETOP was not significant (Figure 7.1.4, compare black and grey bar 7 to black and grey bars 8). These results suggest that CM affects both glucocorticoid and p53 pathways and possibly contributes to drug resistance in a cell dependent and type of treatment dependent manner through unknown mechanisms.

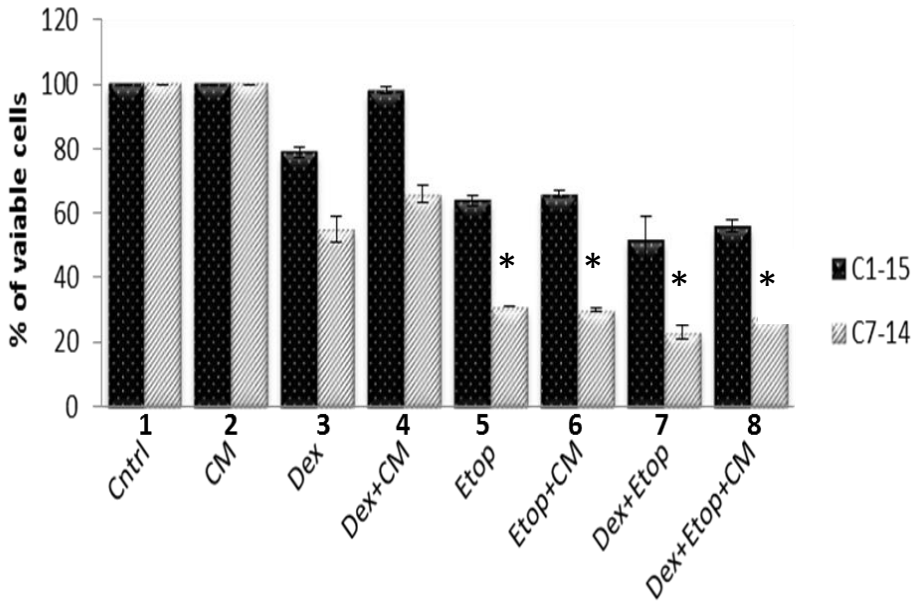


Figure 7.1.4: **Cytotoxic effect of combined treatment of Dexamethasone, Etoposide and CM**. CEM-C1-15 (black bars)and CEM-C7-14(grey bars) cells were treated with 1µM Dex (36 hrs) and10µM ETOP (24hrs) and CM (48hrs) as indicated. Cell viability was determined using Trypan blue assay and a hemocytometer. Results are representative of 3 independent experiments. Error bars represent standard deviation. Histograms compare as (C1-15 Dex with C1-15 Dex+CM), (C7-14 Dex with C7-14 Dex+CM), (C1-15 ETOP with C1-15 ETOP+CM), (C7-14 ETOP with C7-14 ETOP+CM), (C1-15 Dex+ETOP with C1-15 Dex+ETOP+CM), (C7-14

Dex+ETOP with C7-14 Dex+ETOP+CM). Error bars indicate ± standard deviation. Asterisk indicates a significant difference at $P < 0.05$.

7.1.2 Analysis of ALL cell cycle progression and apoptosis

Although trypan blue viability test is a simple and rapid assay, it provides limited information in terms of mechanisms initiating cell death. Flow cytometry technique was chosen to study cell cycle progression and cell fate in detail and investigate whether cell death occurred through apoptosis, necrosis or autophagy.

In order to determine the effects of the microenvironment and drug treatments on cell cycle profiles of ALL cells we employed flow cytometry to determine population of cells in the different phases of the cell cycle (Figure 7.1.5). The data showed that sensitive CEM-C7-14 cells exhibited lower proliferation after combined treatment with Dex and CM or ETOP either alone or combination of all three treatments (Figure 7.1.5 D, compare grey bars to 4, 5 and 8 to grey bar 1). Similar effect was observed in G2/M cell cycle phase except that most marked effect was observed in cells treated with ETOP and CM (Figure 7.1.5 E, compare grey bars to 4, 5, 6 and 8 to grey bar 1). G1 phase was not significantly affected in either cell line (Figure 7.1.5 C, compare black and grey bars 2-8 to black and grey bars 1). This analysis revealed that CM enhances the Dex and ETOP antiproliferative effect on CEM-C7-14 cells and in particular the CM mediated increase in Dex antiproliferative effect is exerted in the S phase of the cell cycle, wherase that effect on ETOP was not prominent in G2/M, The trend suggesting that microenvironment affects selectively GR actions on S phase whereas potential action on ETOP induced actions was most obvious in G2/M phase of the cell cycle.

CEM-C7-14 treated with 1 μM Dex for 36 hrs demonstrated substantial increase in sub-G1 peak while CEM-C1-15 showed lower apoptotic rate (Figure 7.1.5 A and B, compare bars 3).

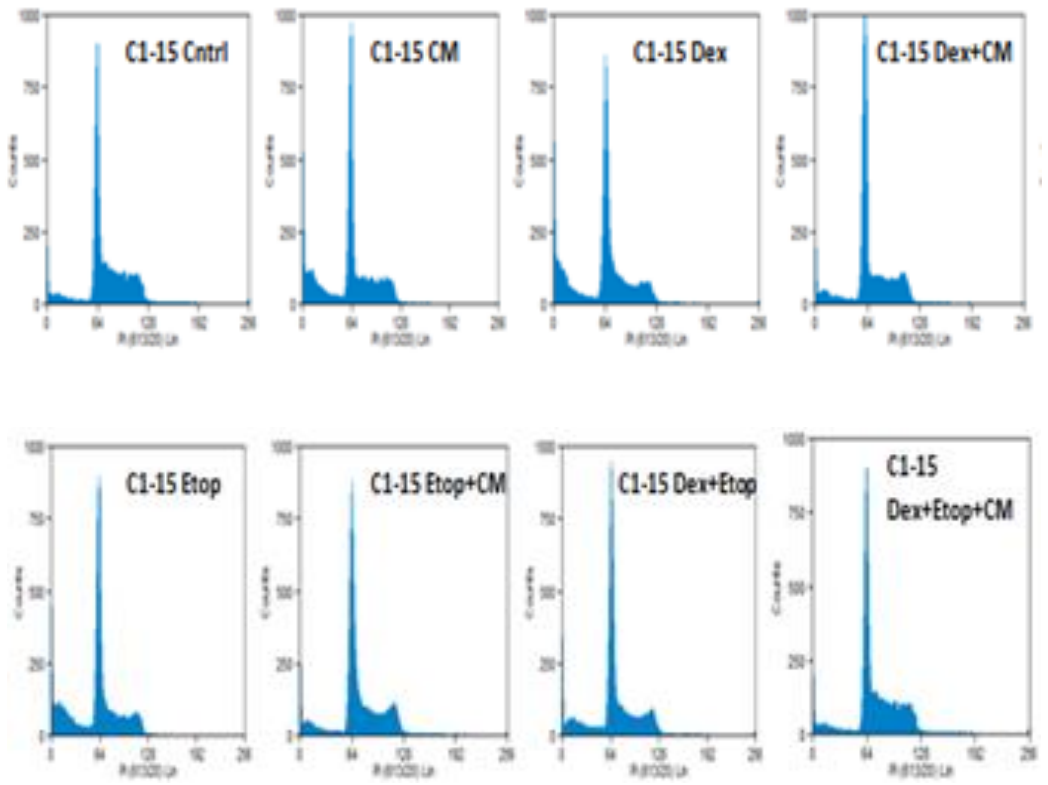
Combination of Dex and ETOP resulted in significant increase of the population of the CEM-C7-14 cells in the sub G1 phase compared to Dex and ETOP individual treatment (Figure 7.1.5 A and B, compare 3, 5 and 7). In addition, the population of the CEM-C7-14 cells in Sub G1 phase was higher than that of the CEM-C1-15 cells treated the same way (Figure 7.1.5 A and B, compare lane 5 of both cell lines).

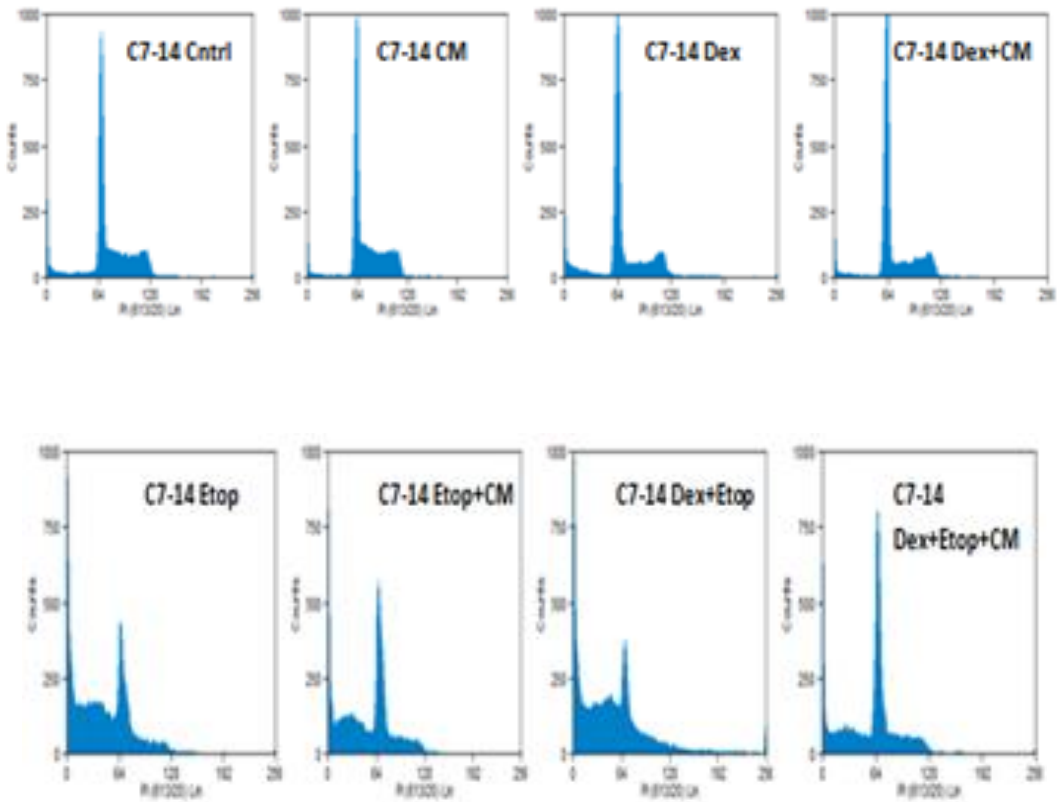
Decreased population in the sub G1 phase was observed after the addition of CM compared to non-treated cells. Reduced cell death in both cell lines was also detected in

Dex and ETOP treated cells after the addition of CM with more pronounced effect recorded in CEM-C7-14 cells (Figure 7.1.5 A and B, compare lane 7 and 8). Treatment with ETOP or Dex alone caused high percentage of cell death (Figure 7.1.5 A and B, compare lane 3 and 5), which was significantly reduced in cells treated with CM (Figure 7.1.5 A and B, compare profile and lane 3 and 4, 5 and 6 and 6 and 7).

Taken together these data showed that the addition of conditioned medium to ALL cells treated with Dex and ETOP increased cell viability as determined by two different essays implying that bone marrow derived soluble factors potentially confer resistance to leukemic cells through unknown mechanism(s).

A


GC resistant cells (C1-15)
C1-15 Cntrl
C1-15 CM
C1-15 Dex
C1-15 Dex+CM
C1-15 Etop
C1-15 Etop+CM
C1-15 Dex+Etop
C1-15
Dex+Etop+CM


GC sensitive cells (C7-14)
C7-14 Cntrl
C7-14 CM
C7-14 Dex
C7-14 Dex+CM
C7-14 Etop
C7-14 Etop+CM
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C7-14
Dex+Etop+CM

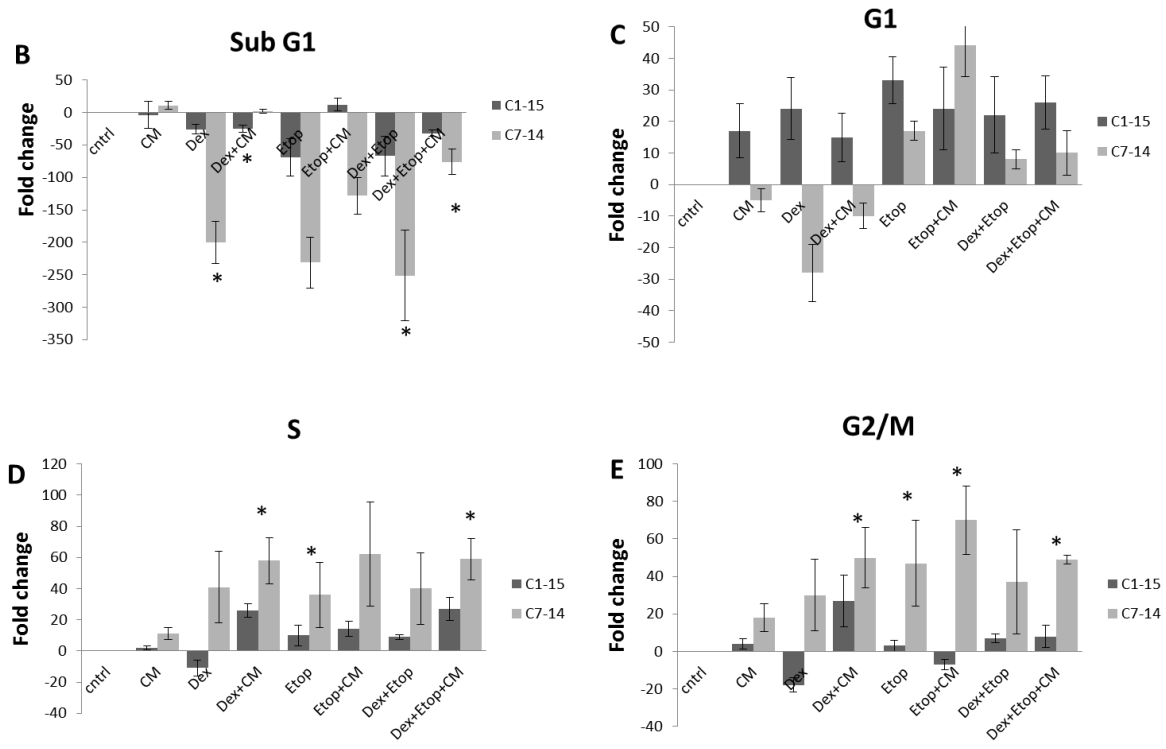


Figure 7.1.5: **Cell cycle pattern of CEM C1-15 and C7-14 cells.** Figure shows the effect of CM on CEM clone. (A) Shows the profile of the cell cycle CEM clone (B) shows the histograms of the sub G1 phase. (C) Shows the G1 phase. (D) Shows S phase. (E) Shows the G2/M phase. The cells were treated with of Dex (1μM) (36hrs) and Etop (10μM) (24hrs) and CM (48hrs). The results were analyzed by flow cytometry. Results are representative of 3 independent experiments that yielded similar data. Error bars indicate ± standard deviation. Asterisk indicates a significant difference at $P < 0.05$.

7.1.3 Analysis of the effect of CM on ALL cell death using Annexin V.

FACS analysis in combination with Annexin V staining was used to confirm that cell death detected with trypan blue and the percentage of cells in the sub G1 phase was apoptosis. An early event in apoptosis is phosphotidylserine (PS) eversion of the lipid from the inner to the outer surface of the cell membrane. PS can be detected by the fluorescence dye Annexin V

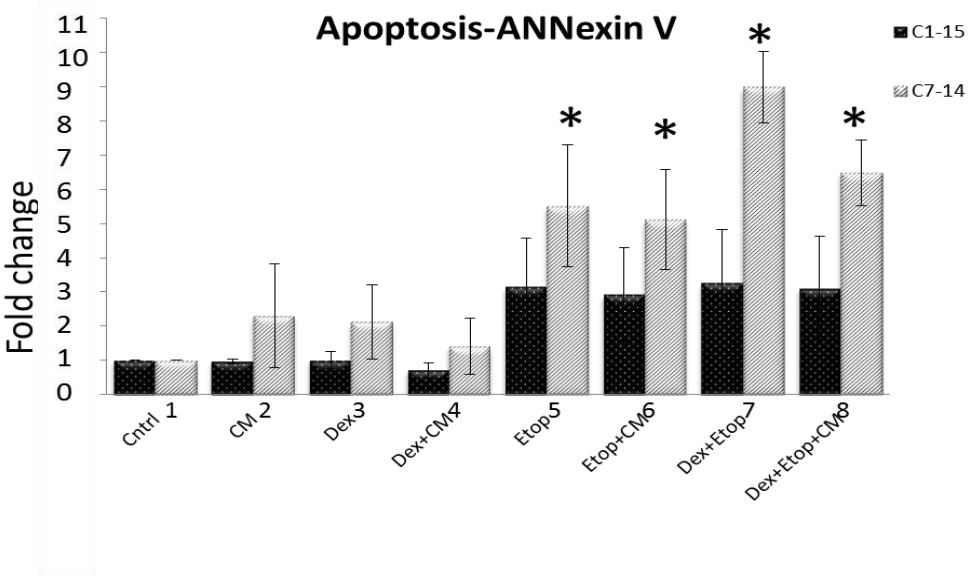


Figure 7.1.6: **FITC-Annexin V/PI flow cytometry of ALL cells.** CEM-C1-15(black bars) and CEM-C7-14 cells (grey bars) treated with1μM Dex for 36 h, 10 μM ETOP for 24 hrs and CM for 48hrs were stained with annexin-V FITC and PS eversion was assessed.Errors bars indicate ± standard deviation. Asterisk indicates statistically significant difference ($P < 0.05$), Histograms compare as (C1-15 Dex with C1-15 Dex+CM), (C7-14 Dex with C7-14 Dex+CM), (C1-15 ETOP with C1-15 ETOP+CM), (C7-14 ETOP with C7-14 ETOP+CM), (C1-15 Dex+ETOP with C1-15 Dex+ETOP+CM), (C7-14 Dex+ETOP with C7-14 Dex+ETOP+CM).

Varying degrees of apoptosis in leukemia cell lines were detected with FITC-Annexin V/PI in which Ann V-ve/PI-ve fraction represented viable cells; Ann V + ve/PI-ve fraction early apoptotic cells; Ann V + ve/PI + ve fraction late apoptotic; and Ann V - ve/PI + ve fraction represented dead cells.

Early and late apoptotic fractions (Ann V + ve/PI-ve and Ann V + ve/PI + ve) increased in the glucocorticoid sensitive CEM-C7-14 cells in response to Dex treatment whereas CEM-C1-15 showed limited response to Dex (Figure 7.1.6, compare grey bar 3 to grey bar 1). ETOP exerted stronger effect on CEM-C7-14 in early and late apoptosis, while CEM-C1-15 were less responsive as the cell viability increased in early and late apoptosis (Figure 7.1.6, compare grey and black bars 5 to grey and black bars1). Combination of Dex and ETOP displayed strong synergistic effect inducing robust early and late apoptosis in CEM-C7-14 cells and weaker effect on CEM-C1-15 cells (Figure 7.1.6,

compare grey and black bars 7 to grey and black bars 1).

Quantitation of early and late apoptosis of Dex treated CEM-C1-15 cells revealed reduction of the number of apoptotic cells in the presence of CM (Figure 7.1.6, compare black bars 4 to black bars 3). Addition of CM to CEM-C7-14 cells treated with combination of Dex and ETOP reduced apoptosis (Figure 7.1.6, compare grey bar 8 to grey bar 7).

Taken together results obtained by three different methodologies demonstrate that combined Dex and ETOP treatment is more effective than individual treatment Furthermore, CM generated from the bone marrow promotes survival and contributes to resistance to Dex and ETOP treatment of leukemia cells.

7.1.4 Investigation of the molecular basis of drug resistance of leukaemia cells

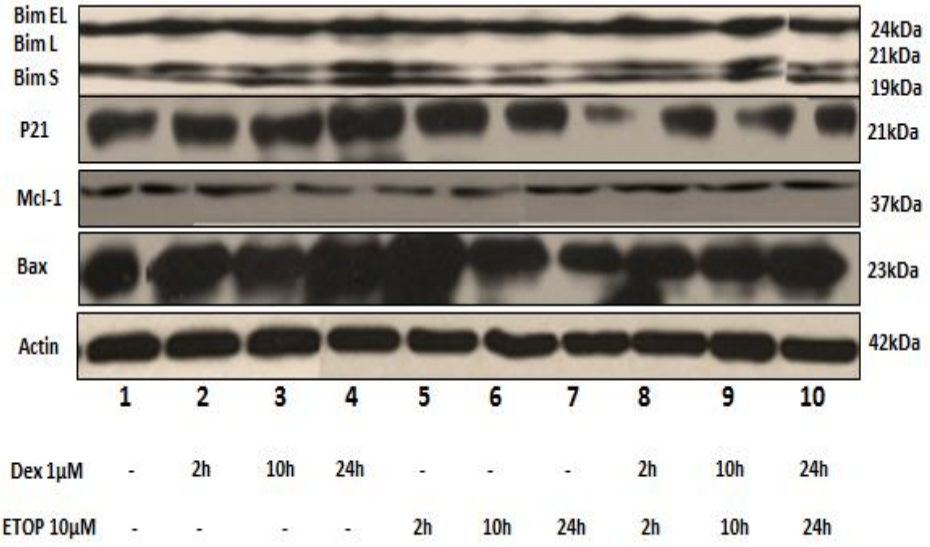
Known glucocorticoid receptor and p53 targets were analyzed as indicators of leukemia cell response to dexamethasone and anthracyclines treatment respectively. GR post-translational modifications were also followed as an additional control mechanism implicated in the regulation of ALL sensitivity to glucocorticoid induced apoptosis (Cardoso *et al.*, 2008). The effects of Dex and ETOP treatment on ALL cells was monitored following the protein levels of GR and p53 as well as their post translational modifications employing SDS PAGE followed by western blot analysis. The protein levels of GR and p53 downstream targets were also estimated by western blot analysis. Mcl-1 and Bim were chosen because they play crucial role in the regulation of the GR mediated apoptosis in leukemia. The p53 transcription targets Bax and p21 were analyzed as they play important role in mediating p53 induced apoptosis and cell cycle arrest respectively.

Analysis of protein levels of GR and p53 downstream targets

The endogenous Mcl-1, Bax, Bim and p21 protein levels were recorded in CEM-C1-15 and CEM-C7-14 cells treated with 1μM Dex, or 10μM ETOP either individually or in combination for 2, 10 and 24 hours and correlated with those of the glucocorticoid receptor and p53 (Figure 7.1.7 A). The highest p21 protein levels were detected in CEM-C1-15 cells treated with Dex and ETOP individually or in combination (Figure 7.1.7 A, lane 4). Mcl-1 and Bim protein levels remained relatively unchanged irrespectively of the duration or the type of the treatment (Figure 7.1.7 A). Increased Bax protein levels were noted after 2hrs treatment with ETOP and 24hrs with combination of Dex and ETOP (Figure 7.1.7 A, compare lanes 5 and 10 to 1).

A

CEM-C1-15



B

CEM-C7-14

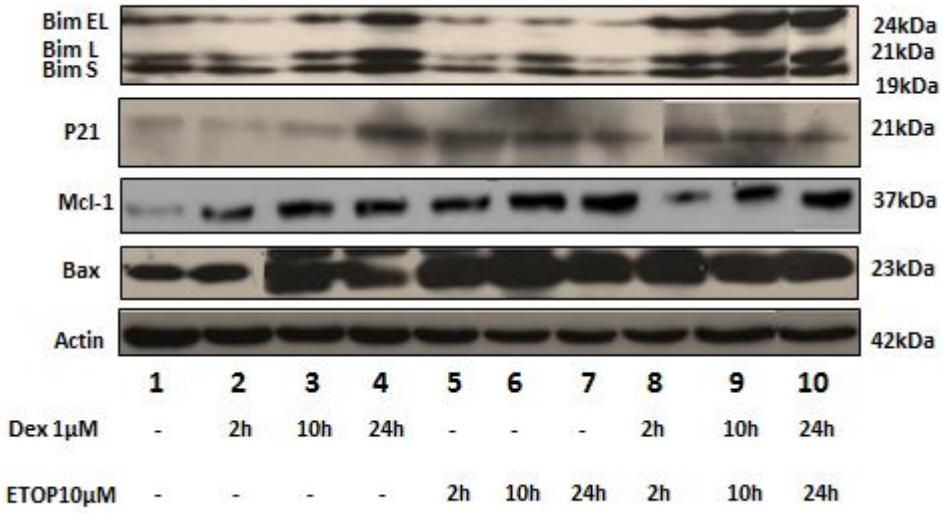


Figure 7.1.7: **Bax, Bim, Mcl-1 and p21 protein levels in CEM-C1-15 and CEM-C7-14 cells treated with Dexamethasne and Etoposide.** CEM-C1-15 (A) and CEM-C7-14 (B) cells were treated with Dex and ETOP individually or in combination for 2hrs, 10hrs and 24hrs, lysed and total cell protein extracts were subjected to western blot analysis. Bax, Bim, Mcl-1 and p21 were detected using specific antibodies against these proteins. Actin was used as loading control. As the proteins to be detected were of very close molecular weight, 2 gels were prepared for one experiment and the blots were stripped out for one time after the protein and its corresponding actin been detected. The first blot was been cut into 2 parts upper and bottom parts from between 35 and 40KDa point carefully. The upper part was propped with actin antibody. The first bottom part was propped first with Mcl-1, stripped and then propped with Bax antibody. The second blot was been cut as well into two parts as mentioned. The upper part was propped with actin as well. The second bottom blot was propped with Bim, stripped and propped with p21.

In CEM-C7-14 cells, Bim protein levels gradually increased with Dex alone or Dex and ETOP treatment, whereas the ETOP alone did not have significant effect (Figure 7.1.7 B). Gradual increase of p21 protein levels with longer duration of the incubation with Dex was observed. Individual ETOP or combination with Dex treatment of CEM-C7-14 cells resulted in elevation of p21 protein levels that gradually declined with longer incubation time (Figure 7.1.7 B). Mcl-1 protein was expressed at higher level in CEM-C7-14 rather than CEM-C1-15 and it increased in cells treated with Dex and ETOP individually and in combination (Figure 7.1.7 B). Bax protein levels were elevated in CEM-C7-14 treated with Dex for 10 hrs and 24hrs as well as in cells treated with ETOP alone or in combination with Dex (Figure 7.1.7 B).

To conclude, in glucocorticoid sensitive cell line Mcl-1, Bim, and Bax were upregulated whereas p21 was relatively unchanged after 24 hrs of combined treatment. On the other hand in the glucocorticoid resistant cell line Mcl-1 and Bim protein levels remained unchanged, whereas p21 levels were downregulated after 24 hrs of combined treatment. Bax upregulation was detected in CEM-C1-15 cells after 24hrs of combined treatment but to a lesser extents than in CEM-C7-14.

7.1.5 *Analysis of mRNA levels of GR and p53 downstream targets*

In order to analyze if changes in protein levels of target genes observed above reflect control mechanisms operating at mRNA levels, resistant/sensitive CEM cell lines were treated with dexamethasone/anthracyclines individually and in combination and qRT-PCR technology was used to measure Bim, Mcl-1, p21 and Bax gene expression.

Glucocorticoids dependent regulation of Bim was affected by microenvironment and DNA damage

To assess the role of the CM on two of the Bcl-2 family members involved in the regulation of glucocorticoid-induced apoptosis, Bim (pro-apoptotic) and Mcl-1 (anti-apoptotic) mRNA levels were measured. A five fold induction of Bim mRNA was observed in sensitive CEM-C7-14 cell line, whereas the resistant CEM-C1-15 cells showed 2 folds induction of Bim gene expresion (Figure 7.1.8, compare black and grey bars 3 to black and grey bars 1). On the other hand, addition of CM to the Dex treated CEM-C1-15 cells lead to decreased induction of Bim (Figure 7.1.8, compare black bars 4 and 3). In general, Bim mRNA did not change with the ETOP treatment alone or combined with CM (Figure 7.1.8, compare black and grey bars 5 to black and grey bars

6), while, combined treatment of Dex and ETOP did not significantly induce Bim in the presence of absence of CM (Figure 7.1.8, compare black and grey bars 7 to black and grey bars 8). As the cells death rate is higher with the combined than individual treatment with Dex and ETOP this means that combined treatment switches cell death pathway from Bim mediated to unknown pathway (Figure 7.1.8, compare black and grey bars 7 to black and grey bars 3).

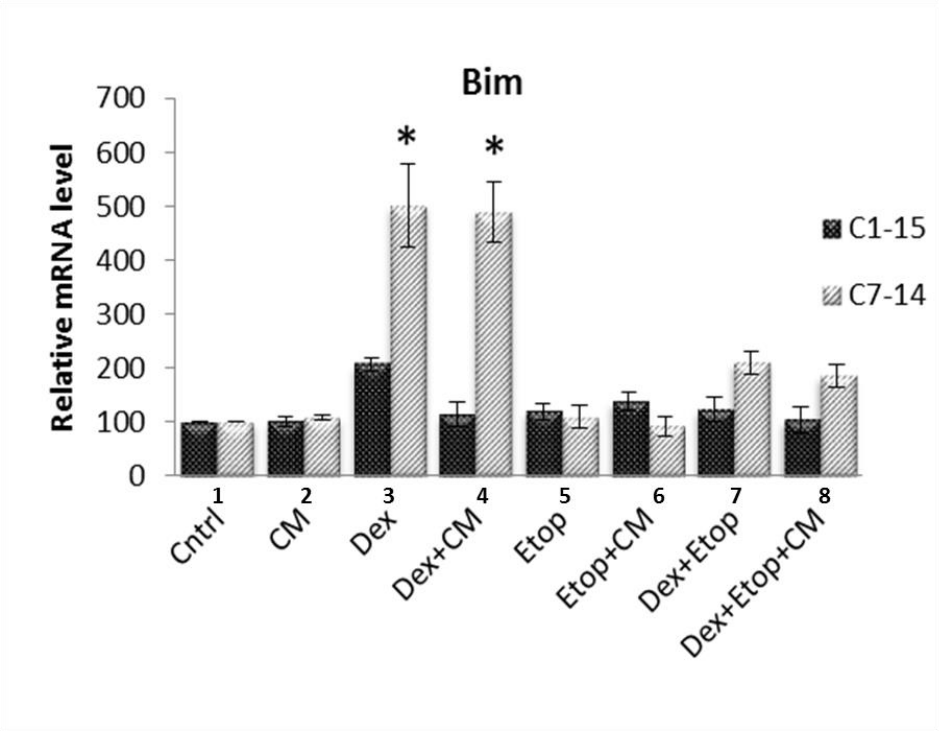


Figure 7.1.8: **Bim gene expressions in CEM-C1-15 and CEM-C7-14 cells .Cells were** treated with dexamethasone (1 μM) for 36 hrs, etoposide (10μM) for 24hrs and CM (48hrs). Cells were lysed and total mRNA was extracted, reverse transcribed and Bim mRNA levels were followed with qRT-PCR. Data shown are representative of three independent experiments. Errors bars indicate standard deviation. Asterisk indicates statistical significance ($P < 0.05$).Comparisons made were as follows: C1-15 Dex with C1-15 Dex+CM, C7-14 Dex with C7-14 Dex+CM, C1-15 ETOP with C1-15 ETOP+CM, C7-14 ETOP with C7-14 ETOP+CM, C1-15 Dex+ETOP with C1-15 Dex+ETOP+CM, C7-14 Dex+ETOP with C7-14 Dex+ETOP+CM.

Regulation of Mcl-1gene expression by glucocorticoids, etoposide and CM

Four-fold increase of Mcl-1 mRNA levels was detected in CEM-C1-15 cells following glucocorticoid treatment consistent with the fact that Mcl-1 is an anti-apoptotic factor and CEM-C1-15 are resistant to glucocorticoid-induced apoptosis. (Figure 7.1.9, compare black bar 3 to black bar 1). No significant changes were recorded in any other treatments. On the other hand Mcl- protein detection result in western blot showed higher levels of

Mcl-1 in CEM-C7-14 rather than CEM-C1-15 and it increased in cells treated with Dex and ETOP individually and in combination (Figure 7.1.7 B) which disagree with mRNA detection results. This could be due to presence of different isoforms of Mcl-1 which can show different levels of stabilization and degradation of the protein.

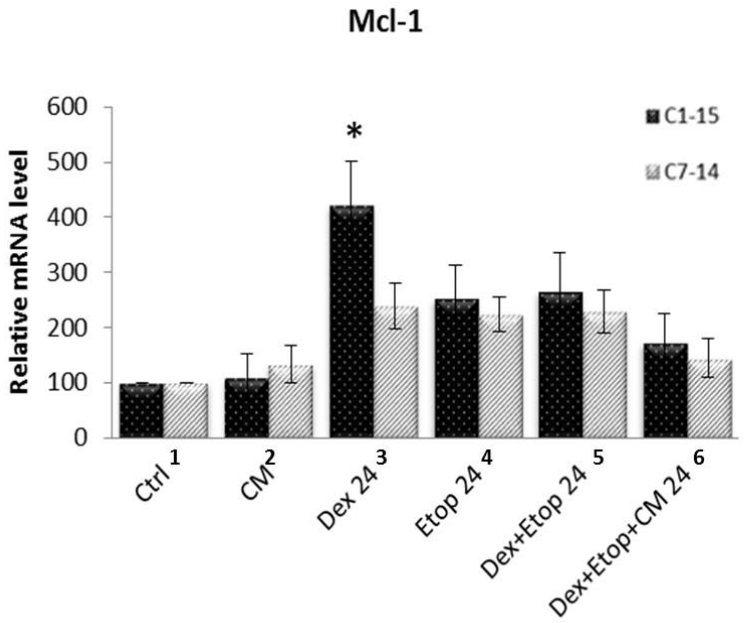


Figure 7.1.9: **Mcl-1 gene expression in CEM-C1-15 and CEM-C7-14 cells**. Cells were treated with dexamethasone (1 μM) for 36 hrs, etoposide (10μM) for 24hrs and CM (48hrs). Cells were lysed and total mRNA was extracted, reverse transcribed and Mcl-1 mRNA levels were followed with qRT-PCR. Data shown are representative of three independent experiments. Errors bars indicate standard deviation. Asterisk indicates statistical significance ($P < 0.05$). Comparisons made were as follows: C1-15 Dex to C1-15 Ctrl, C7-14 Dex to C7-14 Ctrl, C1-15 ETOP to C1-15 Ctrl, C7-14 ETOP to C7-14 Ctrl, C1-15 Dex+ETOP to C1-15 Ctrl, C7-14 Dex+ETOP to C7-14 Ctrl and C1-15 Dex+ETOP+CM to C1-15 CM, C7-14 Dex+ETOP+CM to C7-14 CM.

Regulation of Bax and p21 mRNA levels in CEM-C1-15 and CEM-C7-14 cells

The effect of CM on apoptosis and cell cycle control was assessed following the mRNA levels of the pro-apoptotic Bcl-2 family member Bax which is involved in the p53-induced apoptosis and the p53 transcription target p21 which mediates cell cycle arrest in the G1 phase of the cell cycle to allow DNA repair (Lakin and Jackson, 1999; Nicholas and Jackson , 1999).

Three fold induction of Bax was observed in CEM-C7-14 cells treated with etoposide (Figure 7.1.10, compare grey bar 5 to grey bar 1) whereas no change was observed in the resistant CEM-C1-15 cells under the same conditions (Figure 7.1.10, compare black bar 5 to black bar 1). Bax mRNA did not change with either glucocorticoid treatment alone or

combination of CM with glucocorticoid reflecting the fact that Bax is not a direct transcriptional target of glucocorticoids. Combined Dex and ETOP treatment significantly induced Bax (Figure 7.1.10, compare grey bar 7 to grey bar 1). Apoptosis in ALL cells treated with combination of Dex and Etop may not depend on Bim but on Bax. However, the microenvironment exerts inhibitory effect on Bax induction by the Dex and ETOP treatment (Figure 7.1.10, compare grey bar 8 to grey bar 7).

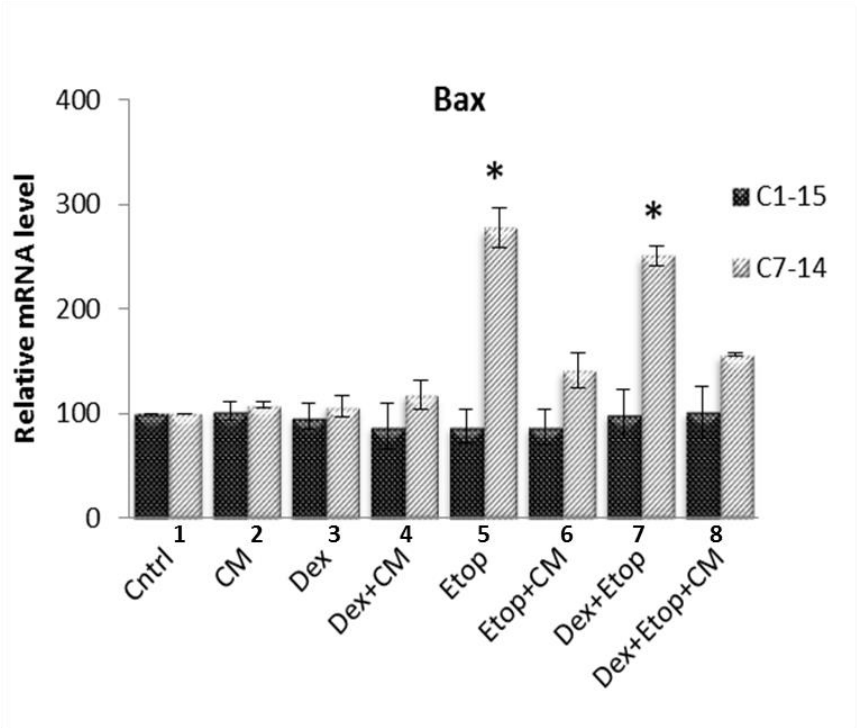


Figure 7.1.10: **Bax gene expression in CEM-C1-15 and CEM-C7-14 cells**.Cells were treated with dexamethasone (1 μM) for 36hrs, etoposide (10μM) for 24hrs and CM (48hrs). Cells were lysed and total mRNA was extracted, reverse transcribed and Bax mRNA levels were followed with qRT-PCR. Data shown are representative of three independent experiments. Errors bars indicate standard deviation. Asterisk indicates statistical significance ($P < 0.05$). Comparisons were made as follows: C1-15 Dex with C1-15 Dex+CM, C7-14 Dex with C7-14 Dex+CM, C1-15 ETOP with C1-15 ETOP+CM, C7-14 ETOP with C7-14 ETOP+CM, C1-15 Dex+ETOP with C1-15 Dex+ETOP+CM, C7-14 Dex+ETOP with C7-14 Dex+ETOP+CM.

Regulation of p21 mRNA levels in CEM-C1-15 and CEM-C7-14

Three folds increase in p21 mRNA levels was detected in the CEM-C1-15 cells treated with ETOP (Figure 7.1.11, compare black bar 5 to black bar 1). Dex and ETOP combined treatment resulted in a four folds induction of p21 mRNA levels in the CEM-C1-15 cells (Figure 7.1.11, compare black bar 7 to black bar 1) whereas in the glucocorticoid sensitive CEM-C7-14 cells the same treatment reduced p21 mRNA levels (Figure 7.1.11,

compare grey bar 7 to grey bar 1). Reduced p21 mRNA levels were observed in CEM-C7-14 compared to CEM-C1-15 cells treated with Dex alone (Figure 7.1.11, compare grey bar 3 with black bar 3). The p21 mRNA levels were not affected significantly in cells treated with Dex and Etop individually or in combination in the presence of CM (Figure 7.1.11, compare bars 4, 6 and 8). Taken together results shown in Figure 7.1.11 indicate that p21 mRNA levels is more prominent in glucocorticoid resistant cell line CEM-C1-15 and it is induction was not significantly affected by the CM.

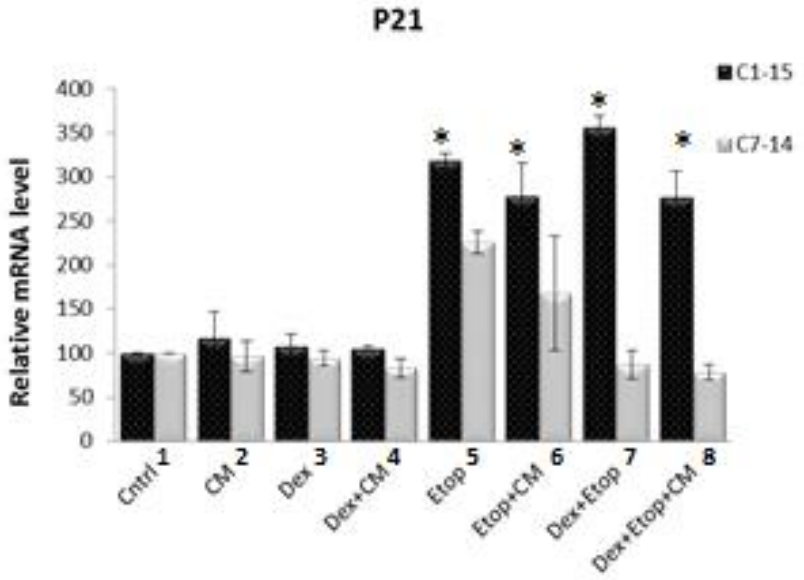


Figure 7.1.11: **p21 gene expression in CEM-C1-15 and CEM-C7-14 cells**. Cells were treated with dexamethasone (1μM) for 36hrs, etoposide (10μM) for 24hrs and CM (48hrs). Cells were lysed and total mRNA was extracted, reverse transcribed and p21mRNA levels were followed with qRT-PCR. Data shown are representative of three independent experiments. Errors bars indicate standard deviation. Asterisk indicates statistical significance ($P < 0.05$). Comparisons were made as follows: C1-15 Dex with C1-15 Dex+CM, C7-14 Dex with C7-14 Dex+CM, C1-15 ETOP with C1-15 ETOP+CM, C7-14 ETOP with C7-14 ETOP+CM, C1-15 Dex+ETOP with C1-15 Dex+ETOP+CM, C7-14 Dex+ETOP with C7-14 Dex+ETOP+CM.

In summary, increased Bax mRNA levels were observed in the GC sensitive CEM-C7-14 cells treated with ETOP alone and elevated Bim mRNA levels in the same cells treated with Dex alone. Moreover, increased Mcl-1 and p21 mRNA levels were detected in the GC resistant CEM-C1-15 cells treated with Dex and ETOP respectively. Interestingly, a shift from Bim mediated to Bax dependent apoptosis was observed in the GC sensitive

CEM-C7-14 cells treated with combination of Dex and ETOP.

7.1.6 Conclusion

Combined treatment of Dex and etoposide lead to increased levels of apoptotic ALL cells reflecting the importance of combined therapy. CM media was used to simulate the microenvironmental conditions of the bone marrow and investigate its effects on ALL cells and their response to combined Dex and etoposide treatment. The obtained data suggest that CM causes reduction of the combined effect of dexamethasone and etoposide on ALL cells apoptosis implying that the bone marrow microenvironment is a potential source of resistance. Support for this notion is provided by the expression pattern of the GR transcriptional target genes Mcl-1, Bim, Bax and p21. In particular, induction of gene expression of the pro-apoptotic genes Bim and Bax was observed in the glucocorticoid sensitive CEM-C7-14 cells, whereas gene expression of the anti-apoptotic p21 was reduced in these cells. In contrast, in the glucocorticoid resistant CEM-C1-15 cells the expression of the cell cycle arrest p21 gene was induced. Furthermore, a switch from Bim to Bax mediated apoptosis was observed in the glucocorticoid sensitive CEM-C7-14 cells treated with combination of dexamethasone and etoposide. These results can be useful in clinical oncology as they provide an insight of potential ways to overcome resistance in ALL treatment.

7.2 GR and p53 post-translational modifications are affected by the microenvironment

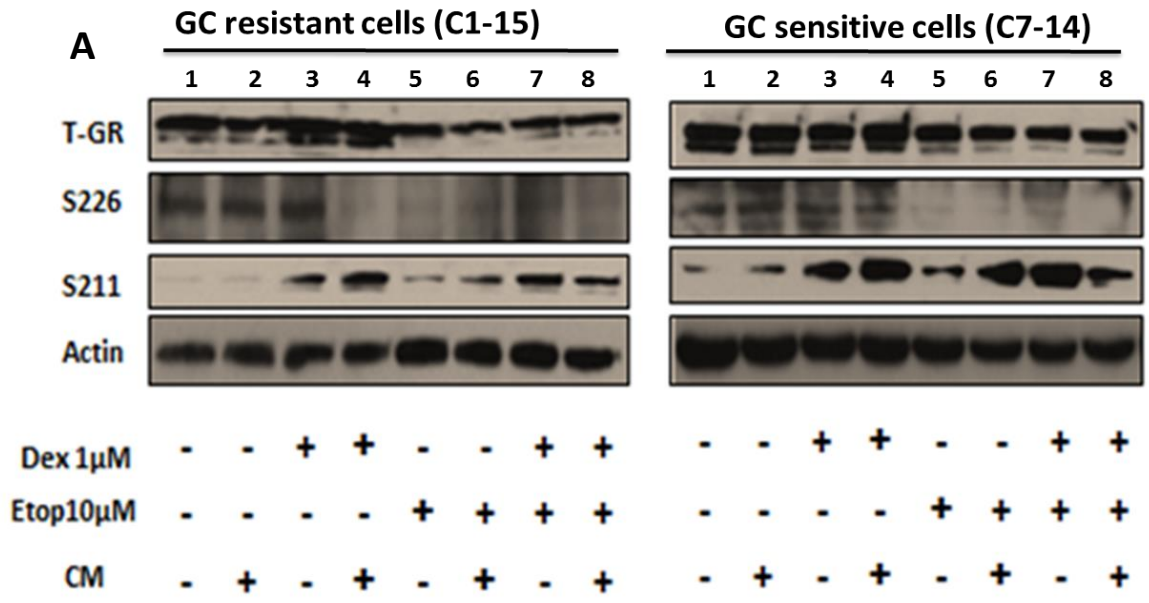
7.2.1 Bone marrow microenvironment affects GR phosphorylation

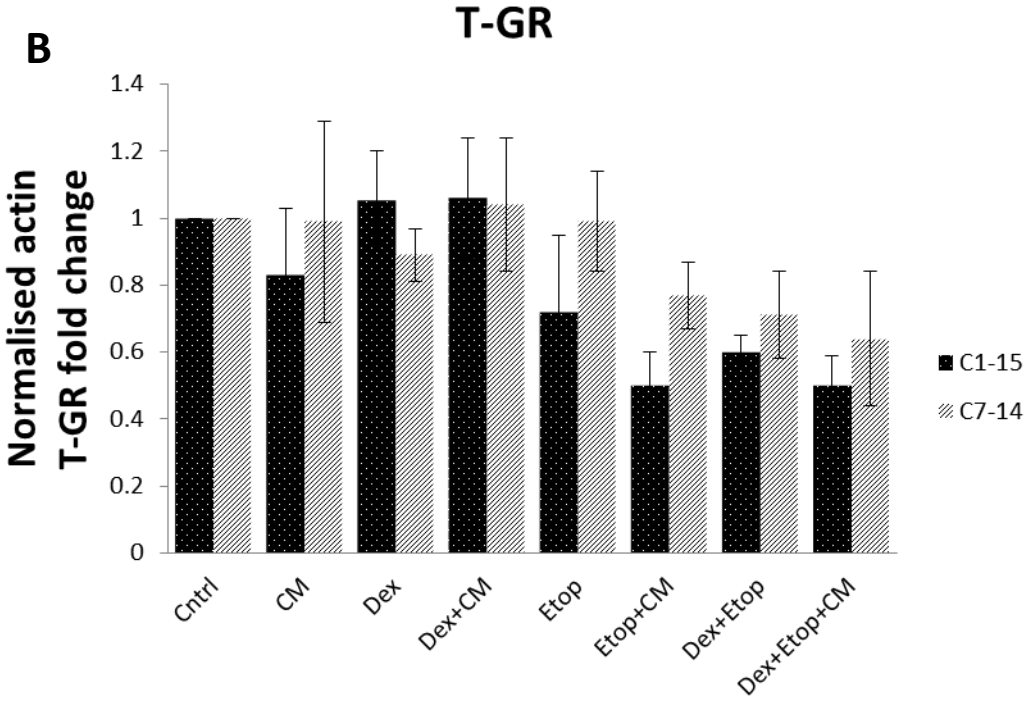
Dexamethasone and etoposide exert their cellular effects via the transcription factors glucocorticoid receptor and p53, respectively. GR posttranslational modifications are an additional control mechanism implicated in the regulation of ALL sensitivity to glucocorticoid induced apoptosis (Rogatsky and Ivashkiv, 2006; Cardoso *et al.*, 2008; Galliher-Beckley and Cidlowski, 2009; Kfir-Erenfeld *et al.*, 2010). On the other hand, the effect of CM on the posttranslational modifications of GR and p53 has not yet been elucidated. To determine the phosphorylation pattern of the GR and p53 in glucocorticoid sensitive and resistant ALL cell lines and analyse the effect of CM on the phosphorylation of these proteins, antibodies specifically detecting total and phosphorylated GR and p53 proteins were used. GR is highly phosphorylated by several kinases at multiple sites in response to diverse types of cytotoxic stress but the precise effects on GR transcriptional activity remain controversial. GR has multiple phosphorylation sites including Ser211 and Ser226 which are located within the transactivation domain-1 (AF-1) (See Figure 4.13.1). Since the GR phosphorylation is suggested to regulate cell fate either positively or negatively, we decided to investigate the effect of the combinatory drug treatment and CM on GR phosphorylation status. Ser211 is hyperphosphorylated early after hormone binding, for that Dex treatment was applied for 24hrs only instead of 36hrs as at 24 hours protein level can be detected. Phosphorylation of GR on Ser226 is usually associated with nuclear export, GR sumoylation and negative effect on its activity. The ratio of GR phosphorylation at Ser211 versus Ser226 is an important determinant of GR activity in ALL and brain cells and higher level of activity is expected if this ratio is higher. GR phosphorylated at Ser211 is targeted by cyclin dependent kinases and p38MAPK pathway, while Ser226 is targeted by c-Jun N-terminal kinases (JNK) (Galliher-Beckley and Cidlowski, 2009; Kfir-Erenfeld *et al.*, 2010; Lynch *et al.*, 2010).

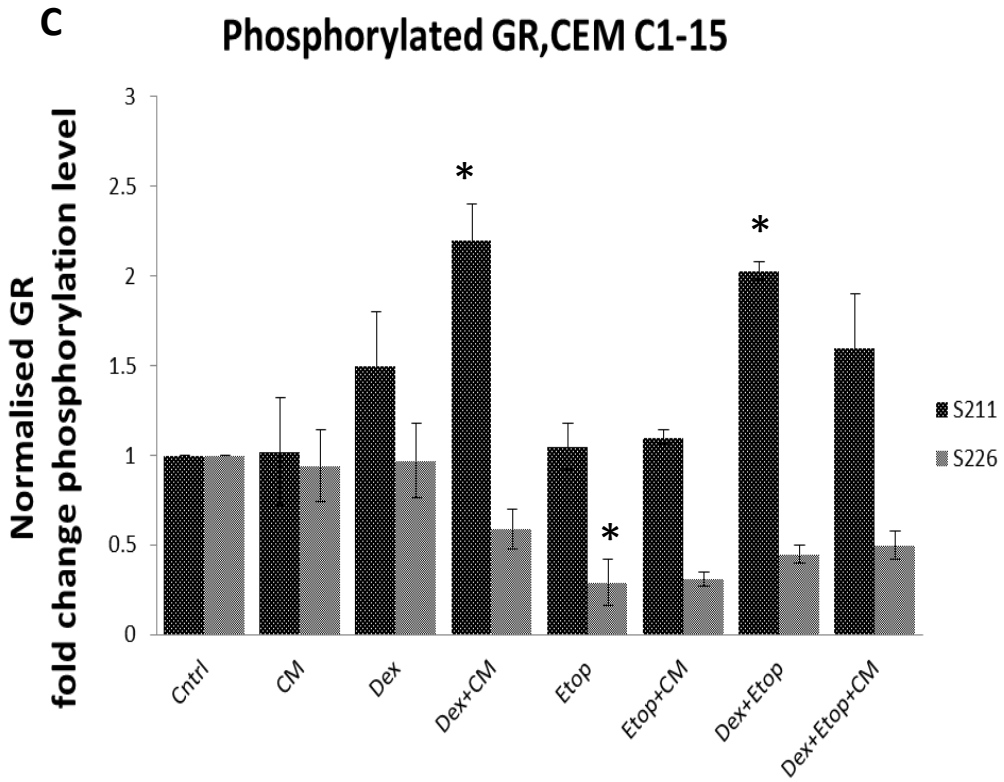
To identify whether GR modifications were differentially regulated in glucocorticoid sensitive and resistant ALL cell lines and how microenvironment affects this process GR protein level was measured, following glucocorticoid, topoisomerase II inhibitor (ETOP) and CM treatment of ALL cell lines. The protein patterns of the extracts of

CEM-C1-15 and CEM-C7-14 were analysed. Higher total GR proteins levels were detected in cells treated with Dex compared to non-treated control cells (Figure 7.2.1 A, lane 3). Densitometric analysis of total GR showed the agreement of the activation of GR with almost all treatments (Figure 7.2.1 B and specifically with Dex treatment as alone or combined with CM and ETOP (Figure 7.2.1 B lane 3, 4, and 7) and mainly higher GR protein level with the sensitive clone, whereas CM alone or in combination with Dex, ETOP and both combinations treatment lead to slight reduction in total GR levels in all cases and mainly higher GR protein level with the sensitive clone, whereas CM alone or in combination with Dex, ETOP and both combinations treatment lead to slight reduction in total GR levels in all cases Actin protein levels were used as a loading control.

In terms of phosphorylation, there is higher phosphorylation at Ser211 in glucocorticoid sensitive cells CEM-C7-14 which reflects higher GR activation (Figure 7.2.1 A lane 3 and 4). In addition, consistent increase of GR Ser211 phosphorylation in Dex and CM treated GC sensitive and resistant CEM cells compared to cells treated with Dex alone was identified (Figure 7.2.1, A compare lane 4 with lane 3).




B
T-GR
Normalised actin
T-GR fold change
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Cntrl
CM
Dex
Dex+CM
Etop
Etop+CM
Dex+Etop
Dex+Etop+CM
C1-15
C7-14


C
Phosphorylated GR,CEM C1-15
Normalised GR
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3
2.5
2
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Dex
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S211
S226

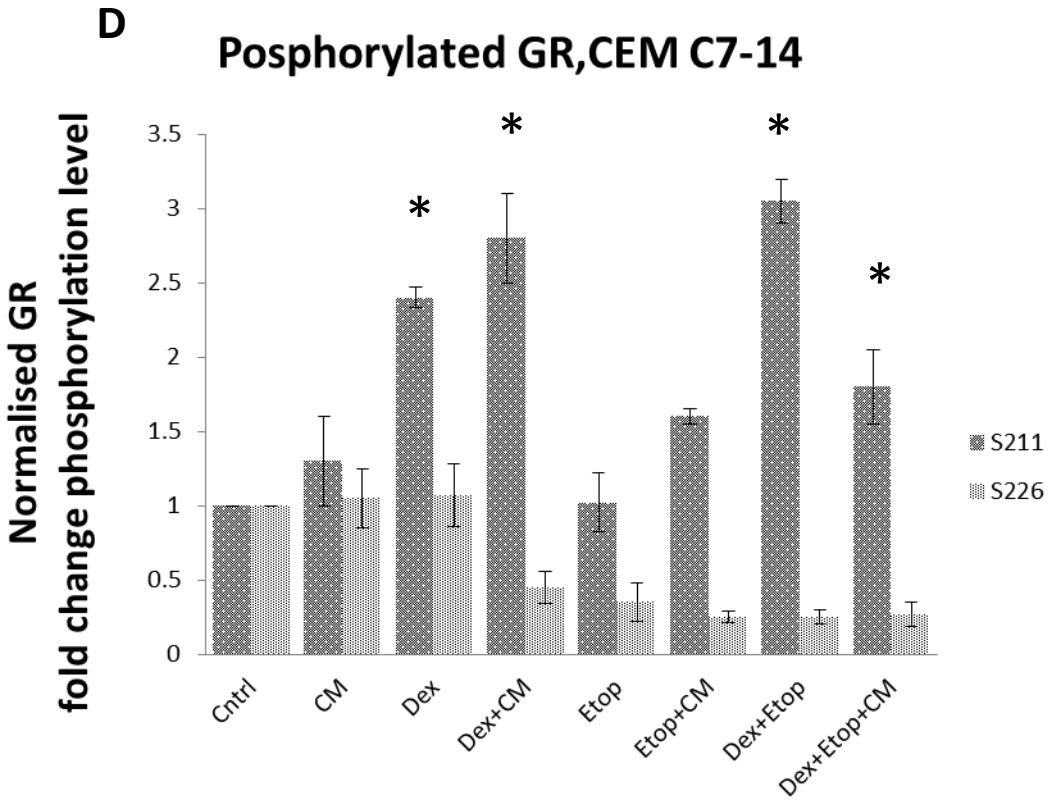


Figure 7.2.1: **Regulation of GR phosphorylation at Ser211 and Ser226 in CEM-C1-15 and CEM-C7-14 cells.** (A) Regulation of GR phosphorylation at Ser211and Ser226 was followed in CEM-C1-15 and CEM-C7-14 cells treated with dexamethasone (1μM), etoposide (10μM) and CM. Cells were lysed and total cell protein extract was subjected to western blot analysis with phosphospecific antibodies detecting total and GR phosphorylated at S211 and Ser226. Actin was used as a loading control. Data are representative of three independent experiments. (B) Quantitative analysis of the GR phosphorylation of total GR density. (C) Phosphorylated GR at Ser211 and Ser226 density in CEM C1-15, and (D) phosphorylated GR at Ser211 and Ser226 density in CEM-C7-14 density. The graph represents the intensity of the bands that correspond to the P-GR against total GR levels, normalized to the actin values. Errors bars indicate standard deviation. Asterisk indicates statistical significance ($P < 0.05$) compared to the CM. 2 gels were prepared for one experiment and the blots were stripped out for one time after the protein and its corresponding actin been detected. The first blot was been cut into 2 parts upper and bottom parts from the 72KD point. The upper part was propped with Ser226 antibody first, stripped and propped with H300 antibody. The first bottom part was propped first with Ser15, stripped and then propped with p53 antibody. The second blot was been cut as well into two parts as mentioned. The upper part was propped first with Ser211, stripped and propped with H300. The second bottom blot was propped with Ser20, stripped and propped with Actin. The bottom blots were shown in Figure 7.2.2.

The densitometric analysis of the GR phosphorylation levels and normalization of phosphorylation levels to the total GR levels in Figure 7.2.1 C for CEM-C1-15 and D for CEM-C7-14 indicated prevalence of GR phosphorylation at the Ser211 residue versus the Ser226 in the CEM-C7-14 cells with Dex treatment as alone or with Dex

and CM, and Dex with ETOP (Figure 7.2.1 D lane 3, 4 and 7). In addition, densitometric scanning of these results and normalization of phosphorylation levels to the total GR levels indicated that Ser226 phosphorylation was the highest in glucocorticoid resistant cells and revealed overall predominant of phosphorylation of the Ser226 over the Ser211 GR phosphorylation (Figure 7.2.1 C).

In conclusion, the quantification data suggested that GR phosphorylation at Ser226 is higher than that of GR Ser211 in glucocorticoid resistant cell CEM-C1-15 (Figure 7.2.1 C), whereas GR phosphorylation at Ser211 is prevalent in the glucocorticoid sensitive CEM-C7-14 cells (Figure 7.2.1 D).

The fact that GR Ser226 phosphorylation was substantially downregulated by CM in Dex treated CEM-C1-15 cells and not in CEM-C7-14 cells under the same conditions suggests that CM might play a role in determining the site of GR phosphorylation in resistant and sensitive to glucocorticoid CEM cells. In addition the observation that ETOP treatment of CEM-C7-14 and CEM-C1-15 resulted in marked reduction of GR Ser226 phosphorylation in both cell lines whereas combinatory Dex, ETOP and CM treatment did not have the same effect implied that CM could potentially contribute to resistance of ALL to glucocorticoid treatment. Overall conditioned media increased GR Ser211 phosphorylation and decreased Ser226 phosphorylation in both cell lines. Moreover, the result indicated a differential role of the GR phosphorylation in the two cell lines as detected by altered phosphorylation and kinetic patterns of CDK and JNK dependent GR target residues.

7.2.2 The effect of microenvironment on p53 phosphorylation pattern

In non-stressed cells p53 is not active and is activated in response to various stresses that damage the DNA (Bai e Zhu, 2006). Although the detailed mechanisms entailing p53 protein stabilization and transcriptional activation have not yet been fully understood it has been shown that multisite phosphorylation of p53 plays important role in the regulation of both processes. The two p53 phosphorylation sites shown to be crucial are the Ser15 targeted by ATM/ATR and the Ser20 targeted by chk1/chk2 (Khanna and Jackson, 2001; Lukas and Bartek, 2004; Efeyan and Serrano, 2007). Experiments were carried out to investigate the effect of conditioned media on p53 phosphorylation in ALL cells treated with Dex and ETOP. Ser15 and Ser20 p53 phosphorylation selectively directs this transcription factor to its pro-apoptotic

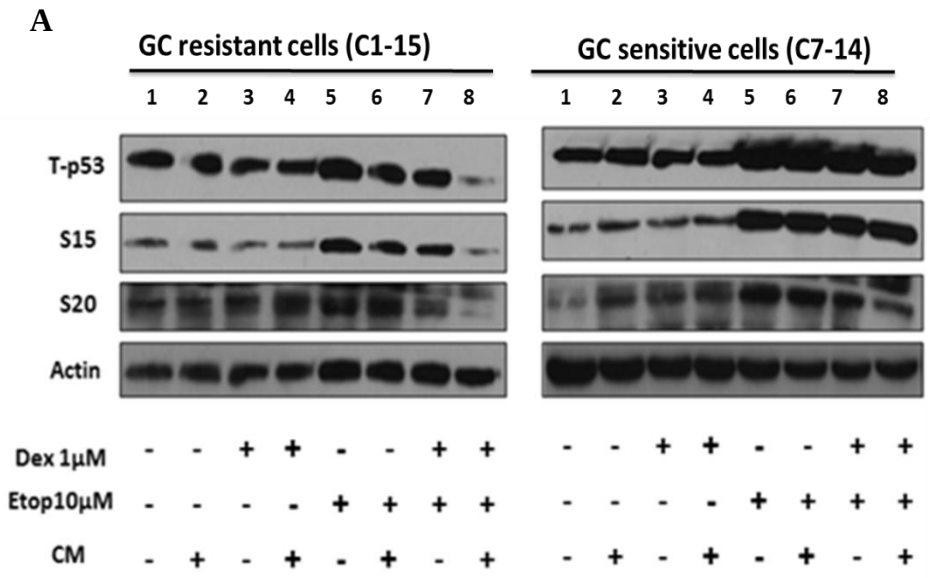
transcription targets and usually this leads to cell death. Reports show that Ser15/Ser20 phosphorylation reduces p53 affinity for its primary negative regulator Mdm2 and promotes the recruitment of transcriptional coactivators (Dai and Gu, 2010).

To identify whether p53 is regulated differentially in glucocorticoid sensitive and resistant cell lines and how bone marrow microenvironment affects this regulation, total p53 protein levels were measured, following glucocorticoid, topoisomerase II inhibitor and CM treatment. The p53 protein levels and phosphorylation patterns in the CEM-C1-15 and CEM-C7-14 cellular extracts were analysed. Increased levels of total p53 protein were detected in CEM-C7-14 cells treated with ETOP alone or in combination with Dex and CM (Figure 7.2.2 A , compare lanes 5-8 to lanes 1-4 respectively and B grey bars 5-8 to grey bars 1-4 respectively) whereas in CEM-C1-15 cells ETOP treatment did not exhibit any significant effect (Figure 7.2.2 A, compare lanes 5-8 to lanes 1-4 respectively and B black bars 5-8 to black bars 1-4 respectively). In the sensitive CEM-C7-14 and resistant CEM-C1-15 cell lines there was no significant effect in total p53 protein levels detected after treatment with the CM alone (Figure 7.2.2 A, compare lanes 2 to lanes 1 and B, black and grey bars 2 to black and grey bars 1) or in combination with either Dex (Figure 7.2.2 A, compare lanes 4 to lanes 3 and B black and grey bars 4 to black and grey bars 3) or with ETOP (Figure 7.2.2 A, compare lanes 6 to lanes 5 and B black and grey bars 6 to black and grey bars 5). In both ALL cell lines treated with Dex, ETOP and CM significant reduction of total protein p53 levels was observed in (Figure 7.2.2 A, compare lanes 8 to lanes 7 and B black and grey bars 8 to black and grey bars 7).

The pattern of the p53 phosphorylated at Ser15 indicates increase in CEM-C1-15 and CEM-C7-14 cells treated with ETOP (Figure 7.2.2 A, compare lanes 5 to lanes 1 and C and D dark and light grey bars 5 to dark and light grey bars 1). Combination of Dex ETOP and CM decreased p53 Ser15 phosphorylation more distinctly in CEM-C1-15 cells (Figure 7.2.2 A, compare lane 8 to lane 7 and C dark grey bar 8 to dark grey bars 7).

Phosphorylation of p53 at Ser20 decreased in both CEM-C1-15 and CEM-C7-14 cells treated with ETOP Dex and CM (Figure 7.2.2 A, compare lanes 8 to lanes 7 and C and D dark and light grey bars 8 to dark and light grey bars 7). Data shown in Figure 7.2.3 indicated reduction of total p53 protein levels and phosphorylated p53 at Ser15

and Ser20 in the resistant CEM-C1-15 cells treated with Dex ETOP and CM. On the other side in the sensitive CEM-C7-14 cells reduced levels of p53 phosphorylated at Ser20 were observed after treatment with Dex ETOP and CM.



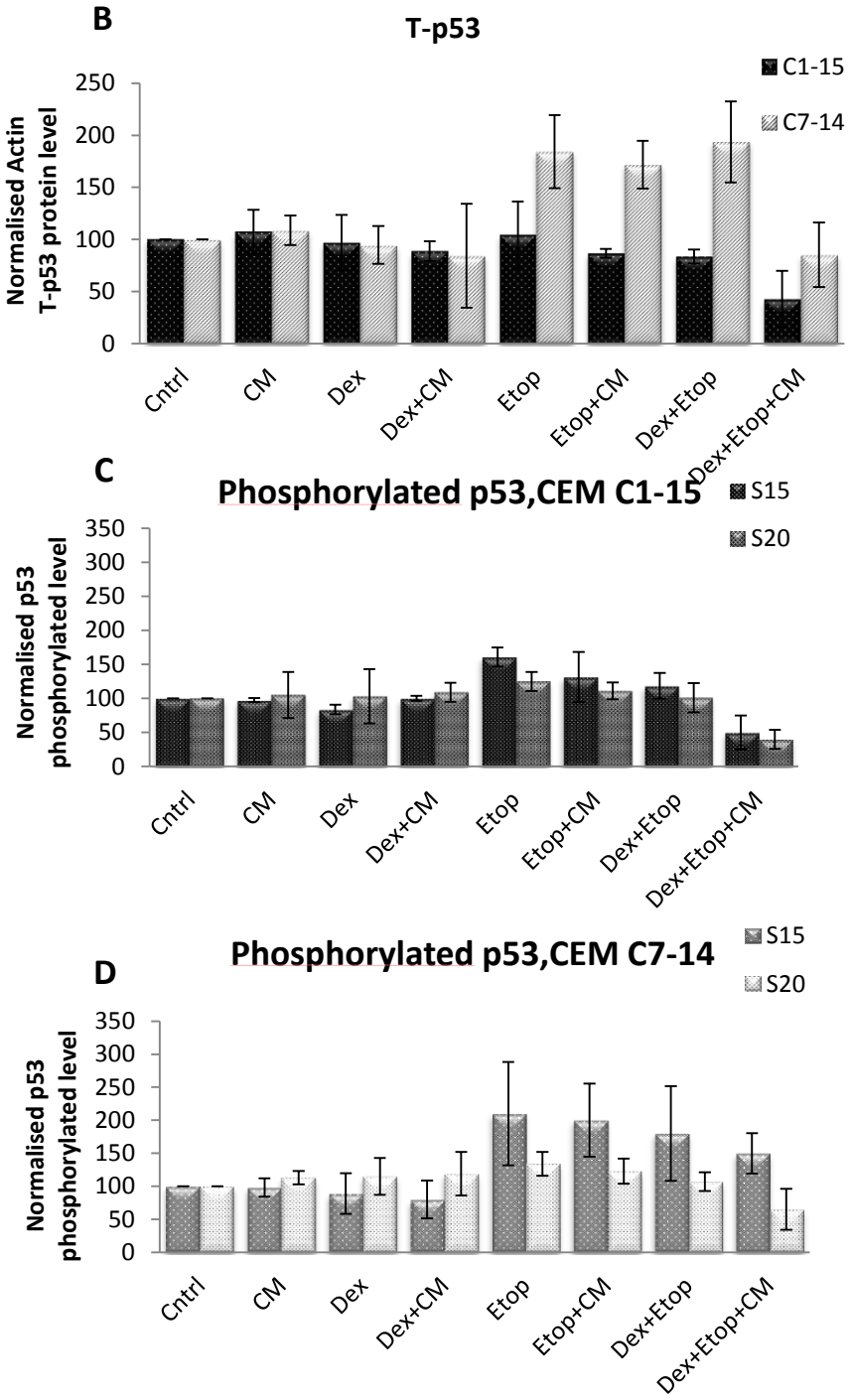


Figure 7.2.2: **Regulation of p53 phosphorylation at Ser15 and Ser20 in CEM-C1-15 and CEM-C7-14 cells.** (A) Total and phosphorylated p53 at Ser15and Ser20 protein levels. Cells were treated with dexamethasone (1µM) for 24hrs for protein level detection, ETOP (10µM) for 24hrs and CM for 48hrs. Cells were lysed and total cell protein extracts were subjected to western blotting. The data is representative of three independent experiments. Quantitative analysis of total p53 and p53 phosphorylation was conducted. (B) Graph representing the total p53 density. (C) Graph representing the phosphorylated p53 at Ser15 and Ser20 density in CEM-C1-15, and (D) Graph representing the phosphorylated p53 at Ser15 and Ser20 density in CEM-C7-14. The graph represents the intensity of the bands that correspond to the

P-GR against total GR levels, normalized to the actin values. The blot loading plan was clarified in Figure 7.2.1

7.2.3 Analysis of signalling pathways mediating drug and microenvironment response

Glucocorticoid (GC) receptor (GR) is a ligand dependent transcription factor phosphorylated at multiple serine/threonine residues (Galliher-Beckley and Cidlowski, 2009). The importance of phosphorylation of the GR in mediating GC signaling has been shown to alter its transcriptional activity positively or negatively, suggesting that GR phosphorylation status may play an important role in the effectiveness of current GC therapies. The AF-1 transcriptional activation domain of GR is highly phosphorylated by kinases such as MAPKs, CDKs, and JNK and these modifications modulate the transcriptional activity of the GR within cells (Krstic *et al.*, 1997; Kino *et al.*, 2007; Chen *et al.*, 2008; Davies *et al.*, 2008). In order to determine pathways that lead to altered phosphorylation of GR and p53 in drug and CM treated cells, we used kinase inhibitors. Given that JNK, p38 and PI3 kinases have been shown to mediate GR, p53 and stem cell signalling we used inhibitors of these kinases. Here, we investigate the phosphorylation status of the GR in cells treated with those inhibitors in order to identify which pathway maybe involved in stem cell effects.

MAPK signalling pathways can function to alter GR activity

Effect of p38MAPK on GR phosphorylation at Ser211

Human GR is phosphorylated by p38 MAPK at Ser211 in leukemia cells, both in vitro and vivo (Miller *et al.*, 2005; Garza *et al.*, 2009). To investigate the effect of this kinase on GR function in ALL cells GC-resistant and sensitive human acute lymphoblastic CEM cells were treated with p38 MAPK inhibitor (SB203580) 2 hours before of the of Dex and ETOP treatment. Western blot analysis was performed and both total and phosphorylated GR and p53 were monitored using specific antibodies for GR phosphorylated at Ser 211 and Ser226 as well as p53 phosphorylated at Ser15 and Ser20. Here, the effectiveness of the inhibitor on the cell death pathway has not been shown. The

Total GR protein levels increased in CEM-1-15 but not in CEM-C7-14 cells treated with SB203580 (Figure 7.2.3 compare lanes 1 to lanes 2 in the left and the right panels). In SB203580 treated CEM-C1-15 cells no significant differences in the

pattern of GR phosphorylation at Ser226 were observed (compare lanes 2-5 in the Figure 7.2.3 left panel with lanes 1 to 4 in Figure 7.2.1 left panel). In contrast, in SB203580 treated CEM-C7-14 cells increase in Ser226 GR phosphorylation was observed in the presence of CM irrespectively of the treatment with hormone (compare lanes 2-5 in the Figure 7.2.3 right panel with lanes 1 to 4 in Figure 7.2.1 right panel).

In the SB203580 treated CEM-C1-15 cells CM increased the GR Ser211 phosphorylation in the presence of combination of Dex and etoposide in contrast to the decreased GR Ser211 phosphorylation observed in the same cells in the absence of the inhibitor (compare lanes 8 and 9 in Figure 7.2.3 left panel with lanes 7 and 8 in the Figure 7.2.1 left panel respectively). Similar results were obtained in the CEM-C7-14 cells under the same conditions (compare lanes 8 and 9 in Figure 7.2.3 right panel with lanes 7 and 8 in the Figure 7.2.1 right panel respectively).

S203580 treatment of the CEM-C1-15 cells in combination with Dex, ETOP and CM reversed the downregulation of the total p53 protein levels observed in these cells treated the same way in the absence of the inhibitor (compare lanes 8 and 9 in Figure 7.2.3 left panel with lanes 7 and 8 in the Figure 7.2.2 left panel respectively). On the other hand in CEM-C7-14 cells p38MAPK inhibition did not have any major effect on the total p53 protein levels (compare lanes 2-9 in Figure 7.2.3 right panel with lanes 1-8 in the Figure 7.2.2 right panel respectively). The p53 Ser15 and Ser20 phosphorylation patterns followed to a great extent that of the total p53 protein levels.

In conclusion p38 kinase downregulates total GR protein levels in CEM-C1-15 cells. In addition, the effect of the combinatory Dex, ETOP and CM treatment on phosphorylation of GR at Ser211 and Ser226 in these cells is p38 MAPK dependent. Inhibition of the p38 MAPK pathway increased p53 protein abundance in CEM-C1-15 cells. Moreover, it seems that the inhibition of p38MAPK pathway in combination with Etoposide promotes cell death pathway.

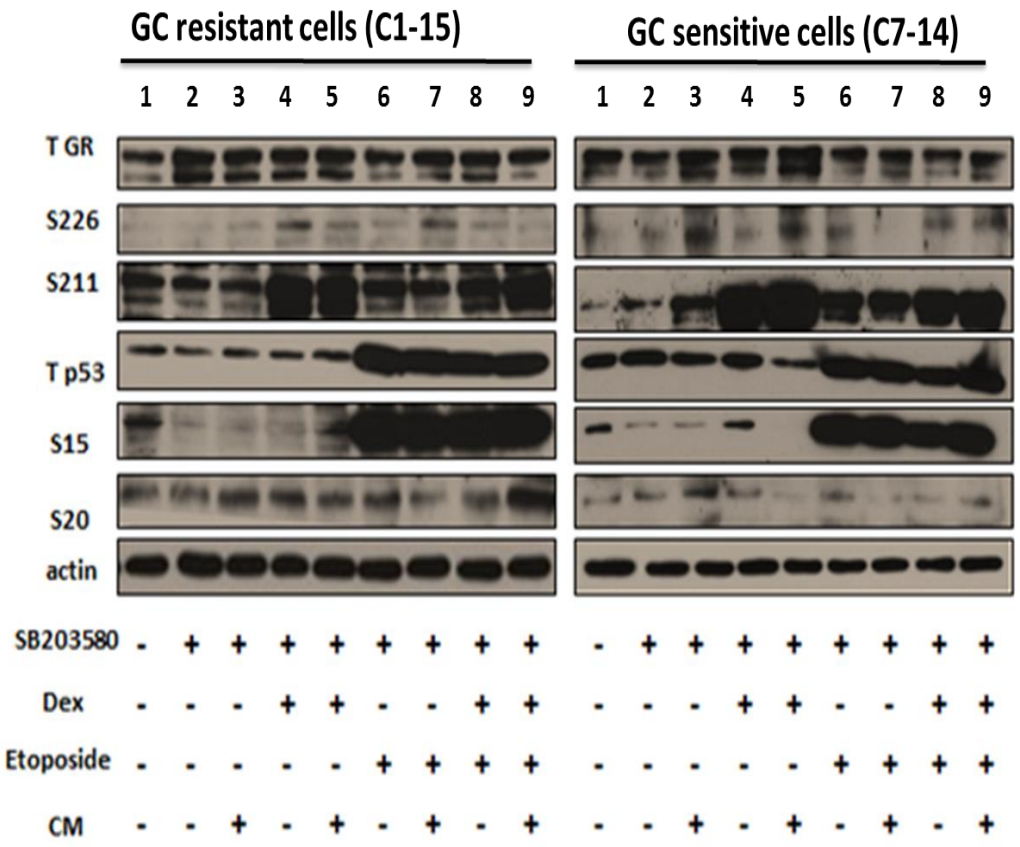


Figure 7.2.3: **Effects of the 38MAPK inhibitor SB203580 on GR and p53 phosphorylation profiles,** CEM-C1-15 (left panel) and CEM-C7-14 (right panel) cells were pre-treated with SB203580 (10μM) for 2 hours prior to the addition of Dex and ETOP. Cells were lysed, cellular extract were isolated and subjected to western blot analysis. Actin was used as a loading control. Data are representative of three independent experiments.2 gels were prepared for one experiment and the blots were stripped out for one time after the protein and its corresponding actin been detected. The first blot was been cut into 2 parts upper and bottom parts from the 72KD point. The upper part was propped with Ser226 antibody first, stripped and propped with H300 antibody. The first bottom part was propped first with Ser15, stripped and then propped with p53 antibody. The second blot was been cut as well into two parts as mentioned. The upper part was propped first with Ser211, stripped and propped with H300. The second bottom blot was propped with Ser20, stripped and propped with Actin.

Effect of JNK on GR and p53 phosphorylation

JNK has been shown to phosphorylate the human GR at Ser226 and JNK-mediated GR phosphorylation acted to attenuate GR transcriptional activity (Krstic *et al.*, 1997; Rogatsky, Logan and Garabedian, 1998). JNK-mediated phosphorylation of human GR at Serine 226 was also found to blunt hormone signaling by enhancing nuclear export of the GR (Krstic *et al.*, 1997; Kfir-Erenfeld *et al.*, 2010). Given that JNK regulates apoptosis and GR phosphorylation it was one candidate to mediate CM effects on GR. Therefore, the co-administration of JNK inhibitor with a synthetic GC

could reduce GR phosphorylation on Ser226 and potentially increase GR activity and the effectiveness of GCs in treating disease. To test this hypothesis, GC-resistant and sensitive human acute lymphoblastic CEM cells were pre-treated with JNK inhibitor (SP600125) prior to Dex and ETOP. Then, the western blot was performed and both total and phosphorylated GR and p53 were investigated using specific antibodies of total and phosphorylated GR at Ser211 and Ser226 and total and phosphorylated p53 at Ser15 and Ser20.

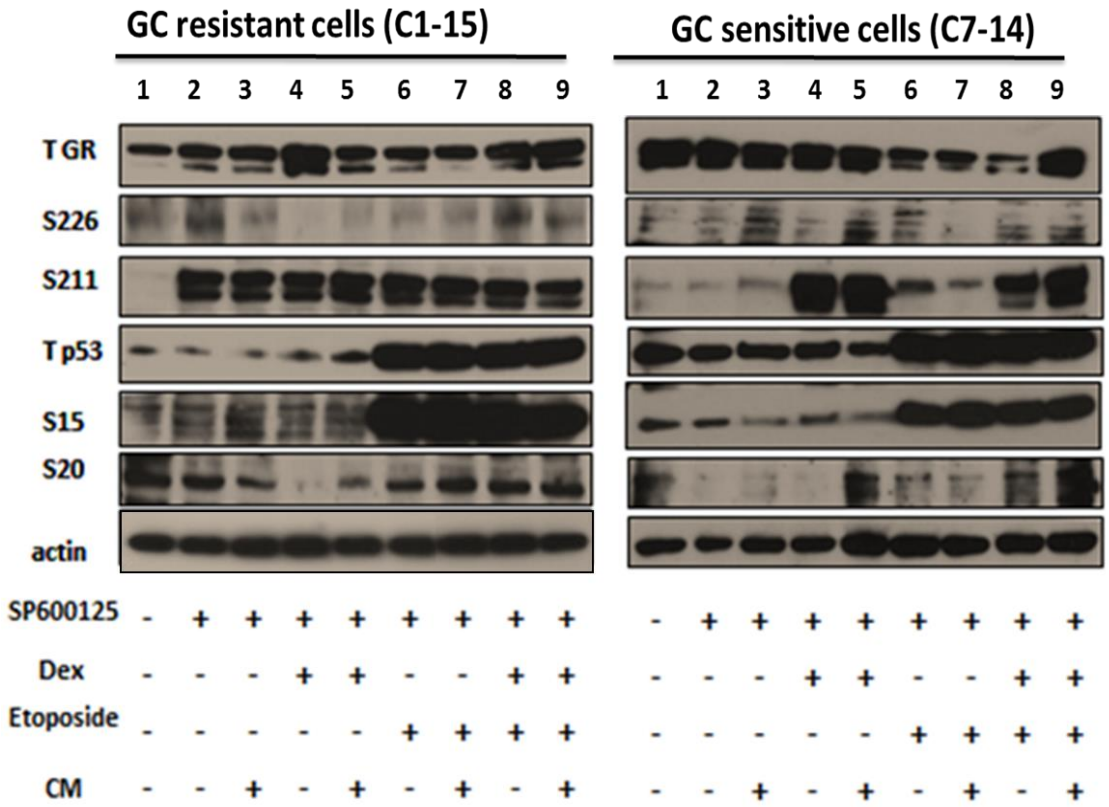


Figure 7.2.4**: Effect of JNK inhibitor SP600125 on GR and p53 phosphorylation profiles,** CEM-C1-15 (left panel) and CEM-C7-14 (right panel) cells were pre-treated with SP600125 (10μM) for 2 hours prior to the addition of Dex and ETOP. Cells were lysed, cellular extract were isolated and subjected to western blot analysis. Actin was used as a loading control. Data are representative of two independent experiments. 2 gels were prepared for one experiment and the blots were stripped out for one time after the protein and its corresponding actin been detected. The first blot was been cut into 2 parts upper and bottom parts from the 72KD point. The upper part was propped with Ser226 antibody first, stripped and propped with H300 antibody. The first bottom part was propped first with Ser15, stripped and then propped with p53 antibody. The second blot was been cut as well into two parts as mentioned. The upper part was propped first with Ser211, stripped and propped with H300. The second bottom blot was propped with Ser20, stripped and propped with Actin.

Increased total GR protein levels were observed in both CEM-C1-15 and CEM-C7-14 cells treated with ETOP, Dex and CM in the presence of the JNK inhibitor compared to that treatment in the absence of the inhibitor (compare lanes 8 and 9 in Figure 7.2.4

left and right panels with lanes 7 and 8 in the Figure 7.2.1 left and right panels respectively). Furthermore, GR phosphorylation at Ser226 decreased in Dex and SP600125 treated CEM-C1-15 cells compared to these cells treated with Dex alone (compare lane 4 to lane 1 in Figure 7.2.4 left panel with lane 3 to lane 1 in the Figure 7.2.1 left panel respectively). Increased GR phosphorylation at Ser226 was detected in CM and JNK inhibitor treated CEM-C7-14 cells either alone or in combination with Dex (compare lanes 3 and 5 to lane 1 in Figure 7.2.4 right panel with lanes 3 and 4 to lane 1 in the Figure 7.2.1 right panel respectively). GR phosphorylation at Ser211 increased with the addition of JNK inhibitor in CEM-C1-15 cells irrespectively of the type of treatment (Figure 7.2.4 compare lanes 2-9 to lane 1 left panel). In CEM-C7-14 cells increased GR phosphorylation at Ser211 was observed in the presence of all treatments (compare lanes 8 and 9 in Figure 7.2.4 right panel with lanes 7 and 8 in the Figure 7.2.1 right panel respectively).

SP600125 treatment of the CEM-C1-15 cells in combination with Dex, ETOP and CM reversed the downregulation of the total p53 protein levels observed in these cells treated the same way in the absence of the inhibitor (compare lanes 8 and 9 in Figure 7.2.4 left panel with lanes 7 and 8 in the Figure 7.2.2 left panel respectively). On the other hand in CEM-C7-14 cells JNK inhibition did not have any major effect on the total p53 and phospho p53 Ser15 protein levels (compare lanes 2-9 in Figure 7.2.4 right panel with lanes 1-8 in the Figure 7.2.2 right panel respectively). The p53 Ser20 phosphorylation pattern differed in both Dex and Dex/ETOP/CM treated ALL cell lines in the presence of JNK inhibitor following the opposite pattern observed in the absence the inhibitor (compare lanes 4 and 9 in Figure 7.2.4 left and right panels with lanes 3 and 8 in the Figure 7.2.2 left and right panels respectively).

Together, these data suggest that, p38 MAPK has dual function in lymphoid cells. By the inhibition of p38MAP in GC resistance cells CM increased the GR Ser211 phosphorylation in the presence of combination of Dex and etoposide in contrast to the decreased GR Ser211 phosphorylation observed in the same cells in the absence of the inhibitor. Additionally, the treatment of JNK inhibitor together with GC leads to increase in total GR protein levels in both CEM-C1-15 and CEM-C7-14 cells treated with ETOP, Dex and CM in the presence compared to that treatment in the absence of the inhibitor. Furthermore, GR phosphorylation at Ser226 decreased in Dex and JNK inhibitor treated CEM-C1-15 cells compared to these cells treated with Dex alone. JNK affect GR phosphorylation and the effect of CM highlighting the

importance of post-translational modifications mediated by these kinases in the function of GR and hence the response to hormone treatment. Bone marrow generated conditioned media affects GR phosphorylation most likely through both p38 MAPK and JNK pathways suggesting that disregulation of these signal transduction pathways are general mechanisms leading to GC-resistance in hematological malignancies.

Effect of PI3K on GR and p53 phosphorylation

Another important signal transduction pathway that is activated by a variety of growth factors is the PI3K pathway. Class I phosphatidylinositol 3-kinase (PI3K) and its downstream effectors have emerged in recent years as a major proliferation- and survival-related pathway (Bar *et al.*, 2005). The phosphatidylinositol-3 kinase (PI3K) pathway in normal cells responds to exposure to growth factors and regulates a cascade of proliferation and survival signals (Luo, Manning e Cantley, 2003). As the PI3K pathway is abnormally active in many cancers, we analysed the possible role of PI3K signaling in GR and p53 activation and function. Cells were exposed to LY294002, a potent PI3K inhibitor in the absence or presence of Dex and CM.

Data showed that PI3 kinase inhibitor LY294002 did not have a major effect on total GR protein level and phospho Ser226 levels in CEM-C1-15 cells. Hormone dependent GR phosphorylation at Ser211 was not observed in CEM-C7-14 and CEM-C1-15 cells treated with LY294002 (Figure 7.2.5 compare lanes 5 and 7 to lanes 6 and 8, left and right panels and Figure 7.2.1 compare lanes 3 and 4 to lanes 1 and 2 left and right panels). In CEM-C7-14 cells LY294002 downregulated the receptor's protein levels in the presence and absence of conditioned media and Dex (Figure 7.2.5 compare lanes 2, 6 and 8 to lanes 1, 5 and 7).

Total and phosphorylated level of p53 at Ser15 and Ser20 were reduced whenever the cells were treated with the PI3K inhibitor generally in both cell lines (Figure 7.2.5 compare lanes 2, 4, 6 and 8 to lanes 1, 3, 5 and 7). To conclude most marked effects of the PI3 kinase inhibitor are on the downregulation of the total GR protein levels in the CEM-C7-14 cells whereas the effects of this inhibitor in the CEM-C1-15 cells seems to be on the GR Ser211 phosphorylation. These findings invoke a negative role of PI3K in GR and p53 activation, through selective effects on the protein and phosphorylation levels.

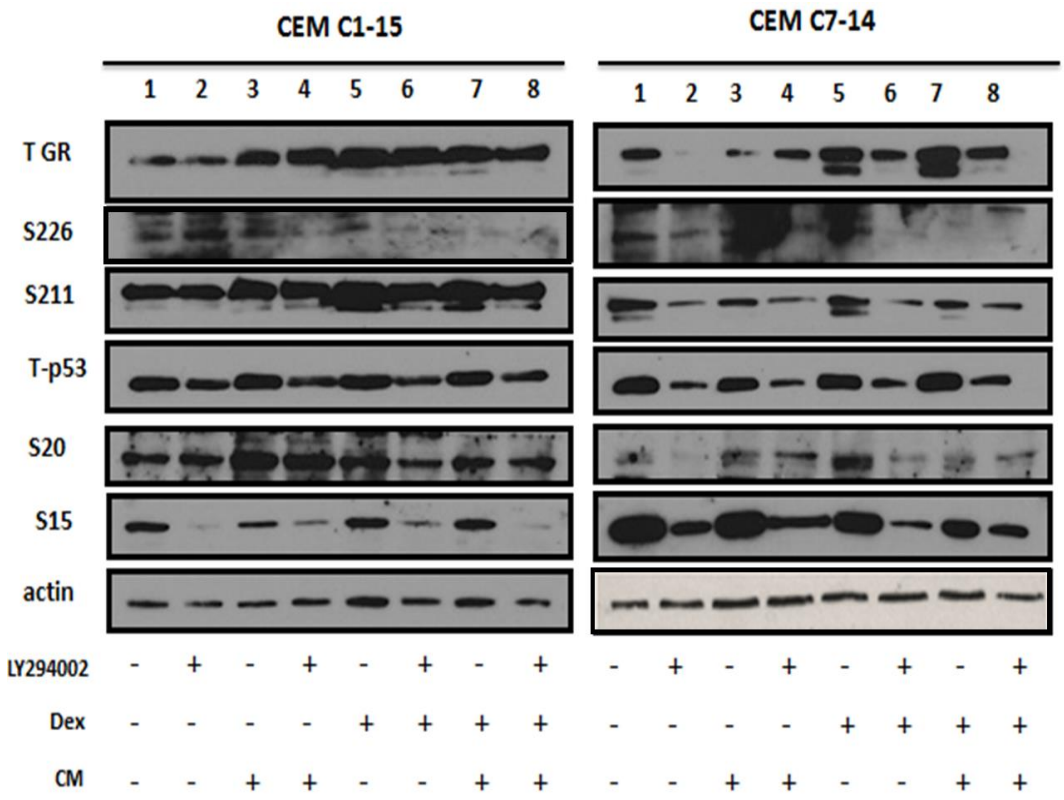


Figure 7.2.5: **Effect of PI3K inhibitor LY294002 on GR and p53 phosphorylation profiles,** CEM-C1-15 and CEM-C7-14 cells were pre-treated with LY294002 (10μM) for 2 hours prior to Dex and ETOP. Cells were lysed, total cell protein extracts were normalized and subjected to western blotting. Actin was used as a loading control. Data are representative of 3 independent experiments. 2 gels were prepared for one experiment and the blots were stripped out for one time after the protein and its corresponding actin been detected. The first blot was been cut into 2 parts upper and bottom parts from the 72KD point. The upper part was propped with Ser226 antibody first, stripped and propped with H300 ntibody. The first bottom part was propped first with Ser15, stripped and then propped with p53 antibody. The second blot was been cut as well into two parts as mentioned. The upper part was propped first with Ser211, stripped and propped with H300. The second bottom blot was propped with Ser20, stripped and propped with Actin.

PI3K inhibition increased apoptotic activity in leukemia cells

The effect of the PI3K inhibitor LY294002 on GR and p53 status was tested in a number of additional experimental conditions. To examine whether inhibition of PI3K promotes apoptosis in leukemia cells, the cell cycle distribution of pre-treated PI3K inhibitor (LY294002) leukemia cells was analyzed. Cells were stained with propidium iodide (PI) and analyzed by flow cytometry. Results shown in Figure 7.2.6 revealed that there was a marked increase in the percentage of dead cells in the subG1 phase of the cell cycle in cells treated with LY294002, Dex and CM compared with cells treated with Dex and CM without the inhibitor (Figure 7.2.6 , compare A and B bar 7

to bar 8). Moreover, increased percentage of dead cells was observed selectively in CEM-C7-14 cells treated with CM plus PI3K inhibitor compared to cells treated with CM only (Figure 7.2.6, B, compare bar 3 to bar 4). As expected, high percentage of dead cells was observed in response to Dex treatment in the glucocorticoid sensitive cell line compared to glucocorticoid resistant cell line under the same conditions (Figure 7.2.6, compare A and B bars 5 to bars 1).

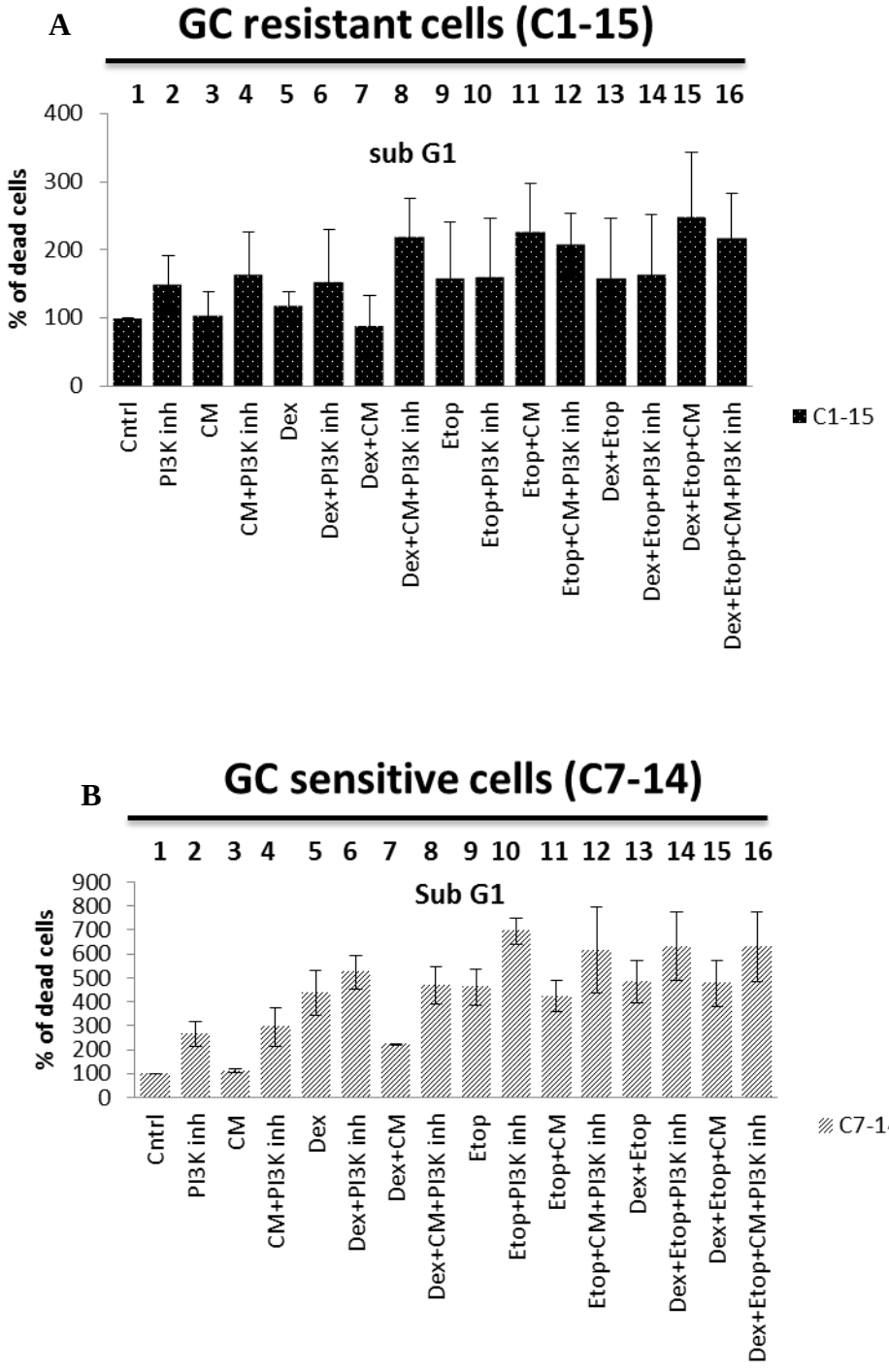


Figure 7.2.6: **Regulation of ALL cell death by the PI3K inhibitor, LY294002.** CEM-C1-15 (A) and CEM-C7-14 (B) cells were pre-treated with LY294002 (10μM) for 2 hours prior to Dex and ETOP and submitted to flow cytometry. The number of cells in the Sub-G1 phase of the cell cycle after the indicated treatment was plotted. Results shown are representative of 3 independent experiments.

The role of the PI3K pathway in the regulation of apoptosis and autophagy

Our previous results indicated that several potentially different PI3K dependent pathways may lead to cell death in glucoccorticoid sensitive and resistant cell lines. We therefore analysed the role played of PI3K on the death pathway and which potential type of cell death is involved, apoptosis or autophagy .For that, the two cell lines, CEM-C1-15 and CEM-C7-14 cells were cultured in DCC-treated media with or without the PI3K inhibitor LY294002 (10μM) for 2 hours prior to Dex and ETOP and then extract of the proteins been subjected to western and probed with very well known autophagy and apoptosis biomarkers which are Beclin (early autophagy), LC3 (late autophagy) and cleaved caspase3(apoptosis).

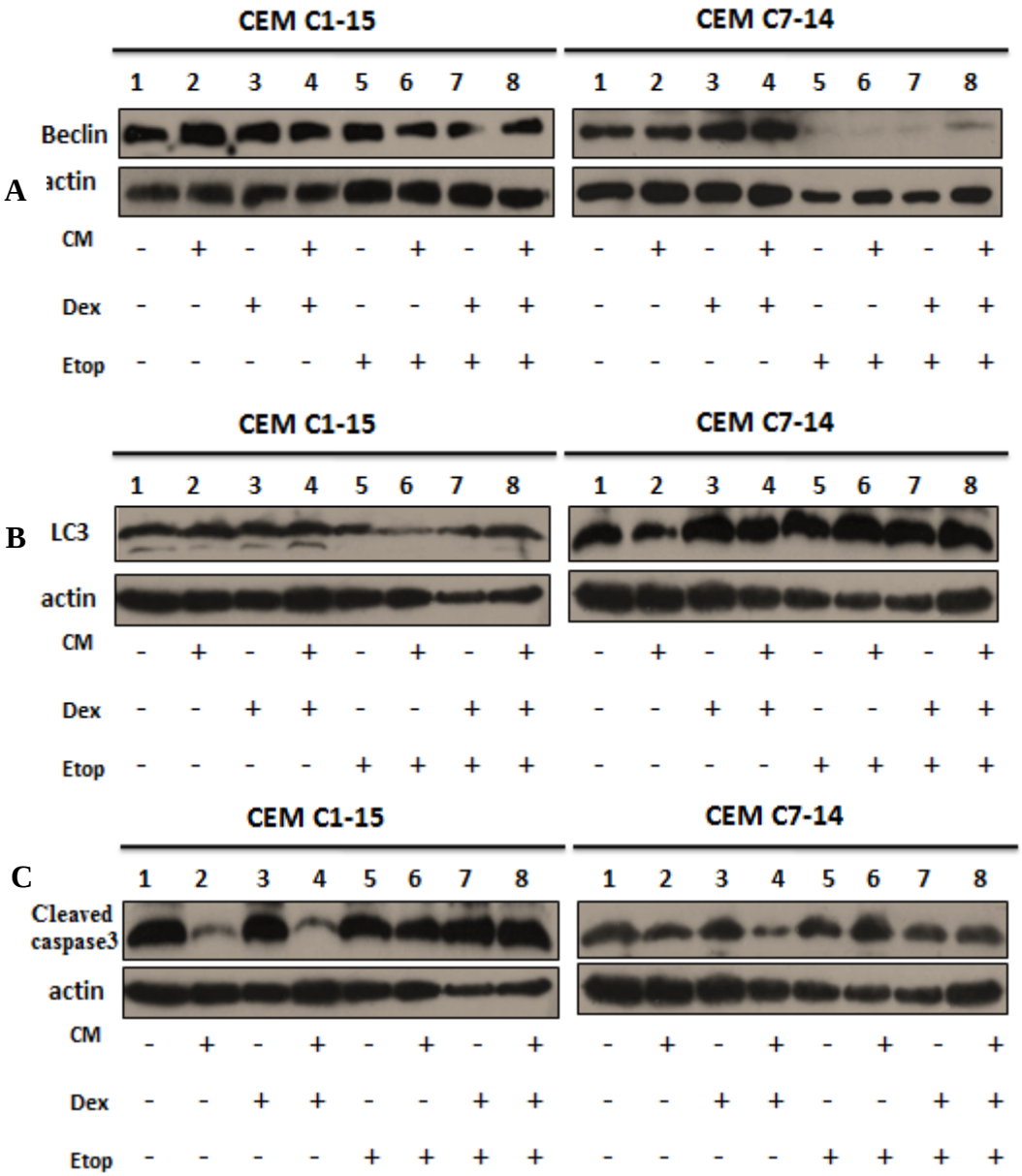
In figure 7.2.7 (A) The data showed unecpectedly upregulation of beclin protein levels in cells treated with Dex or combination with Dex and CM in lane no 3 and 4 in both cell lines with effect being stronger in CEM-C7-14 cell lines. Beclin is downregulated with ETOP, in CEM-C7-14 cells only (Figure 7.2.7, lanes 5, 6, 7 and 8).

In Figure 7.2.7 (B) another autophagy target protein was detected which is LC3 and it is known as late autophagy marker protein (Tanida *et al* , 2008). The data showed that LC3 is upregulated with Dex treatment (Figure 7.2.7 B lane 3) in both cell lines and similarly to beclin effect is more prominent in CEM-C7-14 cell line. On the other hand, the protein level of LC3 are downregulated in both cell types treated with CM (Figure 7.2.7 B lane no 4).

Cleaved caspase 3 showed the same effect of the treatment as LC3. The data showed that cleaved caspase 3 is upregulated with Dex treatment (Figure 7.2.7 C lane no 3) in both cell lines. On the other hand, treatment of both cell lines with CM results in downregulation of the protein levels of cleaved caspase 3 (Figure 7.2.7 C lane 4). This result highlights the role of the cell death pathway, the effect of CM on it, and how it may contribute to ALL resistance.

In an attempt to further analyse the role of PI3K in the death pathway, CEM-C7-14 cells were treated with the LY294002 inhibitor of PI3K activity in the presence or absence of Dex, ETOP and CM as shown in Figure 7.2.7 D and then the membrane

probed with Beclin, LC3 and cleaved caspase3 antibodies. The data showed differential effects in two cell lines. In CEM-C1-15 inhibition of the PI3K leads to increase autophagy protein levels (Beclin and LC3) and reduction of apoptosis (cleaved caspase 3) (Figure 7.2.7 D lane no 3 of C1-15 cells). On the other hand, the glucocorticoid sensitive CEM-C7-14 cells showed increased in apoptosis (cleaved caspase 3) and reduction of autophagy protein levels (Beclin and LC3) after inhibition of the PI3K pathway (Figure 7.2.7 D lane 3 of CEM-C7-14 cells).This reflects the different regulation of the PI3K pathway in both cell lines which affect the response to the treatment.



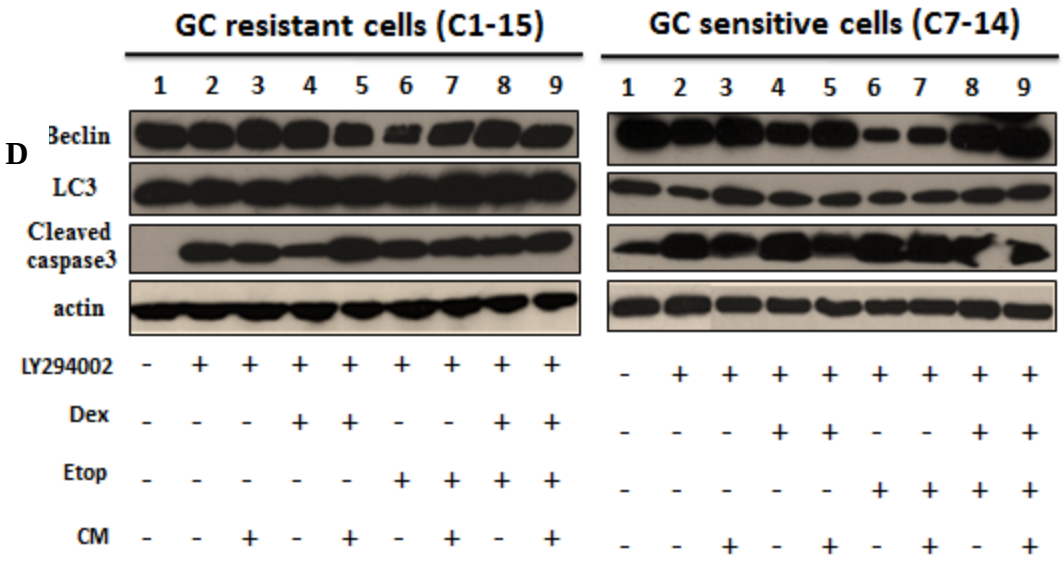


Figure 7.2.7: **The PI3K pathway differentialy regulates apoptosis and autophagy.**CEM-C1-15 and CEM-C7-14 cells were treated with Dex(1μM), ETOP (10μM) and CM. Cells were lysed and probed with Beclin antibody (A), LC3(B) and cleaved caspase 3(D).CEM C1-15 and C7-14 cells were pre-treated with LY294002 (10μM) for 2 hours prior to dex and ETOP(D). Cells were lysed and probed with Beclin, LC3 and cleaved caspase 3 antibodies. Actin was used as a loading control and the results are representative of 3 independent experiments. Note: Actin in figure B and C is the same, as the membrane was first probed with LC3 and then cleaved caspase 3.

7.2.4 Conclusion

GR is highly phosphorylated by several kinases in multiple sites and affects the cell fate negatively or positively. Investigation of CM role on GR phosphorylation status indicates a complex crosstalk may be involved in this regulation. Moreover, CM has significant effects on GR phosphorylation profile, by increasing Ser211 phosphorylation and decreasing Ser226 phosphorylation. GR phosphorylation at Ser226 was more intense than Ser211 in glucocorticoid resistant cell CEM-C1-15 which explains the resistance of CEM-C1-15 toward the treatment. On the other hand, GR phosphorylation at Ser211 is more intense in glucocorticoid CEM-C7-14 which promotes cell death. In order to determine molecular basis for CM effects, we investigated the pathways that may alter phosphorylation of GR and p53. Data suggests that in lymphoid cells, p38 MAPK-induced Ser211 phosphorylation of GR to promote GC sensitivity as apoptosis while JNK activity decreased the amount of Ser211 phosphorylation resulting in enhanced GC resistance JNK by improving the nuclear export of the GR. Additionally, PI3 kinase pathway invokes a negative role of PI3K in GR and p53 activation, and suggests that the efficacy of PI3K inhibitors in

cancer therapy may be greatly affected by the GR and p53 status. PI3K inhibitor increase apoptosis. This reflects the different regulation of the PI3K pathway in both cell lines which affects the response to the treatment. On the other hand an effect of CM on p53 phosphorylation status is limited. Thus, by gaining a better understanding of GR phosphorylation profiles, we can develop better therapies to combat GC resistance. A better and more complete understanding of GC signaling will aid in the improvement of current GC therapies and hopefully lead to the enhanced clinical outcomes of patients.

7. 3 Transcriptome alterations in cells treated with Dex, ETOP and CM

To further investigate the role of combined treatment and BMCM in cell resistance and to determine molecular basis of a potential crosstalk between dexamethasone and ETOP induced pathways and to analyze novel target genes, transciptome analysis has been performed to compare differences in gene expression profiles when individual treatments by dexamethasone and ETOP are compared to combined Dex, and ETOP treatment in the presence and absence of conditioned medium. Microarray experiments will determine the dissimilarities in gene networks in ALL cells during individual and combined treatments with dexamethasone and topoisomerase II inhibitors essential for understanding of molecular and cellular basis of drug action which can result in therapy improvement. The control was used here is untreated sensitive cells CEM C7-14.

Microarray analysis was performed using U133 plus 2 array from Affymetrix in CEM C7-14 cells resulting in differential expression of 2632 genes (P value < 0.05 and fold change ≥ 2) (Figure 7.3.1). Profile filtering method was used to cluster genes into 8 categories based on similarity of expression profiles across the dataset using a k-means clustering algorithm (Brown *et al.*, 2005). Clustering was performed on the means of each sample group (log 2) that had been z-transformed (for each probeset the mean set to zero, standard deviation to 1). K-means clustering was done on the basis of similarity of profiles (Manhattan Distance) across the dataset. This method clusters all genes that have the same trend of expression across different treatments. Background correction, quantile normalization, and gene expression analysis were performed using RMA in Bioconductor. Functional annotation of the genes in clusters was performed using DAVID. This type of microarray analysis depends on using 1) gene enrichment, 2) clustering, and 3) identification of biologically related gene groups.

Gene enrichment highlighted 2632 genes with significant CM and/or GC-mediated changes in leukemia expression. Clustering partitioned these into eight gene clusters based on expression profile similarity (8 clusters and each treatment expression values involved in the CD attached at the back of the thesis). EASE analysis was used to identify biologically related gene groups based on overrepresentation of GO terms (Figure 7.3.1). These ontology terms indicate biological processes or cellular

functions that are specifically altered in the leukemia cells according to the effect of the treatment. Some EASE analysis for each cluster is available in Supplementary table in the appendices.

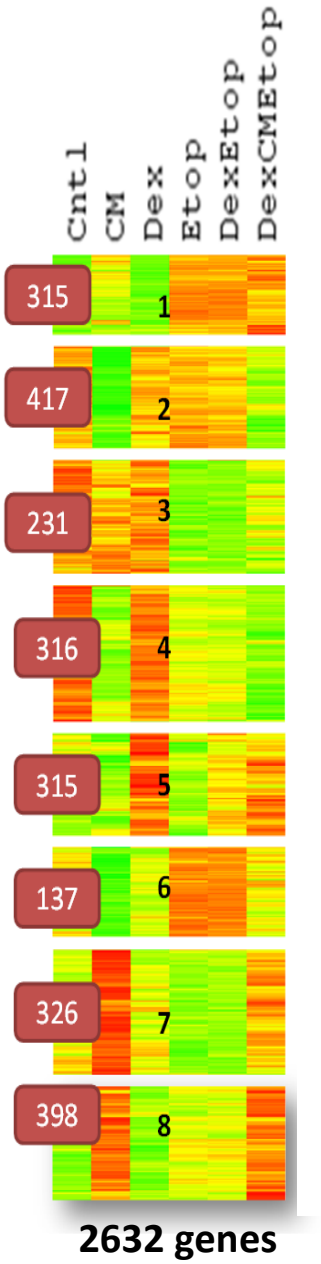


Figure 7.3.1: **Heatmap of mRNA expression in CEM-C7-14 cells obtained by microarray analysis.** The heat map of 2632 genes filtered (P value < 0.05 and fold change ≥ 2), showing

the 8 clusters. Levels of expression are indicated on a colour scale where red represents the up-regulated genes and green the down-regulated genes.

7.3.1 Heat maps showing the hierarchical clustering of relevant genes

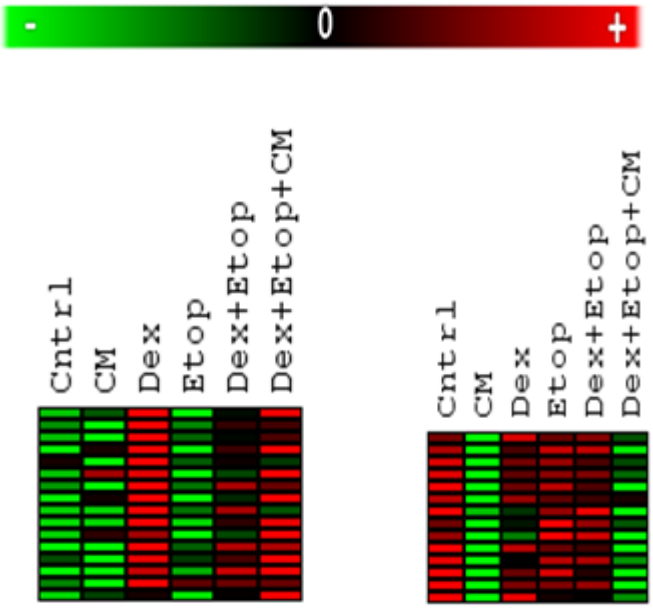
To further investigate the role of CM in ALL resistance and to determine molecular basis of a potential crosstalk between Dex and ETOP induced pathways, a series of microarray analysis were carried out under selected treatment. CEM-C7-14 cells were treated with CM, Dex and ETOP on its own or in combination with one another. There were in total 6 treatments, these are; (1) control, (untreated GC sensitive cells) (2) CM, (3) Dex, (4) ETOP,(5) Dex & ETOP (6) Dex, ETOP and CM, 133 plus 2 Affymetrix microarrays gene expression from the eight clusters was further investigated according to the association with the cellular process.All treatments were compared to control and aftr that we clustered genes from above mentioned heatmap according to the cellular process. We considered apoptosis, autophagy, cytoplasmic vesicles, cell cycle progression and inflammation.

Genes involved in control of apoptosiss

Since the resistance to apoptosis is a key process in leukaemia development and resistance to the treatment, we needed to know the effect of stem cell microenvironment on apoptotic genes. For that, heatmap of apoptosis genes was analyzed (Figure 7.3.2 A; Table 17). Our data revealed that most of apoptotic genes are upregulated with Dex treatment and combination of Dex, ETOP and CM. The only significantly marked effect is the downregulation of Bim (BCL-2-interacting mediator of cell death) (Figure 7.3.2, A line 2 and Table 17) and Fos-L2 (Figure 7.3.2, A line 5 and Table 17) genes by treatment of CM and the combination. Bim (BCL-2-interacting mediator of cell death), a BCL-2 homology 3-only pro-apoptotic protein, is upregulated by Dex treatment in acute lymphoblastic leukaemia cells and has an essential role in Dex-induced apoptosis (Thompson and Johnson, 2003; Strasser, 2005). It has been indicated that Dex induced Bim is regulated mainly by transcription through indirect effects most likely involving Jun induction by GR. However, the molecular mechanisms including responsible transcription factors are unclear (Chen *et al.*, 2012). The second downregulated gene is Fos-L2 or (FOS)-related antigen 2 (FRA2). It is a member of the FOS family activator protein-1 (AP1) transcription factors, these genes encode leucine zipper proteins that can dimerize with proteins of

the JUN (aviansarcoma virus 17 oncogenehomolog JUN family) mainly (JUND), thereby forming the transcription factor complex AP-1. AP-1 activation leads to downregulation of pro-apoptotic genes such as p53, Bim and Fos-L2 and upregulates the pro-oncogene MYB. As such, the FOS proteins have been implicated as regulators of cell proliferation, differentiation, and transformation (Kerppola *et al*, 1993; Molven *et al*, 1996).

On the other hand, anti-apototic genes (Figure 7.3.2 B; Table 18) are downregulated with the all treatment and with CM alone, which excluded the role of CM on the anti-apoptotic proteins. As a conclusion, stem cells microenvironment is contributing to resistance to apoptosis mainly through decreasing Bim and Fos-L2 proteins and less through increase in anti-apototic proteins.Decreasing mRNA level of Bim through CM treatment came in agreement in CEM C7-14 cells (Figure 7.1.8). Understanding the mechanisms of resistance to apoptosis may lead to the development of therapies that can reprogram cell death.



(A) Pro-apoptosis **(B) Anti-apoptosis**

Figure 7.3.2: **Heatamp of different treatment expression values of (A) apoptosis (B) anti-apoptosis genes**. Heatmap was generated using online tool for genomic data exploration, Multiple Experiment Viewer (MeV)http://www.tm4.org/.

Symbol	**GENE NAME**
AKAP13	A kinase (PRKA) anchor protein 13
BCL2L11	BCL2-like 11 (apoptosis facilitator)
CD24	CD24 molecule
DDIT4	DNA-damage inducible transcript 4
FOSL2	FOS-like antigen 2
GRAMD4	GRAM domain containing 4

BIRC3	Baculoviral IAP repeat containing 3
ELMO2	Engulfment and cell motility 2
LOC643733	Hypothetical LOC643733
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor
PTEN	Phosphatase and tensin homolog
SRGN	Serglycin
SGK1	Serum/glucocorticoid regulated kinase 1
SLC18A2	Solute carrier family 18 (vesicular monoamine), member 2
TRIM35	Tripartite motif-containing 35
TNFRSF10D	Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated dea

Table 17: **List of pro-apoptotic genes**. List corresponds to apoptosis heatmap shown above.

Symbol	**GENE NAME**
BAG3	BCL2-associated athanogene 3
BAG4	BCL2-associated athanogene 4
BNIP1	BCL2/adenovirus E1B 19kDa interacting protein 1
DNAJA3	DnaJ (Hsp40) homolog, subfamily A, member 3
ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha
TRIAP1	TP53 regulated inhibitor of apoptosis 1
CASP2	Caspase 2, apoptosis-related cysteine peptidase
F3	Coagulation factor III (thromboplastin, tissue factor)
DLX1	distal-less homeobox 1
HELLS	helicase, lymphoid-specific
FAM164A	Family with sequence similarity 164, member_A
NME1	non-metastatic cells 1, protein (NM23A) expressed 1
C8orf44/// SGK3	Chromosome 8 open reading frame 44 /// serum/glucocorticoid regulated kinase fam
ZC3HC1	Zinc finger, C3HC-type containing_1

Table 18: **List of anti-apoptosis genes List corresponds to the** heatmap shown in Figure 7.3.2 B.

Autophagy

Second studied process is autophagy (Figure 7.3.3; Table 19). Autophagy is a type of cellular catabolic degradation response to nutrient starvation or metabolic stress and p53 is the main upstream regulator of the autophagic pathway (Maiuri *et al.*, 2010). Numerous recent reports have indicated the link between autophagy and cancer (Furuya *et al*, 2004). However, it is still not clear whether autophagy is a main strategy for cell survival, or if it also serves as a trigger for cell death. Although it is still controversial about whether autophagy kills cancer cells or sustains their survival under stressful conditions, numerous recent reports provide data to support the notion that autophagy promotes cancer cell survival after chemotherapy or radiation therapy.

The functional relationship between apoptosis ('self-killing') and autophagy ('self-eating') is complex, under certain circumstances, autophagy constitutes a stress adaptation that avoids cell death (and suppresses apoptosis), whereas in leukaemia, it constitutes an alternative cell-death pathway (Maiuri *et al.*, 2007). Autophagy and apoptosis may be triggered by common upstream signals, and sometimes these results in combined autophagy and apoptosis. On a molecular level, this means that the apoptotic and autophagic response machineries share common pathways that either link or polarize the cellular responses. Experimental evidence for autophagy playing a role in the regulation of programmed cell death is limited (Figure 7.3.3; Table 19). Gene's titles of autophagy were adapted from DAVID; EASE analysis was used to identify biologically related gene groups based on overrepresentation of GO terms. These ontology terms indicate biological processes or cellular functions called lysosome. Since lysosome is the main structure in autophagy, lysosome consider as autophagy. Moreover, although DAVID has listed the genes identified as being included in the "autophagy" group, they are not necessarily restricted to that group.Microarray data analysis has shown that the autophagy genes e.g. Atg, DRAM1, DRAM2, Cathepsin and Beclin are regulated in CEM-C7-14 and p53 is the main upstream regulator of the autophagy. This demonstrates the autophagy is involved together with the apoptosis in ALL response. So, the combination of ETOP with the glucocorticoid triggered autophagy dependent cell death as well.

Moreover, gene expression analysis from cells treated with the ETOP and Dex revealed that, 7% of up-regulated genes belongs to autophagy and 15% of genes belongs to apoptosis, of which apoptosis is the most prominent type of cell death in T-ALL (Figure 7.3.3; Table 19).

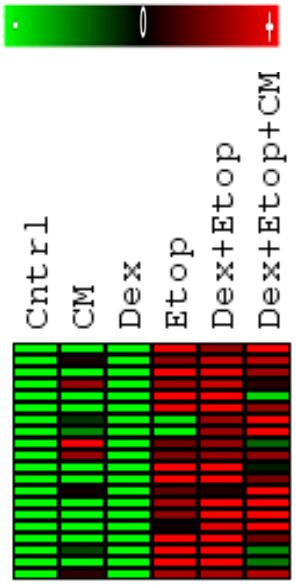


Figure 7.3.3: **Heatmap of different treatment expression values of genes involved in autophagy which indicated that p53 is the main upstream regulators of the autophagic pathway.** Heatmap was generated using online tool for genomic data exploration, Multiple Experiment Viewer (MeV) http://www.tm4.org/.

Symbol	GENE NAME
ABCA5	ATP binding cassette, sub-family A (ABC1), member 5
CD63	CD63 molecule
NAAA	N-acylethanolamine acid amidase
AGA	Aspartylglucosaminidase
CTSH	Cathepsin H
CTSK	Cathepsin K
C1orf85	Chromosome 1 open reading frame 85
GLB1	galactosidase, beta 1
GGH	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)
GUSB	Glucuroidase, beta
LGMN	Legumain
MFSD8	Major facilitator superfamily domain containing 8
HLA-DMA /// HLA-DMB	Major histocompatibility complex, class II, DM_alpha /// major histocompatibilit
EGFL8 /// PPT2	EGF-like domain, multiple 8 /// palmitoyl-protein thioesterase 2
PRCP	Prolylcarboxypeptidase (angiotensinase C)
STX3	Syntaxin 3
TM9SF1	Transmembrane 9 superfamily member 1
TMEM55A	Transmembrane protein 55A
TMEM9	Transmembrane protein 9
VAMP4	Vesicle associated membrane protein 4

Table 19: **List of autophagy genes included in autophagy heatmap.**

Exosomes

The tumour microenvironment plays a critical role in cancer progression, but how it promotes resistance in leukaemia is poorly understood. Exosomes are small

membrane vesicles that originate in large multivesicular bodies (MVBs) and are released in the extracellular milieu upon fusion of MVBs with the plasma membrane. They are secreted by many cell types and enable a mode of intercellular communication. Currently, it is unknown whether BMSCs secrete exosomes and whether these microvesicles support leukaemia cell survival. Previous report indicated that stromal derived exosomes were enriched in micro RNA (miRNA) expression (Théry *et al*, 2002). Inappropriate release of miRNAs from exosomes may cause significant alterations in biological pathways that affect disease development, supporting the concept that miRNA-containing exosomes could serve as targeted therapies for particular diseases (Fevrier and Raposo, 2004; Taylor and Gercel-Taylor, 2008). Our data shows overrepresentation of the genes with the assigned GO term cytoplasmic vesicles, which arise from the leukaemia cells CEM-C7-14 T-ALL cells after treatment with CM alone or combined with ETOP and Dex. Proteins that are involved in vesicle formation are shown in Figure 7.3.4 and Table 20. Among these gene products are RAB4A, a family member of RAS oncogene which has been found upregulated in breast cancer metastasis (Iorio *et al.*, 2005). Moreover, a family member of phosphatidylinositol-4-phosphate 3-kinase which is PIK3C2B is upregulated with the CM treatment, it is known to inhibit apoptosis through blockage of apoptosis effector protein cleaved caspase 3 (Martini *et al.*, 2013). In addition, ATG1 (autophagy related 1 homolog) is upregulated in T-ALL, and increased ATG1 can inhibit autophagy (Scott *et al*, 2007). Among upregulated genes is the mitogen-activated protein kinase associated protein 1(MAPKAP1), which is a subunit of mTORC2, which regulates cell growth and survival in response to hormonal signals. Insulin-like growth factor binding protein 2 (IGFBP2) showed increased expression and is associated with adverse outcome in childhood leukemia (Crofton *et al.*, 2000).

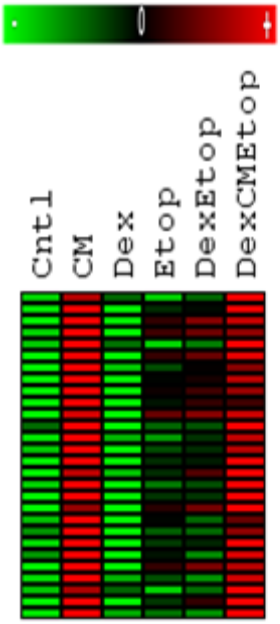


Figure 7.3.4: **Heatmap of different treatment expression values of genes linked to exosomes which indicated involvement of Ras, PI3K, MAPK pathways in bone marrow regulating leukaemia cells.** Heatmap was generated using online tool for genomic data exploration, Multiple Experiment Viewer (MeV) http://www.tm4.org/.

Gene symbol	Gene Title
ATP7A	ATPase, Cu++ transporting, alpha polypeptide
ATP8A1	ATPase,aminophospholipid transporter (APLT), class I, type 8A, member 1
RAB4A	RAB4A, member RAS oncogene family
WIPF1	WAS/WASL interacting protein family, member 1
ARSA	Arylsulfatase A
BSG	Basigin (Ok blood group)
CANX	Calnexin
ERP29	Endoplasmic reticulum protein 29
HSP90B1	Heat shock protein 90kDa beta (Grp94), member 1
IGFBP2	Insulin-like growth factor binding protein 2, 36kDa
LAMP1	Lysomal-associated membrane protein_1
LAMP2	Lysosomal-associated membrane protein_2
MAPKAP1	Mitogen-activated protein kinase associated protein 1
NAP1L1	Nucleosome assembly protein 1-like 1
OPTN	Optineurin
PRF1	Perforin 1 pore forming protein)
PIK3C2B	phosphoinositide-3-kinase, class 2, beta polypeptide
SERPINF1	Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium der
NEU1	Sialidase 1 (lysosomal sialidase)
SLC1A5	Solute carrier family 1 (neutral amino acid transporter), member 5
SLC40A1	Solute carrier family 40 (iron-regulated transporter), member 1
SPG21	Spastic paraplegia 21 (autosomal recessive, Mast syndrome)
SREBF1	Sterol regulatory element binding transcription factor 1
SNAP23	Synaptosomal-associated protein, 23kDa

SYTL1	Synaptotagmin like 1
TMEM187	Transmembrane protein 187
ULK1	Unc 51 like kinase 1 (C.elegans)
VAMP1	Vesicle associated membrane protein 1 (synaptobrevin 1)

Table 20: **List of genes linked to exosomes included in heatmap**

Cell cycle progression proteins

The genes related to cell cycle progression were clustered as shown in figure 7.3.5 and table 21. In this heatmap, all the records were downregulated with the CM treatment together with Dex and ETOP such as BRCA2, CDKN1A and CDC45 cell division cycle 45-like (S. cerevisiae) (fold change > 2.0; $p < 0.05$).

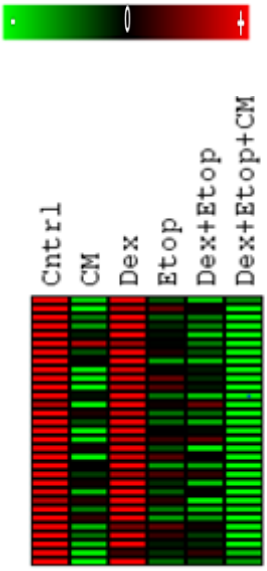


Figure 7.3.5: **Heatmap of genes liked to cell cycle progression.** Heatmap was generated using online tool for genomic data exploration, Multiple Experiment Viewer (MeV) http://www.tm4.org/.

Gene symbol	**Gene Title**
BCCIP	BRCA2 and CDKN1A interacting_protein
CDC45L	CDC45 cell division cycle 45 like (S.cerevisiae)
HAUS2	HAUS augmin like complex, subunit 2
HAUS8	HAUS augmin like complex, subunit 8
NIPBL	Nipped B homolog (Drosophila)
RAD21	RAD21 homolog (S.pombe)
RAD51C	RAD51 homolog C (S.cerevisiae)
RAD51L1	RAD51 like1 (S.cerevisiae)

SEH1L	SEH1 like (S.cerevisiae)
UHMK1	U2AF homology motif (UHM) kinase 1
ANXA1	Annexin A1
ASPM	Asp (abnormal spindle) homolog, microcephaly associated (Drosophila)
BCAT1	Branched chain aminotransferase 1, cytosolic
CDC2	Cell division cycle 2,G1 to S and G2 to M
CEP72	Centrosomal protein 72kDa
C13orf34	Chromosome 13 open reading frame 34
CLSPN	Claspin homolog (Xenopus laevis)
CUL1	Cullin 1
CUL4A	Cullin 4A
CCNA2	Cyclin A2
CCNE2	Cyclin E2
ESCO1	Establishment of cohesion 1 homolog 1(S.cerevisiae)
GSG2	Germ cell associated 2 (haspin)
KIF23	Kinesin family member 23
MNAT1	Ménage a trois homolog 1, cyclin H assembly factor (Xenopus laevis)
NCAPH	Non-SMC condensing I_complex,_subunit_H
PA2G4///PA2G4P4	Proliferation-associated 2G4, 38kDa /// proliferation-associated 2G4 pseudogene
PSMD1	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1
RBL1	Retinoblastoma-like 1
SUV39H2	Suppressor of variegation 3-9 homolog 2 (Drosophila)
TBRG4	Transforming growth factor beta regulator 4

Table 21: **List of cell cycle progression genes**. The genes included in cell cycle progression heatmap in figure 7.3.5.

Inflammation

Although inflammation has long been known as a localized protective reaction of tissue to irritation, injury or infection, characterized by pain, redness, swelling, and sometimes loss of function, there has been a new realization about its role in a wide variety of diseases, including cancer (Coussens and Werb, 2002; Dalgleish and O'byrne, 2006). While acute inflammation is a part of the defence response, chronic inflammation can lead to cancer, diabetes, cardiovascular, pulmonary and neurological diseases (Dalgleish and O'byrne, 2006).

Several pro-inflammatory gene products have been identified that mediate a critical role in suppression of apoptosis, proliferation, angiogenesis, invasion, and metastasis. Among these gene products are TNF and members of its superfamily, IL-1a, IL-1b, IL-6, IL-8, IL-18, chemokines, MMP-9 and VEGF. The expression of these genes is mainly regulated by the transcription factor NF-kB, which is active in most tumors (Dolcet *et al.*, 2005). Moreover, a primary response to inflammation is an increased

survival of the target cell. Several pathways have been identified that promote maintenance of the cell. The principal mechanism for the extended survival is through induction of anti-apoptotic Bcl-2 family proteins. Bcl-2 was the founding member of this family with five additional members, Bcl-XL, Bcl-W, Bcl-B, Bfl-1, and Mcl-1, discovered mostly in haematological malignancies, that is why studying inflammation is crucial for resistance due to stromal support.

On the other hand, some of genes are downregulated with CM as alone which are CD55 molecule, decay accelerating factor for complement (CD55), chemokine (C-C motif) receptor 2 and 4 (CCR2) and (CCR4), lymphocyte antigen 96 (LY96), complement component (C), Interleukin 10 (IL10), NF-kB. On the other hand Caspase6 (Casp6) and tumor necrosis factor (TNF) are downregulated with the treatment combination and overexpressed with CM as alone (Figure 7.3.6; Table 22).

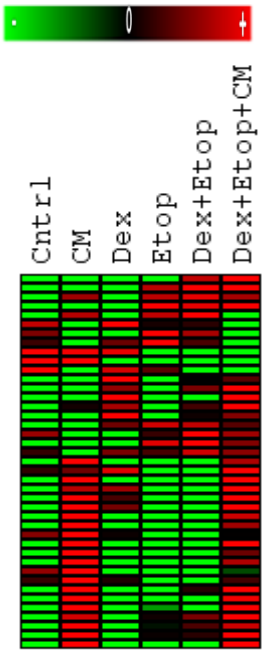


Figure 7.3.6: **Heatmap of different treatment expression values of inflammation**. Heatmap was generated using online tool for genomic data exploration, Multiple Experiment Viewer (MeV) http://www.tm4.org/.

Gene symbol	Gene Title
CD55	CD55 molecule, decay accelerating factor for complement (Cromer blood group)
AOC3	amine oxidase, copper containing 3 (vascular adhesion protein 1)
F8	coagulation factor VIII, procoagulant component
IRF7	interferon regulatory factor 7
IL15	interleukin 15
SCYE1	aminoacyl tRNA synthetase complex-interacting multifunctional protein 1

F3	coagulation factor III (thromboplastin, tissue factor)
CFD	complement factor D (adipsin)
CASP6	caspase 6, apoptosis-related cysteine peptidase
CCR7	chemokine (C-C motif) receptor 7
ANXA1	annexin A1
CD24	CD24 molecule; CD24 molecule-like 4
CCR2	chemokine (C-C motif) receptor 2
IRGM	immunity-related GTPase family, M
LY96	lymphocyte antigen 96
TLR5	toll-like receptor 5
HDAC5	histone deacetylase 5
SCG2	secretogranin II (chromogranin C)
TICAM2///TMED7-TICAM2	transmembrane emp24 protein transport domain containing 7; toll-like receptor adaptor molecule 2
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog
GPR68	G protein-coupled receptor 68
CCR4	chemokine (C-C motif) receptor 4
C1RL	complement component 1, r subcomponent-like
C9	complement component 9
HDAC4	histone deacetylase 4
IL10	interleukin 10
ITCH	itchy E3 ubiquitin protein ligase homolog (mouse)
KRT1	keratin 1
NFATC3	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 3
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
P2RX7	purinergic receptor P2X, ligand-gated ion channel, 7
STAT5B	signal transducer and activator of transcription 5B
TNF	tumor necrosis factor (TNF superfamily, member 2)
TNFRSF1A	tumor necrosis factor receptor superfamily, member 1A
LY75	CD302 molecule; lymphocyte antigen 75
CD44	CD44 molecule (Indian blood group)
C5	complement component 5
CFP	complement factor properdin
ITGB2	integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)
LIPA	lipase A, lysosomal acid, cholesterol esterase
P2RX1	purinergic receptor P2X, ligand-gated ion channel, 1

Table 22: **Titles of inflammation genes included in inflammation heatmap.**

Jak-Stat pathway

Genes upregulated by CM (clusters 1 and 7) are found in Jak-Stat pathway and belong to family of cytokines (interleukin 10 and 15), cytokine receptors (interleukin 11 receptor alpha, interleukin 7 receptor, interferon (alpha, beta and omega) receptor 1), PI3K and STAT5. All components apart from STAT5 upregulate the pathway such as STAT4 (signal transducer and activator of transcription 4) and IL2 (interleukin 10) .It

appears that genes that express components of the Jak-SAT pathway are upregulated by CM and downregulated by dexamethasone. Genes that belong to the Jak-STAT pathway in cluster 1 are upregulated by CM and downregulated by dexamethasone treatment. Characteristic example is the interleukin 15 which is 2 folds upregulated by CM and 2 folds dowreguated by dexamethasone. Analysis of cluster one indicates that in principle genes belonging to this cluster is upregulated by CM and GR has the opposite effect and the net result is that Dex downregulates the pathway and CM activates it. Given that pathway may have overall proliferative effect this may explain antiapoptotic effect of CM and cause of resistance (Figure 7.3.7; Table 23).

Genes downregulated by CM (clusters 2 and 4) found in Jak-STAT pathway include protein tyrosine phosphatase, non-receptor type 6, phosphoinositide-3-kinase, catalytic, gamma polypeptide, sprouty homolog 4 (Drosophila), suppressor of cytokine signaling 4.

Genes upregulated by GR and not affected by CM (cluster 5) include interleukin 7 receptor, signal transducer and activator of transcription 4, suppressor of cytokine signaling 1, thyroid peroxidase and oncostatin M receptor. Three major classes of negative regulators of the Jak-STAT pathway have been described including the SOCS, PIAS and SHP-1. SOCS family members (SOCS1) are upreguated by dexamethasone and not affected by CM treatment (cluster 5) (Figure 7.3.7; Table 23).

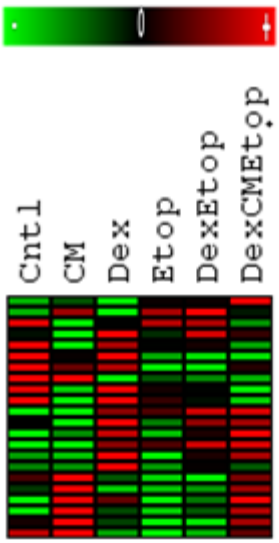


Figure 7.3.7: **Heatmap of different treatment expression values of Jak/Stat pathway.** Heatmap was generated using online tool for genomic data exploration, Multiple Experiment Viewer (MeV) http://www.tm4.org/.

Gene Symbol	Gene Title
IL10	Interleukin 10
STAT4	Signal transducer and activator of transcription 4
SOCS1	Suppressor of cytokine signaling 1
IL11RA	Interleukin 11 receptor, alpha
IL7R	Interleukin 7 receptor
SOS2	Son of sevenless homolog 2 (Drosophila)
IL6ST	Interleukin 6 signal transducer (gp130, oncostatin M receptor)
IL15	Interleukin 15
STAT5B	Signal transducer and activator of transcription 5B
PIK3R5	Phosphoinositide-3-kinase, regulatory subunit 5
TPO	Thyroid peroxidase
SPRY4	Sprouty homolog 4 (Drosophila)
STAT5A	Signal transducer and activator of transcription 5A
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
SPRY1	Sprouty homolog 1, antagonist of FGF signaling (Drosophila)
FAM164A	Family with sequence similarity 164, member A
PTPN6	Protein tyrosine phosphatase, non-receptor type 6
SOCS4	Suppressor of cytokine signaling 4
PIAS1	Protein inhibitor of activated STAT, 1
PIK3CG	Phosphoinositide-3-kinase, catalytic, gamma polypeptide
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)

Table 23: **List of genes included in the heatmap of Jak/STAT.**

7.3.2 Microarray Analysis of CM, ETOP and Dex regulated genes

For each cluster in figure 7.3.8, overrepresented gene ontology (GO) groups were identified using the expression analysis systematic explorer (EASE) online tool. We reasoned that it should be possible to determine which specific genes or groups of genes were involved in these processes and identify the relative effects of CM. According to downregulated or upregulated genes in CM treated cells, clusters have been divided into 2 major groups for making it easier to study, and those groups are:

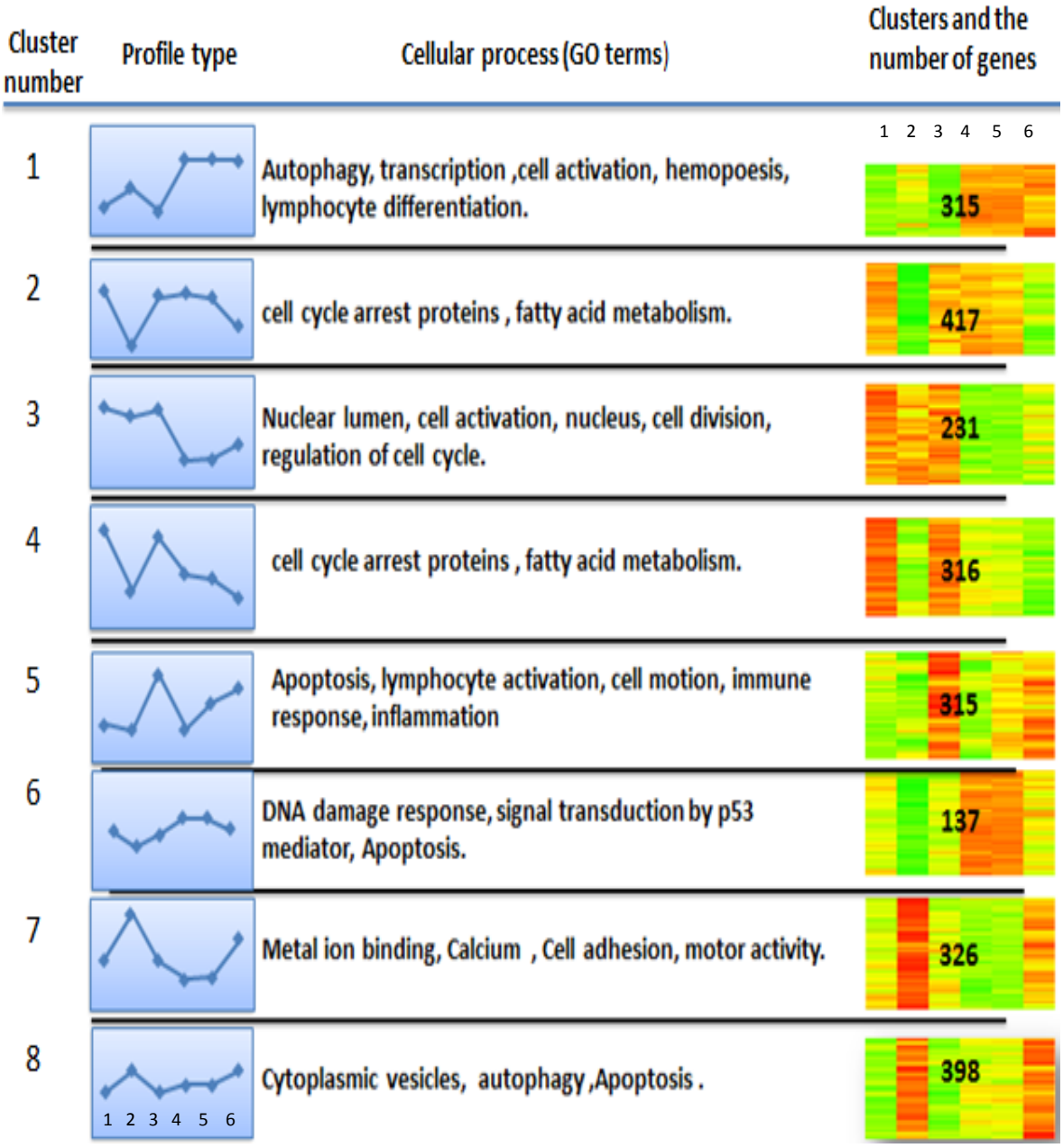


Figure 7.3.8: **Clusters, profiles, and GO groupings of CEM-C7-14 cells.** CEM-C7-14 cells were treated with vehicle (1), CM (2), Dex (3), ETOP (4), Dex and ETOP (5), Dex, ETOP and CM (6).Identified genes were assigned to one of eight distinct clusters using k-means clustering algorithms. On the left, the data for each cluster are represented as a profile of the z-transformed (for each probe set, the mean set to 0 and SD to 1 using maxdView), log (2) values for the mean of each experimental group/condition. Clusters and the number of genes in each cluster are displayed on the right. Red and green indicate positive and negative change from zero, respectively, with colour intensity indicating the degree of deviation. The most significantly overrepresented GO terms are shown for the genes within each cluster.

7.3.2.1 Genes Up-Regulated by CM with ETOP and Dex treatment

In terms of determining genes involved in resistance to treatment, the most important clusters were characterized where CM demonstrated potential interaction with ETOP and Dex controlled pathways that could result in resistance to treatment (Fig 7.3.8; clusters No1, 5, 7 and 8). Via interaction with ETOP and Dex, CM up-regulated groups included: genes associated with autophagy, transcription, hemopoeisis, cell activation and lymphocyte differentiation. In cluster 1 (315 genes or 12%), the genes are mostly upregulated with ETOP treatment alone, ETOP combined with Dex only, and with ETOP combining with Dex and CM.

Cluster 5 included 315 records or 12% of genes representing the genes that are mostly upregulated with Dex treatment alone and with ETOP, Dex and CM together and the genes are associated with apoptosis, lymphocyte activation, cell motion, immune response and inflammation. Cluster No 7 and 8 had similar profile, but in cluster 7 (326 records or 12%) the genes associated with metal binding, cell adhesion and motor activity which are normal biological functions occurring in the bone marrow. On the other hand, cluster 8 (398 records or 15%) involved the function associated with the cell fate that is cytoplasmic vesicles formation, apoptosis and autophagy.

7.3.2.2 Genes Down-Regulated by CM in cells treated with ETOP and Dex

This group contained genes that have low expression in cells treated with CM, ETOP and Dex together (Figure 7.3.8; clusters 2, 3, 4 and 6). Maximal down-regulation was seen in cluster 2 (contains 417 records or 16%). This cluster was enriched with genes that were associated with cell cycle arrest, protein and fatty acid metabolism and it had the same biological function of cluster 4 (316 records or 12%). Cluster 3 (231 records or 9%) included genes that are associated with common biological functions of the cells which are nuclear lumen, cell activation, nucleus, cell division and regulation of the cell cycle. Cluster 6 (137 records or 5%), the genes in this cluster reflects mostly the upregulation with ETOP alone and combined with Dex, but it was downregulated in cells treated with ETOP combined with Dex and CM. the records are associated with DNA damage response, p53 signalling pathway and apoptosis.

Furthermore, to identity the significant representative biological function in each cluster, EASE analysis was performed based on the GO terms of each gene. We classified genes associated with several processes of interest including the relevant

GO terms autophagy, apoptosis, DNA damage, cell cycle, mitochondria, endoplasmic reticulum (ER), cytoplasmic vesicles, phosphorylation and inflammation.

It seems that in Cluster 6, almost the entire cluster was suppressed when undergoing CM treatment, with only LOC648556, MNS1 and C8orf83 activated. In comparisons, repression of cluster 6 is much greater when treated with CM; we assessed the top ten mostly repressed genes in Cluster 6. The most repressed genes were GEM (-5.90241-fold), NFKBIB (-5.40461-fold), STAU2 (-4.40338-fold), RUNX3 (-4.39893-fold), SCG2 (4.37987-fold), FOS (-4.17916-fold), KIAA0895 (-4.05797-fold), ZNF473 (-3.91539-fold), ARL5B (-3.46418-fold) and MXD1 (3.43624). The most repressed gene, GEM (GTP binding protein overexpressed in skeletal muscle), belongs to the RAD/GEM/KIR (RGK) subfamily of Ras-related GTPases and has been linked to cell morphology through the interaction with the kinesin-like protein, KIF9. It was interesting to observe that NFKBIB is severely repressed when treated with CM, as NFKBIB, also known as the I-κB, is an inhibitor of NK-κB (Table 24). There was limited information to be found in STAU2, SCG2, KIAA0895, ZNF473, ARL5B and MXD1. RUNX3 (Runt-related transcription factor 3), has been associated with cell proliferation/apoptosis and has been identified as a key player for gastric epithelial growth, neurogenesis and T-cell differentiation (Subramaniam *et al.*, 2009). In gastric cancer, RUNX3 has been shown to be important in inducing apoptosis by direct binding to its conserved RUNX binding element within the pro-apoptotic Bcl-2 member Bim's promoter region and cooperating with another transcription factor, Foxo3a (Yamamura *et al.*, 2006). Furthermore, FOXO3a and RUNX subfamily member RUNX1 has been associated with autophagy in skeletal muscle (Wang *et al.*, 2005; Sanchez *et al.*, 2012). In the case of leukaemia cells, a recent study showed that RUNX3 is not affected by Dex alone treatment (Heidari *et al.*, 2012), which suggests there may be a specific regulation of CM on RUNX3 expression in leukaemia cells.

7.3.3 Further study of cluster number 6

Amongst the 8 clusters, genes in cluster 6 were found to be most enriched with apoptosis (12%) and since apoptosis is known to be important to leukaemia resistance it would be interesting to study the genes differentially expressed in response to CM to identify any potential key biomarkers. To visualize the genes in Cluster 6 versus various treatments in the context of biological pathways, we mapped Cluster 6 against

the KEGG database with the use of KEGGanim, which allowed us to visualize the changes of the genes in the signalling pathway under various treatments.

Several pathways were highlighted and a number of genes in Cluster 6 were found to be deregulated in various conditions. In the process of apoptosis, a strong repression was found in RIP-1 gene expression (repression -2.01697 log-2-fold) and BAD (repression -1.6065 log-2-fold) when cells were treated with CM only (Table 34). In MAPK pathway, RELB, FOS and CRK were found in Cluster 6, a significant repression of FOS was identified when treated with CM only and a combination of CM and Dex (Table 24). As RIP-1 is significantly repressed in cells grown in CM media and it has been linked to not only apoptosis, but also in other forms of cells death. In addition RIP-1 is also involved in the regulation of MAPK and NF-κB, which are pathways thought to be important to GR regulation (Cardoso *et al.*, 2008), it would be interesting to study the role of RIP-1 in CM induced ALL resistance.

Genes	CM Fold change	Dex Fold change	Etop Fold change	Dex+Etop Fold change	Dex+Etop +CM Fold change	Function
RIP-1	-2.01697	1.138804	-0.817664	-0.767831	1.014902	Apoptosis, MAPK regulation, NF-KB
BAD	-1.6065	-1.167854	0.58096	0.631104	0.794759	apoptosis
FOS	-4.179151	0.844418	0.954184	0.685634	0.809122	Proliferation/differ--entiation/survival
Bim	-1.951969	4.740634	0.935221	3.225986	1.685352	Apoptosis
Runx3	-4.398922	0.907268	0.638381	0.473774	0.766257	Proliferation/ apoptosis
IKB	-5.40461	0.995367	0.41972	0.513665	0.676987	Cell survival (NF-KB inhibitor)

Table 24: **Cluster 6 from microarray study indicating key genes.**Table is showing the fold changes of the top six of the most expressing genes that were affected by the CM and the most common function of those genes.

7.4 Microarray results validation

Receptor interacting protein 1 (RIP-1) have been chosen as the gene of interest as mentioned in section (7.3.3); RIP-1 gene expression is the significantly repressed when treated with CM (repression -2.01697 log-2-fold) (Table 34) and it has been linked to apoptosis and to another form of cells death called necroptosis (Chaabane *et al.*, 2013). In addition RIP-1 is also involved in the regulation of MAPK and NF-κB, which are pathways thought to be important to GR regulation (Cardoso *et al.*, 2008), it would be interesting to study the role of RIP-1 in CM induced ALL resistance (Chaabane *et al.*, 2013).

The role of RIP-1 is not known in leukemia and the link to GR or p53 is not clear. It is the only protein involved in necroptosis and likewise there is no clear picture or relation between the apoptosis, nectroptosis or autophagy in leukemia.

For that reason it is important to determine the role played by RIP-1 in leukemia and to clarify the RIP-1 function in cell resistance due to stem cell microenvironment.

7.4.1 The effect of RIP-1 inhibitor on apoptosis and autophagy.

Since RIP-1 role is not known in leukemia and leukemia treatment, Nectrostatin -1 (RIP-1 inhibitor) was used to evaluate the RIP-1 inhibition effects on apoptosis and autophagy. For that cells were treated with necrostatin-1 two hours before all other treatment and then cells were subjected to cell extraction, western blots and detecting Beclin (autophagic protein) and Cleaved caspase 3 (apoptotic protein) proteins by specific antibodies.

In Figure 7.4.1 the data showed upregulation of Beclin protein levels in cells treated with the necrostatin-1 alone, and the protein level of beclin is decreased in cells treated with the inhibitor together with CM (Figure 7.4.1, compare lane 3, 7 and 9 to lane 2). On the other hand the Dex treatment with the inhibitor lead to increase in Beclin protein level (Figure 7.4.1 lane 5) while the necrostatin-1, Dex and CM lead to further induction of Beclin as shown in Figure 7.4.1, lane 4 and 5).

Moreover, the treatment of RIP-1 inhibitor with the ETOP, ETOP with CM and ETOP, Dex and CM revealed equal increase in cleaved caspase3 protein level (Figure 7.4.1, lane 6, 7, 8 and 9).

Our results suggested that in the absence of RIP-1 protein (necroptosis protein), Beclin (autophagy) is induced as a result of dexamethasone treatment whereas the

cleaved caspase3 (apoptosis protein) is induced as result of etoposide treatment. The general tone of the regulation is the increase of an autophagy protein in the absence of RIP-1.

Significantly, with the inhibition of RIP-1and treatment of Dex and CM, Beclin is induced which may indicates that CM can regulates beclin indicating important potentially novel role of beclin in leukaemia. Moreover, with inhibition of necroptosis, Dex can induced beclin as well which may shed the light on beclin as a new target of Dex treatment and that still need more investigations.

On the other hand, we can see equally upregulations of caspase3 with ETOP treatment alone, or with Dex, or with CM, or with ETOP combined with Dex and CM. Inhibition of RIP-1 can leads to apoptosis as a result of anthracycline agents' treatment only.

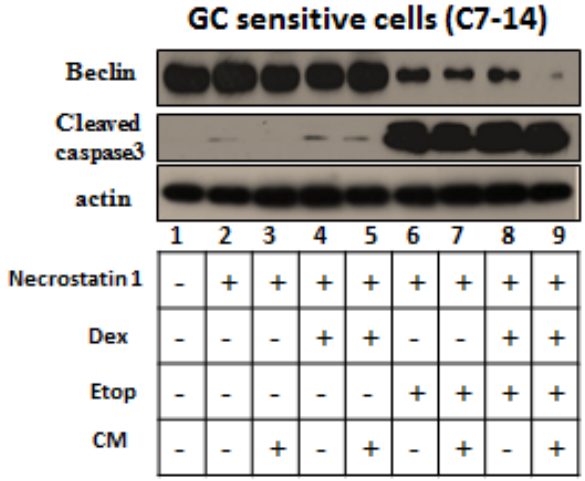


Figure 7.4.1: **The effect of RIP-1 inhibitor (Necrostatin-1) on apoptosis and autophagy pathway.**CEM C7-14 cells were pre-treated with Necrostatin-1 (10μM) for 2 hours prior to Dex and ETOP treatment. Cells were lysed and probed with beclin, and cleaved caspase 3 antibodies. Actin was used as a loading control and the results are representative of 3 independent experiments.

7.4.2 RIP-1 inhibition increased apoptotic activity in leukemia cells

To examine whether inhibition of RIP-1 promotes apoptosis in leukemia cells, the cell cycle distribution of pre-treated RIP-1 inhibitor (Necrostatin1) leukemia cells was analyzed. Cells were stained with propidium iodide (PI) and analyzed by flow cytometry. As shown in Figure 7.4.2 flow cytometry analysis revealed increase in the percentage of dead cells in the sub G1 phase in cells treated with the inhibitor and Dex compared with cells treated with Dex without the inhibitor (Figure 7.4.2, compare lane no 5 and 6). Similarly, treatment with ETOP and with the inhibitor

showed increase in the percent of dead cell when compared to ETOP treatment without the inhibitor (Figure 7.4.2, compare A and B lane no 9 and 10).

Likewise, treatment with Dex, ETOP and necrostatin-1 demonstrated increase as well in the percent of dead cells when compared to the treatment of Dex and ETOP only (Figure 7.4.2, compare A and B lane no 13 and 14) Moreover, there was increase as well in the dead cells percent when the cells were treated with CM with the inhibitor when compared with the CM only treatment (Figure 7.4.2, compare lane no 3 and 4). There was slight increase in the damaged cells population with the treatment of the Dex and CM when compared with Dex, CM and the inhibitor treatment (Figure 7.4.2, compare A and B lane no 7 and 8), strongly indicating that RIP-1 pathway may contribute to leukemia induced resistance. Generally, in this experiment, results showed a common trend of increase in the cell death after addition of the RIP-1 inhibitor when compared to the number of the cell death without the necroptosis. The type of the cell death measured could be any type like, apoptosis, autophagy and necrosis.

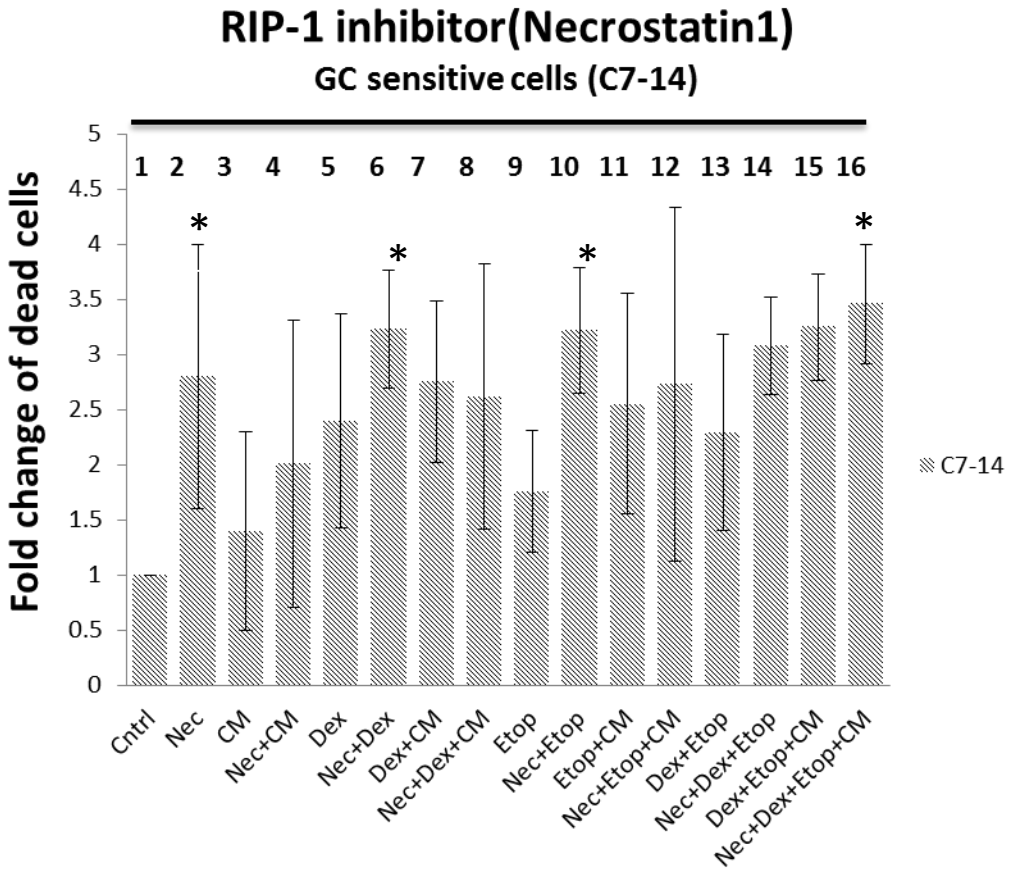


Figure 7.4.2: **Sub G1 cell cycle phase in cells treated with RIP-1 inhibitor, Necrostatin-1.** CEM-C7-14 cells were pre-treated with Necrostatin1 (10μM) for 2 hours prior to Dex and

ETOP and flow cytometry performed and the results are representative of 3 independent experiments.

7.4.3 Autophagy analysis in cells treated with necrostatin-1

To further investigate the role of RIP-1 in cell death and determine effect on autophagy the experiment was designed to investigate the occurrence of autophagy after and before using RIP-1 inhibitor and the interrelationship between autophagy and apoptosis. Moreover, the role of autophagy, in leukemia development and therapy has not been well documented and needs to be investigated further.

For that purpose cells were treated with RIP-1 inhibitor (Necrostatin-1) 2hrs before; Dex (1µM) for 36hrs, ETOP (10µM) for 24hrs and CM for 48hrs, then flow cytometry was performed.

Generally, the results showed that RIP-1 inhibition enhanced the autophagy-induction effect with ETOP and CM treatment but not with dexamethasone. However, Necrostatin-1 alone induced autophagy in 22.17% CEM-C7-14 when compared to control (Figure 7.4.3, compare profile1 and 2).

Moreover, the data showed that the levels of autophagy were very low when the cells were treated with Dex either alone, or combined with Necrostatin-1, or with CM, or when combined with Necrostatin-1 and CM (Figure7.4.3, profiles 5, 6, 7 and 8 respectively). This indicated that other treatments failed to induce autophagy in leukemia cells because of treatment with Dex.

However, with etoposide treatment, the incidence of autophagy increased with the treatment of Necrostatin-1 rather than the ETOP alone. The autophagy incidence of ETOP and Necrostatin1 treatment is increased 5.26% as compared to ETOP only treatment (Figure7.4.3, compare profile 9 and 10). Autophagy incidence showed 10.64% increase when cells were treated with Necrostatin-1, ETOP and Dex when compared to ETOP and CM only (Figure7.4.3, compare profiles 11 and 12).

Again, there was no significant increase in the autophagy incidence in CEM C7-14 cells treated with Dex and ETOP, Dex+ETOP and Necrostatin-1, Dex+ETOP, Dex ETOP, CM and Necrostatin-1(Figure7.4.3, compare profile 13, 14, 15 and 16 respectively).

Thus, we examined autophagy incidence in leukemia cells after treatment with or without Necrostatin-1 (the RIP-1inhibitor). The results showed that inhibition of RIP-1 resulted in increased incidence of autophagy not apoptosis. On the other hand, Dex treatment alone or combined with ETOP and CM tends to exert apoptosis stress on the cells rather than autophagy. Interestingly, In Figure 7.4.1, the result of inhibition of RIP-1 and treatment of Dex was induced Beclin which may indicate Beclin as a new target of Dex treatment and highlight the role played by Dex in autophagy regulation as an additional pathway to avoid resistance toward treatment.

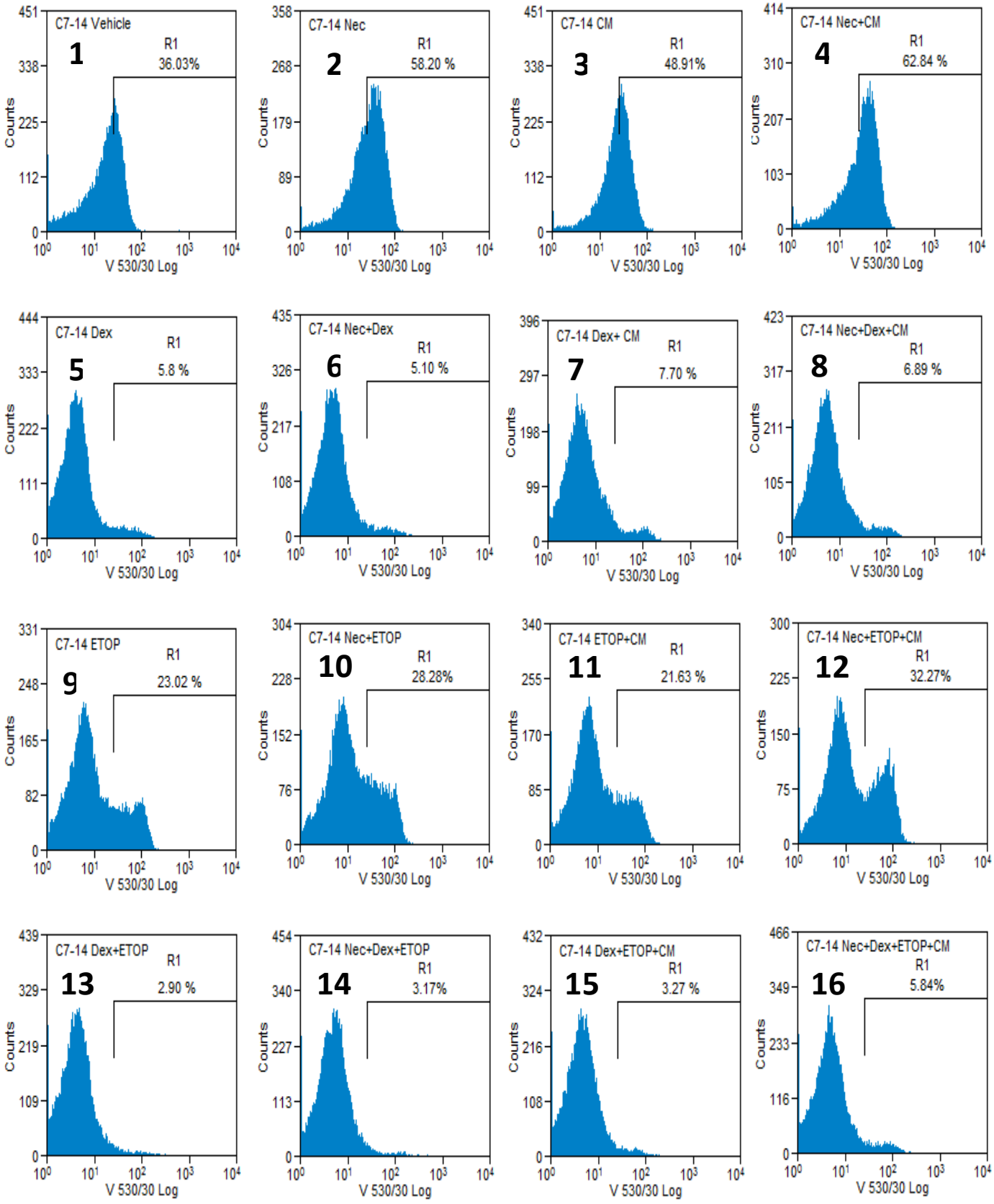


Figure 7.4.3: **Representative profiles obtained by flow cytometry analysis after monodansylcadaverine (MDC) staining to detect Autophagy incidence.** CEM-C7-14 cells were pre-treated with Necrostatin-1 (10μM) for 2 hours prior to Dex and ETOP and flow cytometry performed and the results are representative of 3 independent experiments.

8 Discussion

Acute lymphoblastic leukemia is one of the most common forms of leukemia that affects children. Although a remarkable achievement in cure rate is seen, still the percent of children is facing the risk of death due to the resistance to drug or toxic side effects of treatment (Saha and Kearns, 2011). Glucocorticoids such as dexamethasone are regularly used in the treatment because of its pro-apoptotic effects. Recent studies highlighted the role of bone marrow stem cells in developing resistance and failure of the treatment and subsequently death of the patient (Saha and Kearns, 2011). In this thesis, we demonstrated the importance of considering how combined drug treatment is different from individual drug treatment and how the bone marrow stem cells can affects drug resistance.

8.1 Glucocorticoid receptor and p53 induced apoptosis may not depend on Bim induced apoptosis but on Bax dependent apoptosis

The Glucocorticoids are capable of regulating Bcl-2 family and Bim. Reports suggest they are the most important pro-apoptotic members that have the apoptotic properties (Bouillet *et al.*, 1999). Moreover, the role of GC induced apoptosis is not very well understood and further explorations will allow better treatment and less resistance .

To investigate the underlying mechanism of GC resistance with respect to Bim and Bax regulation in ALL, we conducted a qRT-PCR for leukemia cells with 36hrs treatment of Dex, 24 hrs of ETOP and 48hrs of CM. Moreover genome wide analysis was conducted as well with 24 hrs treatment of Dex, 24 hrs of ETOP and 48hrs of CM. Interestingly both results showed the down regulation of Bim in both the treatments in GC resistance and sensitive clone. Microarray and qRT-PCR data indicated reduction of Bim in GC sensitive cells with the combined treatment of Dex and ETOP, and there is further downregulation of Bim mRNA level after treatment of CM (Figure 7.1.8) .On the other hand, the combined treatment caused upregulation of Bax and CM partially downregulated Bax (Figure 7.1.10).

GC-induced apoptosis requires B-cell lymphoma 2 family members and most importantly pro-apoptotic Bim. Bim activates pro-apoptotic Bax through neutralization of pro-survival B-cell lymphoma 2 like (Bcl-2) members (Wang *et al.*, 2003; Erlacher *et al.*, 2005; Chen *et al.*, 2012). The ability of GCs to selectively induce apoptosis in white blood cells is the main factor contributing to their therapeutic use.

Numerous laboratories have performed genome-wide microarray analysis in order to identify genes differentially regulated during glucocorticoid-induced apoptosis (Medh *et al.*, 1998; Thompson and Johnson, 2003; Wang *et al.*, 2003; Schmidt *et al.*, 2006; Tissing *et al.*, 2007; Chen *et al.*, 2012). However, to date, only a few genes have been assigned a functional role in the regulation of glucocorticoid-induced apoptosis. Most notably, the expression of the pro-apoptotic BH3-only Bcl-2 family member Bim is induced by glucocorticoid treatment in murine lymphoma cell lines, human leukemic cell lines, mouse primary thymocytes and human primary chronic lymphoblastic leukemia (CLL) and acute lymphoblastic leukemia (ALL) samples (Distelhorst, 2002; Wang *et al.*, 2003; Schmidt *et al.*, 2004). The mechanism of induction is expected to be indirect, as there is no GRE in the promoter region of the Bim gene (Bouillet *et al.*, 2001; Wang *et al.*, 2003). One potential mechanism of Bim induction is via the induction of the Foxo3a transcription factor, which is up-regulated by glucocorticoids (Planey *et al.*, 2003). The induction and activation of Bim leads to downstream activation of the apoptotic mediators Bax and Bak (Kim *et al.*, 2009). Once activated, these mediators mediate the destabilization of the mitochondrial membrane potential, a hallmark of the intrinsic mitochondrial apoptosis pathway (Kim *et al.*, 2009). The up-regulation of Bim is mostly an important mediator of glucocorticoid-induced apoptosis. For example, thymocytes from homozygous Bim knock-out mice exhibit decreased sensitivity to glucocorticoid-induced apoptosis (Bouillet *et al.*, 1999). Furthermore, in vitro studies utilizing shRNA or siRNA targeting the various Bim transcripts confirm a substantial role for Bim induction in the progression of glucocorticoid-induced apoptosis (Abrams *et al.*, 2004; Lu *et al* , 2006; Ploner *et al*, 2008).

In agreement with the other group's findings, we confirm the induction of Bim post treatment with Dex in sensitive cell line CEM C7-14 (Figure 7.1.8). Our novel findings indicate the reduction of Bim due to Dex and CM in glucocorticoid resistant cells rather than he sensitive clone and both cell lines showed reduction in Bim mRNA level with Dex, Etop and CM treatments (Figure 7.1.8). These treatments revealed the role of bone marrow microenvironment that contributes to reduction in the cell cytotoxicity in response to treatment. Interestingly, when leukemia cells were treated with a combination of Dex and ETOP (which mimics the current treatment of leukemia), Bim was down-regulated and Bax up-regulated highlighting the possibility of alternative pathways being utilized for apoptosis when both drugs are used.

The role of glucocorticoid-induced leucine zipper (Gilz) is an area of expanding interest in the study of glucocorticoid-induced apoptosis. Gilz was first identified as a glucocorticoid-responsive gene by a systemic screen for genes responsive to glucocorticoids in the thymus (D'adamio *et al.*, 1997). Due to the presence of three GREs in the Gilz promoter, the glucocorticoid induction of Gilz expression is direct and robust (Wang *et al.*, 2004). Gilz is highly up-regulated due to Dex treatment (data is included in the indices) and evidences suggest Gilz antagonize Foxo3a effect by unknown mechanism (Kfir-Erenfeld *et al.*, 2010; Mahdipour *et al*, 2011). Although Bim and Bax are the effector pro-apoptotic members Bim can be excluded from the combined treatment but Bax is involved mostly in the combined treatment as prominent effector protein .

Several studies have shown a correlation between high Bcl-2 expression and poor response to therapy in leukemia (Findley *et al.*, 1997). The anti-apoptotic BCL-2 protein is embedded in the ER, the nuclear envelope and the OMM by a hydrophobic C terminal membrane-spanning domain, with most of its amino acids in the cytosol. Although BCL-2 in any of these subcellular locations can block apoptosis, the functions of BCL-2 at the ER and the nuclear envelope are less clear than those on mitochondria and have recently been reviewed (Kuo *et al.*, 1998). In contrast to BCL-2, Bax is mostly cytosolic and sequesters its hydrophobic C terminal membrane anchor in its BH3-binding pocket with a minor fraction lightly bound to the OMM. Bax appears to exist as a monomer in the cytosol of cells rather than being bound to any anti-apoptotic BCL-2 family members. During apoptosis induction, Bax translocates specifically to mitochondria (Almawi *et al*, 2004).

The apoptosis afforded by pro-survival Bcl-2 proteins is likely to be explicit for the GCs. The Bcl-2 and Bcl-xL blocked GC-triggered apoptosis in T cell does not affect death receptor apoptosis (Almawi *et al*, 2004). Additionally GCs induced apoptosis during reduction in the expression of pro-survival (Bcl-2 and Bcl-xL) proteins in leukemic cells (Almawi *et al*, 2004). The inhibition of pro-survival Bcl-2 members by GCs and limiting GC-triggered apoptosis by over-expression of Bcl-2 indicated an underlying association between GC-triggered apoptosis and Bcl-2 proteins. The above mentioned association could be through a system that requires a precondition of hydrogen peroxide being generated (Tonomura *et al.*, 2003) or modified trans-

membrane transfer. On the other hand GCs can differentially control the expression of Bcl-2 proteins and apoptotic actions that depends on the cell type and stimulus.

8.2 Complex crosstalk of p38/JNK and GR pathways

GR's translocation from the cytoplasm to the nucleus of a cell and the transcriptional activity of the GR depends on the ligand binding. Additionally the ligand binding and the other signaling pathways are capable of integrating and modulating the GR's transcriptional responses by its phosphorylation. The receptor activity can be modulated by GR's phosphorylation by cell-specific kinases such as MAPKs, CDKs, and GSK-3. Hormone-bound GR when present in the nucleus can bind to DNA, co-activators, co-repressors, and transcription factors. The phosphorylation of the receptor alters GR's conformation leading to modifying the DNA binding and cofactor recruitment. Furthermore phosphorylation of the receptor can modify the nuclear or cytoplasmic shuttling, ubiquitin-mediated proteasomal degradation and receptor's half-life. Therefore, phosphorylation of GR can significantly influence the transcriptional activity and cellular response to hormone.

Since the GR phosphorylation is suggested to regulate cell fate either positively or negatively, we decided to investigate the effect of combinatory drug treatment and CM on GR phosphorylation status. Ser211 is major residue hyper-phosphorylated after hormone binding and it is ligand-dependent. Indeed, transcriptional activity of GR correlates with the intensity of Ser211 phosphorylation, suggesting that Ser211 phosphorylation is a biomarker for activated GR. Phosphorylation of GR on Ser226 is usually associated with nuclear export, GR sumoylation and negative effect on its activity. The relative level of phosphorylation at Ser211 versus Ser226 is an important determinant of GR activity in ALL (Kfir-Erenfeld *et al.*, 2010) thereby serving as a marker of GR activity. GR phosphorylated at Ser211 is targeted by cyclin dependent kinases and p38MAPK pathway, while Ser226 is targeted by c-Jun N-terminal kinases (JNK).

To determine phosphorylation pattern of the GR in glucocorticoid sensitive and resistant cell lines and to analyze the effect of CM on phosphorylation, western blot was carried out by using antibodies that specifically detect total and phosphorylated proteins. The antibodies used to study are H300 antibody, and Ser211antibody and Ser 226 antibody specific for phosphorylated indicated residues on GR.

The comparisons between both cell lines at the level of total and phosphorylated GR data showed that GC sensitive clone reflected high induction of GR level after the treatment of Dex. GR phosphorylation at Ser211 showed increased in GC sensitive cells than resistant clone. On the other hand GR phosphorylation at Ser226 is more induced in GC resistant cells which may explain why GC cells are more effective than GC resistance cells. This finding are in agreements with Lynch et al (Lynch *et al.*, 2010), which revealed that the Ser211 phosphorylation was dominant in CEM-C7-14, whereas the opposite was the case in CEM-C1-15 where prevalence of Ser226 GR phosphorylation was observed (Figure 7.1.2) On the other hand, the GR phosphorylation at Ser211 was significant when cells treated with the combination of Dex and ETOP and it was dowregulated as a result of CM with the combination. Moreover, the treatment of ETOP alone did not affect Ser211 regulation of GR which indicated the hormone dependence in the activation.

We wanted to further investigate the role played by the CM in regulating GR phosphorylation since the effect of CM on the post-translational modification of GR is unknown. Our data showed that CM may play a role in positively influencing the GR phosphorylation at Ser211 in glucocorticoid sensitive clone cells only. Such effect however was not detected with the treatment of CM with GC resistant clone. On the other hand, Ser226 phosphorylation is substantially down-regulated by CM in Dex treated resistant cells and not in sensitive cells. Taking the data together, the results indicate that a complex crosstalk may be involved, and the GR phosphorylation status differs when cells were treated with either Dex or ETOP alone or in combination. Additionally, CM has significant effects on GR phosphorylation profile in both cell lines. It seems that overall conditioned media affects Ser211 and Ser226 phosphorylation differently in both cell lines. Moreover, the result indicated a differential role of the GR phosphorylation in the two cell lines as detected by altered phosphorylation and kinetic patterns of MAPK, CDK and JNK dependent GR target residues.

The Wang group found that Ser211 phosphorylation influences the level of transcription therefore, greater the phosphorylation within the receptor, higher the transcriptional activity (Wang *et al*, 2002).

Further Ser211-phosphorylated GR has been shown by ChIP assays to be a transcriptionally active form of the receptor due to its occupancy at several GRE-

contains promoters (Blind and Garabedian, 2008). Additionally the GR has the maximum transcriptional activity when Ser211 phosphorylation is exceeding the Ser226 phosphorylation. For that matter, inhibiting Ser226 phosphorylation enhances the transcriptional reaction of GR (Chen *et al.*, 2008; Galliher-Beckley and Cidlowski, 2009). This confirms a significant role of Ser211 phosphorylation in the transcriptional activity of GR. Some reports confirmed that GC can enhance action of p38MAPK, and have also observed that p38 MAPK can inhibit and protect mouse as well as human lymphoid cells from GC- triggered cell death (Miller *et al.*, 2005; Galliher-Beckley and Cidlowski, 2009) but our data indicated that GC and p38 crosstalk has been demonstrated. Miller et al, 2005 have also successfully demonstrated through use of a phospho-Ser211 antibody that human GR is phosphorylated by p38 MAPK at Ser211, both in vitro and within intact cells (Miller *et al.*, 2005). Further study has sugessted that lack of Ser211 phosphorylation is a mechanism likely to make human lymphoid cells GC resistant (Miller *et al.*, 2007). To test this hypothesis, GC-resistant and sensitive human acute lymphoblastic CEM cells were treated with inhibitors of JNK and p38MAPK functions in order to identify pathways that affect CM and regulates Ser211 phosphorylation of GR. Results have demonstrated that by inhibiting JNK's activity the sensitivity of CEM cells to the apoptotic action of dexamethasone can be resorted (Figure 7.2.4). Furthermore, these inhibitors have potentially enhanced the Ser211 phosphorylation of the GR in the CEM cells (Miller *et al.*, 2007). This suggests the inhibition of JNK activity can enhance the presence of GR phosphorylated on Ser211. JNK phosphorylates Ser226 and not Ser211, so perhaps apoptosis was triggered through inhibiting Ser226 and those crosstalk's to Ser211 or through alternative mechanism (figure 7.2.4). This evidence emphasizes the significance of MAPK activation resulting in varying phosphorylation profiles of the GR that regulate the cellular response to GCs. Evidently reduced Ser211 phosphorylation within GR can reduce its nuclear localization and can also reduce the transcriptional action of the receptor on numerous endogenous genes. Rogatsky et al, 1998 has also demonstrated that phosphorylation of the GR at Ser211 can promote a conformational alteration within the receptor (Rogatsky *et al*, 1998). More investigations are required to comprehend cell- and tissue specific GR Ser211 phosphorylation status and its influence on GR action.

Beside the importance of Ser211 phosphorylation, GR phosphorylation at Ser226 is also known to regulate GR negatively. JNK has been shown to phosphorylate the GR at JNK-mediated GR phosphorylation acted to attenuate GR transcriptional activity (Rogatsky *et al,* 1998). JNK-triggered phosphorylation of human GR at Ser226 was capable of blunting hormone signaling by increasing nuclear export of the GR (Galliher-Beckley and Cidlowski, 2009). These outcomes present evidences that activated MAPK signaling pathways can act to modify GR signaling. Several reports proposed that Ser226 phosphorylation of the GR attenuates GR signaling. In summary, we can conclude that the Ser226 phosphorylated receptor is transcriptionally active and further research is required to determine the precise role of human Ser226 phosphorylation of GR in modulating GC signaling.

8.3 Microarray analysis key findings and cross talk of necroptosis, autophagy and apoptosis

By studying the genes that are most repressed by the treatment of CM, we found some interesting genes to study that may play an important role in the contribution of Bone marrow microenvironment in the resistance of leukemia cells. As been mentioned in section (7.3.3), there were some potential key biomarkers which are linked to apoptosis. These potential biomarkers in cluster 6 were analysed in context of the biological pathways, accordingly the Cluster 6 genes were mapped against the KEGG database with the use of KEGGanim, which allowed us to visualize the changes of the genes in the signalling pathway under various treatments. As a result of the significantly repressed genes in CM treated cells, several genes were studied.

The first gene is nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, beta (NFKBIB) and it was interesting to observe that this gene was severely repressed when treated with CM (-5.4 folds repression), as NFKBIB, also known as the I-κB, is an inhibitor of NK-κB . Inhibition of I-κB by bone marrow microenvironment leads to cell survival, which is one way how CM is generated from bone marrow and can develop resistance.

Second gene that we found to be repressed by CM is Runt-related transcription factor 3 (RUNX3) which is -4.4 folds repressed by CM. RUNX3 and has been associated with cell proliferation/apoptosis and further identified as a key player for gastric epithelial growth, neurogenesis and T-cell differentiation (Subramaniam *et al.*, 2009). In gastric cancer, RUNX3 is key player in inducing apoptosis by direct binding to its

conserved RUNX binding element within the pro-apoptotic Bcl-2 member Bim's promoter region and co-operating with another transcription factor, Foxo3a (Yamamura *et al.*, 2006). Furthermore, FOXO3a and RUNX subfamily member RUNX1 have been associated with autophagy in skeletal muscle (Wang *et al.*, 2005; Sanchez *et al.*, 2012). In case of leukaemia cells, a recent study showed that RUNX3 is not affected by Dex alone treatment (Heidari *et al.*, 2012), which suggests there may be a specific regulation of CM on RUNX3 expression in leukaemia cells.

In the process of apoptosis, the third gene that was strongly repressed is (TNFRSF)-interacting serine-threonine kinase 1 (RIP-1) gene expression (repression -2.01697 log-2-fold), whereas the fourth repressed gene was BCL2-associated agonist of cell death (BAD) (repression -1.6065 log-2-fold) when cells were treated with CM only (Table 34).

RIP-1 is significantly repressed in cells grown in CM media and it has been linked to not only apoptosis, but also with other forms of cells death (Christofferson and Yuan, 2010). In addition RIP-1 is also involved in the regulation of MAPK and NF-κB, which are pathways that play an important role in regulating GR (Chaabane *et al.*, 2013). It would be interesting to study the role of RIP-1 in CM induced ALL resistance and we included preliminary evaluation of its role in ALL development and treatment

8.4 Autophagy is a fundamental cell death mechanism in leukaemia

Cell death is an essential cellular process thereby justifying the existence of more than one form of cell death processes. Principally, apoptotic pathways that cause cell death are of crucial significance throughout the organism growth and for regulation of the immune system while controlling defense response to a disease stimulus. Cell death is inter-connected with both cell- endurance and cell-expansion, through control of molecular pathways (Maddika *et al.*, 2007). It's evident that inter-connection and inter-dependence among death processes can give diverse outcomes.

Among various types of cell death; apoptosis and autophagy are the most interesting area for researchers. The less researched form of cell death is necroptosis. We investigated if necroptosis and protein RIP-1 as its regulator could be inter-connection between apoptosis and autophagy, and their significance with respect to cancer development and treatment.

It's crucial to investigate the role of necroptosis and RIP-1 in the combination with apoptosis and autophagy also because its role in leukemia and leukemia treatment is not well known. Necrostatin-1 (RIP-1 inhibitor was used to evaluate the RIP-1 inhibition effects on apoptosis and autophagy. For that purpose cells were treated with necrostatin-1 two hours before all other treatments with Dex, ETOP and CM either alone or combined. Further the cells were subjected to western blots and detecting beclin (Autophagy target) and cleaved caspase 3(apoptosis target) proteins by specific antibodies. Additionally, cells were subjected post treatment with RIP-1 inhibitor to cell cycle progression test.

In our preliminary data we have found that dexamethasone induces autophagy as well as apoptosis in leukemia cells. CEM C7-14 dexamethasone- sensitive cell line showed induction of beclin upon treatment with dexamethasone for 24 hrs which may highlight beclin as a new target of glucocorticoids (Figure 7.2.7). Beclin induction through Dex is a subject of interest and needs further investigations. Significantly, with the inhibition of RIP-1and treatment of Dex and CM, Beclin in highly induced that indicates CM can regulate beclin in combination with Dex. Moreover, with inhibition of necroptosis, Dex can induce beclin.

Moreover, we examined autophagy in leukemia cells after treatment with or without Necrostatin-1 (the RIP-1inhibitor). The results showed that inhibition of RIP-1 resulted in increased autophagy and not apoptosis (Figure 7.4.1). On the other hand, Dex treatment alone or combined tends to exert apoptotic stress on the cells rather than Autophagy (Figure 7.4.1).

Glucocorticoids have been successful in treating lymphoid malignancies because of apoptotic cell death induced as a mechanism of action. It has recently been shown that dexamethasone initiates autophagy prior to the activation of the apoptotic cascade and has fatal consequences for acute lymphoblastic leukemia (ALL) (Grandér *et al.*, 2009). Activation of autophagy appears to work through Beclin which is activated through dexamethasone treatment and activation of beclin is considered as a reason for efficient killing of the leukemic cells by dexamethasone. These above mentioned findings are in agreement of Laane and his group; this group showed the induction of autophagy along with apoptosis as a result of dexamethasone treatment in acute lymphoblastic leukemia cells and Inhibition of Beclin 1 inhibited apoptosis which is

reflecting the crucial role of autophagy in dexamethasone-induced cell death (Laane *et al.*, 2009). In another study of Grandér group; it has been shown that the inhibition of autophagy by siRNA-mediated silencing of Beclin 1 inhibits apoptosis which as well demonstrating an important role of autophagy in dexamethasone-induced cell death (Grandér *et al.*, 2009).

CM may activate PI3K pathway through Akt. Akt in turn activates mTOR which have inhibitory effects on autophagy by Beclin. In this context an inactive Akt would prevent the activation of mTOR and would allow for autophagy to be initiated in response to dexamethasone.

Autophagy is a physiological process that can regulate tumor progression by supporting the cell survival. In contrast to apoptosis, where the induction is detrimental for the fate of the cancer cell and many of the anti-cancer strategies aim to activate this cell death program, autophagy seems to be involved in both the destruction of the cancer cells and also in facilitating their survival according to cell type. The newly discovered attribute of dexamethasone, i.e., inducing Autophagy by beclin, as the means of killing hematological cancer cells may open up new therapeutic avenues. By allowing the combination of this glucocorticoid agent with autophagy promoting agents such as rapalogues, or inhibitors of the proteasome and PI3K inhibitor that possibly could come over resistance of leukemia and improve the treatment by increasing the survival rate.

8.5 The reversal of glucocorticoid resistance may occurr through activation of autophagy-dependent necroptosis.

The aim of this study is to understand how does GC improve treatment and on the other hand, how does bone marrow environment induce resistance. For that we try to make a scenario; the scenario of cell death mode by glucocorticoid and bone marrow microenvironment is based on all the data that have been collected through this study. Hence, the CM presents the strategy for drug resistance as it inhibits the I-κB, RIP-1 and RUNX3 (microarray result). In details, I-κB CM found to be severely repressed when treated with CM and I-κB is known as an inhibitor of NF-κB. Furthermore, NF-κB is a protein responsible for cytokine production and cell survival and it plays a role in regulating immune response. According to that any improper regulation of NF-κB is connected with cancer and autoimmune disease (Dutta *et al.*, 2006). The other

interesting microarray finding regarding the repression of CM is repression of RIP-1.RIP-1 is significantly repressed in cells grown in CM media and it has been linked to not only apoptosis, but also to autophagy. In addition RIP-1 is also involved in the regulation of MAPK and NF-κB, which are pathways considered important to GR regulation (Chaabane *et al.*, 2013). Moreover, inhibiting of RIP-1 by nacrostatin-1 can increase the autophagy by allowing more induction of beclin due to GC treatment (Figure 7.4.1). So autophagy could potentially be cell death pathway in leukemia rather than cell survival strategy.

So the scenario of GC induced apoptosis and autophagy and CM induced resistance in leukemia cells can occur in this paradigm which is; CM induces sever repression of I-κB which consequently inhibit NF-κB. Likewise, CM represses RIP-1which is linked to apoptosis and to autophagy. In addition RIP-1 is also involved in the regulation of MAPK and NF- κB. Also, RUNX3 is repressed by CM and it has been associated with cell proliferation and apoptosis (Subramaniam *et al.,* 2009). In leukemia cells, RUNX3 is not affected by Dex alone treatment (Heidari *et al.*, 2012), which suggests there may be a specific regulation of CM on RUNX3 expression. RUNX3 has been shown to be important in inducing apoptosis by direct binding to its conserved RUNX binding element within the pro-apoptotic Bcl-2 member Bim's promoter region and co-operating with another transcription factor, Foxo3a (Yamamura *et al.*, 2006).

On the other side, GR is phosphorylated at several serine residues including Ser211, and undergoes dimerization. In GC-sensitive lymphoid cells, GR then translocates to the nucleus. In the nucleus; GR alters the transcription of multiple genes. Some of the GR-responsive genes (e.g. Bim) require FoxO transcription factors for their induction. Inhibition of FoxO by Akt prevents GC-induced up-regulation of these genes. p38 is required for the nuclear FoxO activity. GR also affects the kinome either by up-regulating genes affecting the kinome such as Gilz. Glucocorticoid-induced leucine zipper (Gilz) is transcriptionally activated by GR and has also recently been identified as a protein modulating T-cell receptor activation by repressing FoxO3 transcriptional activity that leads to activation of Bim expression (Asselin-Labat *et al.*, 2004). Up-regulation of IκB, besides Gilz, inhibits NF-κB function. In addition, GR directly interacts with NF-κB, recruiting histone deacetylases, thus preventing their transcriptional activity (Kfir-Erenfeld *et al.,* 2010). On the other hand, glucocorticoid

regulates autophagy through regulation of beclin thus serving as a link between autophagy and apoptosis.

In addition to my study, our research collaborator Prof Vaskar Saha demonstrated the presence of stromal cell derived exosomes in the bone marrow derived CM which produces soluble factors that induce a redox adaptation in ALL cells leading to chemoresistance. Moreover, Prof Saha and his group revealed that exosomes that affect ALL cells response to drugs contain several micro RNA's. That may act through PI3K pathway. This will be one of directions that will be explored in future studies.

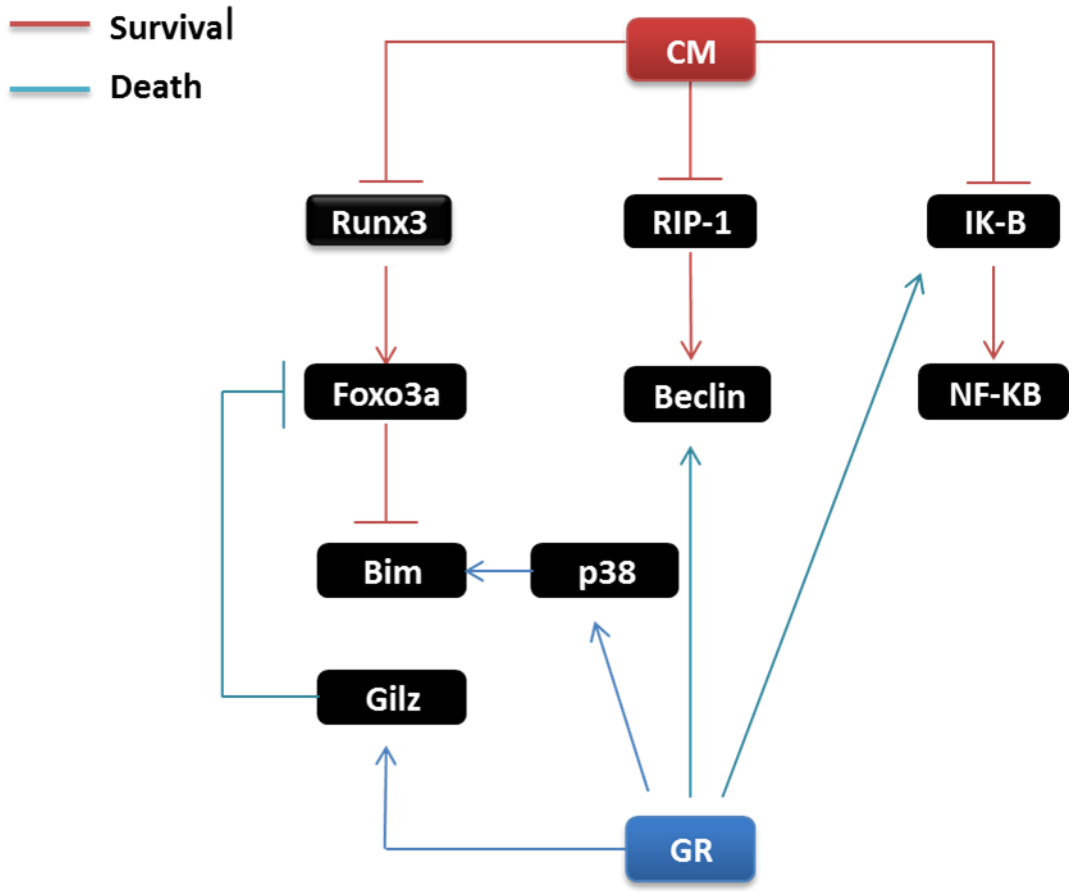


Figure 8.5: **The scenario of GC induced apoptosis and autophagy and CM induced resistance in leukemia cells**. CM induces severe repression of I-κB which consequently inhibits NF-κB. Likewise, CM represses RIP-1, which is linked to apoptosis and to autophagy. In addition RIP-1 is also involved in the regulation of MAPK and NF-κB. Also, RUNX3 is repressed by CM and it has been associated with cell proliferation and apoptosis. RUNX3 has been shown to be important in inducing apoptosis by direct binding to its conserved RUNX binding element within the pro-apoptotic Bcl-2 member Bim's promoter region and co-operating with another

transcription factor, Foxo3a. In parallel, GR gets phosphorylated at several serine residues including Ser211, and undergoes dimerization. In GC-sensitive lymphoid cells, GR then translocates to the nucleus. In the nucleus; GR alters the transcription of multiple genes. Some of the GR-responsive genes (e.g. Bim) require FoxO transcription factors for their induction. Inhibition of FoxO by Akt prevents GC-induced up-regulation of these genes. p38 is required for the nuclear FoxO activity. The GC-target gene Bim is crucial for the apoptotic process. GR also affects the kinome either by up-regulating genes affecting the kinome such as Gilz. Up-regulation of IκB, in additiona to GILZ, inhibits NF-κB function. In addition, GR directly interacts with NF-κB, recruiting histone deacetylases, thus preventing transcriptional activity.

9 Future directions

Further investigation will be focused on answering questions raised by the present study in order to improve our understanding of leukemia development and treatment

We will complete validation of RIP-1 a target of Dex, ETOP and CM by investigating RIP-1 regulationat transcriptional level by measuring mRNA levels using qRTPCR and determining time course and the most effective doses of above mentioned drugs. In addition, we plan to analyse identified pathways regulated by Beclin and RIP-1in samples taken from acute lymphoblastic leukaemia patients to confirm that cell lines reflect pathway regulation in patient's samples. Also, further investigations will be carried out to determine GR location on beclin gene regulatory region by use of chromatin immunoprecipitation (ChIP) and phosphospecific antibodies such as Ser211 and Ser226 antibody. Our data suggested that beclin (autophagy) is induced as a result of dexamethasone treatment and these approaches will determine the role of GR pohosphoisoforms targeted to particular promoters.

Then, it is necessary to investigate the inhibition of PI3K and necrostatin-1 together on the CM induced resistance. As our data showed partial increase in apoptosis after inhibition of PI3K pathway by using (LY294002) or inhibition of RIP-1 by using necrostatin-1. Their clinical significance could be assessed in mice through collaborative efforts.

Finally, we plan to inhibit beclin and RIP-1 function using siRNA in cells treated with Dex, ETOP and CM individually or in combination to firmly establish their role in ALL response to these drugs and development of resistance.

10 Appendices

Appendices contain some results that didn't included in the result section but mentioned throughout the thesis

10.1 Dexamethasone and Etoposide regulates Gilz mRNA levels in different time point (2, 10 and 24h).

Gilz was used as it is an essentially glucocorticoid optimising control that because it is a well-known GR target gene.

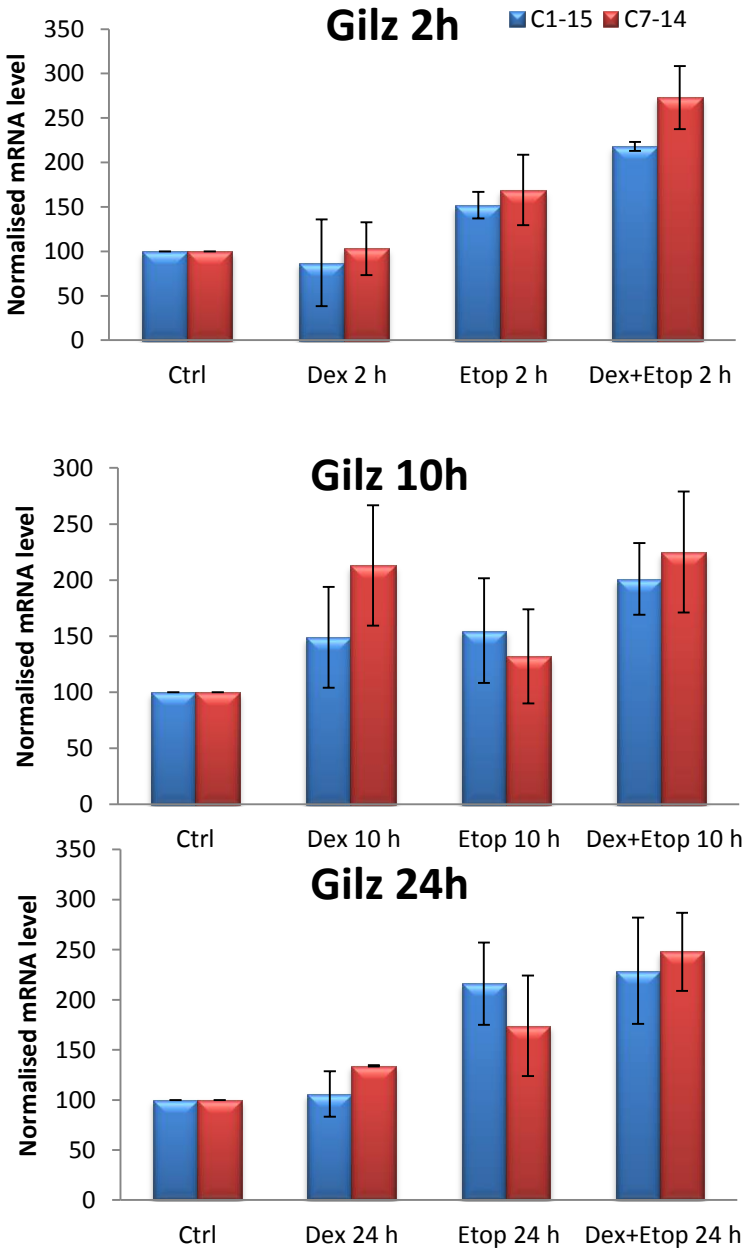


Figure 10.1: **Dexamethasone and Etoposide regulates Gilz mRNA levels in different time point (2, 10 and 24h) in CEM cells**. CEM C1-15 and C7-14 were treated with Dex as 1µM and ETOP as 10 µM for different time point (A) 2hrs treatment, (B) 10 hrs treatment and (C) 24hrs treatment. All results have been normalised to Rpl19. The data shown is representative of three independent experiments.

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