INVESTIGATION OF ABERRANT SIGNAL TRANSDUCTION IN ACUTE MYELOID LEUKAEMIA

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ABSTRACT

Haematopoiesis is the result of tightly regulated signal transduction pathways mediated by cytokines and their receptors. Aberrations in these pathways are an underlying cause for diseases such as leukaemia and other myeloproliferative and lymphoproliferative disorders.

The PI3-Kinase/Akt pathway is central to regulation of cell survival and proliferation. This study found that the PI3-Kinase pathway is activated in AML cells. This activation was reduced or abolished when the cells were incubated with the PI3-Kinase inhibitor, LY294002. Leukaemic cell survival appeared to be dependent on PI3-Kinase activation as incubation with LY294002 resulted in a reduction in cell number and an increase in apoptosis. This was also true for the CD34+/38- leukaemic stem cell population. Further work indicated that activation of Akt alone was sufficient to protect factor dependent cells from cytokine withdrawal induced apoptosis and also from the cytotoxic effects of Ara-C and Etoposide.

The JAK/ STAT Pathway is important for many biological responses including differentiation and proliferation and its dysregulation has also been reported in many malignancies.

It was shown that Gö6976, a selective PKC inhibitor, is a potent inhibitor of JAK 2 in in vitro kinase assays and in whole cell systems and inhibits signaling

downstream of multiple JAK2 coupled cytokines including IL-3, GM-CSF and EPO. Gö6976 was also found to inhibit signalling downstream of disease-associated forms of JAK2 such as the TEL-JAK2 fusion and mutant JAK2 V617F.

The majority of primary AML cells investigated had constitutive STAT activation which was reduced by incubation with Gö6976 in the majority of cases. This reduction was accompanied by a decrease in cell survival and proliferation. This work indicates that both the PI3-Kinase/Akt and the JAK/STAT pathways would be appropriate targets for the development of small molecule inhibitors for use in the treatment of AML.

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TABLE OF CONTENTS

TABLE OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

LIST OF TABLES

COMMONLY USED ABBREVIATIONS

AML	Acute Myeloid Leukaemia
ALL	Acute Lymphoblastic Leukaemia
JAK	Janus Kinases
EPO	Erythropoietin
TPO	Thrombopoietin
GCSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte macrophage colony stimulating factor
IL-3	Interleukin 3
SCF	Stem cell factor
WT	wild type
ITD	internal tandem duplication
IFN	Interferon
STAT	Signal transducer and activator of transcription
FLT3	fms-like tyrosine kinase
MM	Multiple Myeloma
PMA	Phorbol 12 Myristate 13 Acetate
RTK	Receptor Tyrosine Kinase
FTI	Fanesyl Transferase Inhibitor
SDS	Sodium dodecyl sulphate
EDTA/EGTA	Ethelenediaminetetraacetic acid/ Ethelene glycol tetraacetic acid
ALCL	Anaplastic large cell lymphoma

CHAPTER 1-INTRODUCTION

1.1 Haematopoiesis

Haematopoiesis is the continuous process by which blood cells are generated. It is an orderly progression which requires complex regulation by cytokines and their associated receptors. Aberrations in this process lead to the development of diseases such as leukaemia and myeloproliferative disorders. Blood cells originate from a self renewing population of haematopoietic stem cells (HSC) that become committed to differentiate down the erythroid, megakaryocytic, granulocytic, monocytic and lymphocytic lineages. HSCs are found in the Sca-1+ C-Kit + Lin- population in mice and this is likely to be the case in humans. [1] Evidence suggests that these most primitive of cells are largely quiescent with the majority being out of cycle at any one time. [1] Haematopoiesis is predominantly confined to the bone marrow, after birth, except in some pathological conditions where extramedullary haematopoiesis occurs in the liver and spleen. The decision as to whether a HSC undergoes self renewal or lineage commitment is governed by the expression of a number of transcription factors. SCL, GATA 2 and AML-1 are required for definitive haematopoiesis [1] whereas the expression of PU-1 and GATA-1, amongst others, influences lineage commitment.

There are two models as to how cytokines may influence cell fate: 1) The Instructive model which states that haematopoietic cytokines and factors extrinsic to the cell control commitment and differentiation and 2) The permissive model which states that intracellular pre determined factors govern unilineage differentiation and that cytokines provide a supportive role. [2]

Low levels of multiple cytokines modulate HSC transcription factor expression to maintain basal haematopoiesis and short lived amplifications of specific cytokines are produced in response to haematopoietic stresses. The cytokine environment supporting basal haematopoiesis appears to be produced locally by mesenchymal cells within the bone marrow, through the secretion and cell surface presentation of cytokines.

These 'stromal cells' include specialised fibroblasts, endothelial cells, osteoblasts and perhaps adipocytes and produce SCF, TPO, Flt3L and GMCSF. These cytokines are locally concentrated and support HSC survival and proliferation.[1]

1.2 Cytokines and their receptors

Cytokines are soluble polypeptides which form an integrated network of cell to cell communication and humoral interactions regulating growth, differentiation and survival. A particular cytokine may exhibit a wide variety of biological functions plus several cytokines may exert similar and overlapping effects.

Cytokine receptors can be classified broadly according to their structure into receptor tyrosine kinases, class I and II cytokine receptors, protein serine/ threonine kinase receptors, TNF receptors and G protein coupled receptors. Class 1 cytokine receptors and receptor tyrosine kinases will be concentrated on here.

1.2.1 Class I cytokine receptors

Most cytokine receptors consist of a multi-subunit protein complex with a specific ligand binding subunit and a signal transducing subunit which may be shared with other members of the cytokine receptor superfamily and is responsible for transmission of the signals to downstream target proteins.

Cytokine receptors typically have a 210 amino acid extracellular domain consisting of two fibronectin type III modules connected by a hinge region. These proteins have conserved motifs in their N-terminal segment containing cysteine (C) and tryptophan (W) and a tryptophan-serine-X tryptophan serine (W-S-X-W-S) sequence, where X is any non conserved amino acid, in the carboxy-terminus. The W-S-X-W-S motif is in the hinge region and is thought to act as a ligand binding site. The cytokine receptor super family has been further classified based on structural motifs in their extracellular domain into i) The gp130 family which consists of receptors for IL-6, IL-11, ciliary neurotrophic factor, cardiotrophin, leukaemia inhibitory factor and oncostatin M. They all signal through a common β chain called gp-130 and each have a unique ligand binding α subunit. ii) The IL-2

receptor family-the IL-2 receptor consists of 3 subunits α, β and γ. The γ chain is shared by the receptors for IL-4, IL-7, IL-9 and IL-15. The α subunit acts as a ligand binding domain and the β and γ as signal transducing units. iii) The growth hormone family-this family includes receptors for growth hormone, erythropoietin, prolactin, GCSF and thrombopoietin. These receptors exist as homodimers. Binding of the ligand leads to a change in the orientation of the two receptor subunits and this conformational change is transmitted through the juxtamembrane and transmembrane domains, leading to activation. [3]There is no cross reaction between ligands and each receptor transduces signals specific for a cytokine. iv) The interferon (IFN) family-this family is comprised of the IFN-α and IFN-γ receptors together with the IL10 receptor. v) The gp-140 family-this family consists of receptors for IL3, IL5 and GMCSF. They share a common β unit (gp-140) for signal transduction and have unique α ligand binding units. [4]

The cytokine receptor superfamily is characterised by a lack of intrinsic tyrosine kinase activity. They couple ligand binding with tyrosine phosphorylation utilising janus kinases (JAK), cytosolic kinases which are associated with the proximal domains of the cytokine receptor at Box 1 and 2 motifs.[4] The conserved motifs consist of a PxP or PxxP sequence and a sequence LxxL. This region has been shown to be essential for receptor function and critical for the association of JAKs with the receptor complex. [4,5]It is also responsible for the specificity of JAK kinase activation. Jiang et al used mutational analysis and created chimeric receptors by swapping the Box 1 motif of the Epo receptor which transmits signals utilising

JAK2, with that of the β chain of IL-2. This chimeric receptor was able to activate JAK2 on IL2 stimulation, thus confirming the role of the Box 1 motif in influencing JAK specificity. [4,6]

When a ligand binds to its cognate receptor, the receptor dimerises and brings into apposition two JAK molecules, these then auto/transphosphorylate and become activated. The activated JAK molecules phosphorylate the receptor creating phosphotyrosine docking sites for downstream signalling molecules including signal transducers and activators of transcription, STATs.

A cytokine may activate one or more JAKs. Those cytokines which activate more than one JAK may do so independently as in the case of IL-6 which can activate JAK1, 2 and Tyk2 and the absence of one JAK does not preclude activation of the others or as in the case of interferons may show co-dependence i.e. the absence of one JAK prevents activation of the other. Table 1 illustrates the pattern of JAK utilisation amongst the different cytokines.

Table 1 JAKs in cytokine signalling[5]

Ligand	Kinase
Epo	JAK 2
Thrombopoietin	JAK2
Growth Hormone	JAK2
Prolactin	JAK2
G-CSF	JAK2, JAK1
IL2,IL4, IL7,IL9,IL12	JAK3, JAK1
IL3, IL5, GM-CSF	JAK2, JAK1
IFN α, IFN β	JAK1, Tyk2
IFN γ	JAK1, JAK2
IL10	JAK1, Tyk2
IL12	JAK2, Tyk2
IL6, CNTF, LIF, IL11	JAK1, JAK2, Tyk2

1.2.2 Receptor tyrosine kinases

The receptor tyrosine kinases are a family of more than 50 different transmembrane receptors. They differ from the cytokine receptor superfamily in that they possess a tyrosine kinase domain in their intracellular portion. They all share common structural and functional features and apart from the insulin receptor all consist of a single polypeptide chain. This can be divided into an extra cellular and intracellular portion and a short transmembrane domain. Many of the growth factors that bind to these receptors are dimeric molecules and are therefore able to bind two receptors at the same time creating a stable receptor dimer. Dimerisation promotes

auto/transphosphorylation of the receptor and may induce a conformational change leading to receptor activation and the creation of docking sites for the interaction of downstream signalling molecules. This group of receptors includes the receptor for stem cell factor, C-Kit which is expressed on many haematopoietic progenitors and germ cells and the FLT3 receptor.

1.3 JAK STAT Pathway

There are four known members of the JAK family, JAK1, JAK2, JAK3 and Tyk2. They range from 110kDa to 140kDa. The genes localise to 3 chromosomal clusters; JAK1 is encoded by a gene located on human chromosome 1p31.3, JAK2 by a gene on 9p24 and JAK3 and Tyk2 genes are located on chromosome 19 at 19p13.1 and 19p13.2 respectively.[7] JAK1, 2 and Tyk2 are ubiquitously expressed. In contrast JAK3 is predominantly expressed in cells of myeloid and lymphoid lineage. [5,7]

1.3.1 JAK Structure.

Amino acid sequencing of the JAKs reveals that they possess seven highly conserved regions, JH1-JH7, that follow a non-conserved amino terminus of about 30-50 amino acids. They are unusual when compared to other members of the cytosolic protein tyrosine kinase family in that they lack any SH2 or SH3 domains. The other distinctive feature is that the regions JH1 and JH2 both have extensive

homology to tyrosine kinase domains and together they comprise approximately 50% of the JAK molecule. Only the JH1 region appears to be functional. The activation loop of all JH1 domains contains a KE/DYY motif which appears to be a site of autophosphorylation; phosphorylation of the first of these tandem motifs, Y1007 in JAK2, appears to be essential for full kinase activity. [8] The JH2 domain lacks certain critical amino acids and does not appear to be associated with kinase activity. It is now clear that this domain plays a regulatory role. Deletion analysis of JAK2 showed that the pseudokinase domain, but not JH3-JH7, negatively regulated JAK2 catalytic activity as well as STAT 5 activation by JAK2. Furthermore JAK2 kinase inhibition was mediated by an interaction between JAK2 kinase and the pseudokinase domain. [9] Auto/transphosphorylation of conserved tyrosine residues in the JAK activation loop determines levels of catalytic activity. If Tyrosine 980 of JAK3 is mutated to phenylalanine the resultant mutant displays reduced kinase activity, however mutation of tyrosine 981 leads to significantly increased kinase activity. Substrate phosphorylation is dependent on phosphorylation of Y980. [10] Tyrosines not within the activation loop are also autophosphorylated on JAK2. Argetsinger et al found that tyrosines 221 and 570 were autophosphorylated and that mutation of these tyrosines to phenylalanine led to an altered catalytic activity, suggesting that they may have a regulatory role. Phosphorylation of Y221 led to an increased catalytic activity and phosphorylation of Y570 appeared to have an inhibitory effect. These tyrosines fall within a YXXL motif which appears to be a favoured motif for JAK phosphorylation, together with the closely related YXXI/V motifs. [11]

The amino terminal region of JAK molecules spans 550 amino acids and is relatively non conserved between JAK family members. It is thought to be involved in receptor association and specificity of binding. [12] The JH3-JH4 region shows some similarities to SH2 domains and the JH4-JH7 region constitutes a FERM (Four point one, Ezrin, Radixin, Moesin) domain. These domains consist of 3 lobes, the F1 (aa 37-115) in JAK2 has homology to Ubiquitin, the F2 (aa146-258) in JAK2 has homology to acyl coenzyme A binding protein and F3 (aa269-397) has homology to phosphotyrosine binding/ pleckstrin homology domain. [11] The linker between domains F1 and F2 which is at the centre of a hydrophobic core plays an essential role in stabilising the structure of the FERM domain. Zhou et al found that some naturally occurring mutations in the JAK3 FERM domain led to impaired kinase-receptor interaction but also abrogated catalytic activity and ATP binding. [13] They also found that a tyrosine kinase inhibitor which altered the conformation of the kinase domain also significantly affected the binding of JAK3 to the receptor suggesting that the FERM domain and the kinase domain reciprocally affect each others structure and function. This is thought to be because the FERM domain has a permissive role in stabilising the conformation of the activated kinase domain.

1.3.2 JAKs in development

Studies with JAK knockout mice have furthered our understanding of JAK function in mammalian development and growth.

JAK1 is ubiquitously expressed and is widely utilised by interferon receptors and receptors using gp130 and the common γc. JAK1-/- mice have grossly normal nonlymphoid organogenesis, however the JAK1-/- mice die in the perinatal period from an inability to nurse. This neurologic defect is thought to be due to a failure of signalling via cytokines utilising gp130 e.g. LIF, CT-1 and CNTF and thus a failure to promote neuronal survival. [7,14] JAK1 deficiency also leads to reduced numbers of T and B lymphocytes which has been attributed to failure in IL-7 signalling.

JAK2 is also widely expressed, like JAK1. Targeting the JAK2 gene results in embryonic lethality at day 12.5 due to failure of erythropoiesis analogous to that observed in the EPO knockout mouse.[7,12,14] Cells from the JAK2 knockout mouse also show that JAK2 is essential for IL-3, GMCSF, IL-5, TPO and IFNγ but not IL-6 and IFNαβ signalling.

JAK3 selectively associates with the common γ chain and not with other cytokine receptors. Mutation of either the γc or JAK3 in humans results in severe combined immunodeficiency, characterised by lack of T cells and NK cells but not B cells. JAK3 -/- mice are defective in their response to IL-2, IL-4 and IL-7. Consistent with a role in IL-2 signalling JAK3 deficient mice have a defect in peripheral tolerance and lymphoid homeostasis. Failure of IL-7 signalling is thought to be responsible for the reduced numbers of lymphoid cells and the lack of thymic progenitors and the absence of NK cells has been attributed to impairment in IL-15 signalling.[7,12,14]

Tyk 2 contributes to IFN α/β as well as IL-6, IL-10 and IL-12 signalling. Tyk2 -/- knockout mice display relatively subtle defects in IFN α/β signalling. They maintain antiviral responses within the normal range despite having a defect in response to low dose IFNα. IL-10 signalling is relatively normal but IL-12 responses are reduced but not absent. Thus unlike other JAKs many of the in vivo functions of Tyk 2 are redundant. [12,14]

1.3.3 STAT structure and function

STATs are latent transcription factors that play a critical role in signal transduction pathways associated with several cytokines. There are seven members of the STAT family ranging in size from 750-850 amino acids: STATS 1-6 including 5a and 5b which are encoded by two distinct genes on chromosome 17. There are 6 conserved domains; amino terminal, coiled coil, SH2, linker, DNA binding and transcriptional activation domain. The amino terminal consists of 130 amino acids and is conserved amongst STATs. It appears to be critical for STAT function as small deletions were found to eliminate the STATs ability to be phosphorylated. [4] The crystal structure for the first 123 residues of STAT 4 has been resolved and suggests that this domain consists of 8 helices that are assembled in a hook like structure. It has been implicated in various protein-protein interactions affecting transcription and it enables dimerised STAT molecules to polymerise and bind to DNA cooperatively. [15] This region is followed by the DNA binding domain that is located between amino acids 400 and 500. It is highly conserved amongst the STATs and enables the

STATs, with the exception of STAT 2, to differentially bind more than 10 GAS (γ activated sequences) sequences. These sequences are characterised by the consensus motif TTNCNNNAA. [4] Adjacent to the DNA binding domain lays the linker region which is a putative SH3 domain. It is not well conserved and it is therefore uncertain as to whether it functions as an SH3 domain, especially as critical amino acids involved in binding to the PXXP motifs do not appear conserved. [4] The SH2 domain is highly conserved, however, and is situated adjacent to the putative SH3 domain. It plays a critical role in STAT signalling through its capacity to bind with phosphotyrosine residues and is essential for the recruitment of STATs to the receptor complex, for the interaction with JAK kinases and is also required for STAT homo and heterodimerisation. The domain consists of an anti parallel β-sheet flanked by two α helices. This forms a pocket with an absolutely conserved arginine at the base which mediates the interaction with phosphate. [12] Immediately downstream of the SH2 domain at around amino acid 700 is a tyrosine residue present in all STATs which plays a critical role in STAT activation. Phosphorylation of this residue has been found to be essential for activation and dimerisation of STATs and this can be achieved by growth factor receptors, JAK and Src kinases depending on the cell type and the nature of the ligand- receptor interaction. [4] Most vertebrate STATs contain a second phosphorylation site within their C termini on a serine residue. Comparing the C-termini of STATs 1, 3 and 4 it was noted that unlike most amino acids, a PMSP motif between positions 720 and 730 is perfectly conserved. In addition STAT 5a and 5b contain a conserved PSP motif in the same position. Mutation of this serine to alanine leads to a reduced transcriptional activity

in STATs 1, 3 and 4 but in STAT 5 mutation did not affect transcriptional activity. The mechanisms by which this serine phosphorylation occurs and it's biological impact are unclear in most situations. [16]

The transactivation domain is at the carboxy terminus of the STAT molecule. It is poorly conserved among the STATs. Isoforms of STAT 3, 4 and 5 have been identified in which the C-terminal has been truncated; they appear to function as dominant negative regulators. The crystal structure of STAT 1 complexed with DNA has been elucidated by Chen et al. [17] STAT 1 utilises a DNA binding domain with an immunoglobulin fold. The STAT 1 dimer forms a C shaped clamp around the DNA. This is stabilised by mutual highly specific interactions between the SH2 domain of one monomer and the C-terminal phosphorylated tyrosine on the other. The phosphotyrosine binding site of one monomer is joined with the DNA binding domain which suggests a potential role for the SH2-phosphotyrosine interaction in stabilisation of DNA promoter sites.

1.3.4 JAK/STAT Signalling

When a cytokine binds to its cognate receptor, the receptor oligomerises and brings two JAK molecules, associated with the Box1 motif of the receptor, into apposition. They auto/transphosphorylate and become activated. The activated JAK then phosphorylates the receptor and creates docking sites for several down stream signalling molecules including STATs, via their SH2 domains. The STATs are then

phosphorylated on the conserved tyrosine residue in the carboxy terminus which allows them to form stable homo or heterodimers by interactions between the phosphotyrosine residue on one molecule and the SH2 domain of the other. These dimers then translocate rapidly to the nucleus and induce gene expression. A wide range of cytokines activate JAK1, 2 and Tyk2 but only those utilising the common γ chain activate JAK3.The specificity of STAT phosphorylation appears to be determined by the docking sites for STATs on the receptor rather than the JAK kinases. [4] IFNα, β and GCSF all activate JAK1 -however IFNα and β stimulation lead to the phosphorylation of STAT 1 and 2 which form a complex with a third protein p48. In contrast GCSF stimulation leads to the formation of STAT 3 and 5 homodimers, some STAT 1 homodimers and STAT 1/3 and 3/5 heterodimers. This diversity among which STAT complexes are activated may contribute to the cellular responses to a given cytokine or growth factor. [18] The specificity of STAT activation is partially mediated through their recruitment to specific tyrosines on a particular receptor. For instance tyrosine 440 on the IFN γ receptor α chain is responsible for recruitment and activation of STAT 1. Similarly several studies have identified the YXXQ motif as a consensus STAT 3 docking site. It is possible that STAT molecules can be activated by JAK kinases in the absence of receptor docking. It has been proposed that JAK1 and JAK2 can specifically activate and recruit STAT 1 and STAT 5 respectively. This would explain why full activation of STAT 1 by GCSF or GH, or STAT 5 by GCSF and GMCSF can occur in the absence of receptor tyrosines. [18] There is also evidence that STATs can utilise other receptor components as docking sites for instance STAT 1 can bind to STAT 2 already

docked to an activated IFNα/β receptor. It is also likely that the particular JAKs and STATs activated may also depend on the cell type and its state of differentiation. [18]

1.3.5 Regulation of the JAK/STAT Pathway

The JAK/ STAT Pathway is important for many biological responses including differentiation, proliferation and oncogenesis. Various mechanisms therefore exist to modulate this signalling pathway both positively and negatively. These regulatory processes determine the rate at which STAT signals are transduced and these signals can be modulated at various stages of the pathway.

A signalling pathway can be down regulated at the level of the receptor which can occur via the production of soluble receptors which compete for ligand binding or by receptor endocytosis. This is supported by the discovery of a 10 amino acid motif within the intracellular domain of gp130 that regulates endocytosis. [12]

Ubiquitin proteasome dependent degradation may also play a role in downregulation of cytokine signalling. The significance of this remains controversial but there is some evidence to suggest that phosphorylated STATs 4, 5 and 6, but not STATs 1,2 and 3 can be stabilised by proteasome inhibitors. Proteasome inhibitors have also been shown to prolong JAK activity. [12]

JAK activation is dependent on phosphorylation on defined tyrosine residues. Two SH2 containing phosphatases, SHP1 and SHP2 have been found to regulate JAK activity. These enzymes are mainly cytoplasmic; their SH2 domains allow association with phosphotyrosines present on activated receptors or on signalling molecules as well as on activated JAKs. This association triggers activation of the phosphatase domain and subsequent dephosphorylation of the substrate. SHP1 expression is largely restricted to haematopoietic tissues whereas SHP2 is more widely expressed. Evidence suggests that loss of a receptor's ability to recruit SHP1/SHP2 leads to prolongation of JAK activity. In the EPO receptor when receptor tyrosine motifs responsible for the recruitment of SHP are lost, there is prolongation of JAK activation. A naturally occurring mutation on the EPO receptor resulting in its truncation leads to familial erythrocytosis. [19] Hypermethylation of normally unmethylated CpG islands of tumour suppressor genes is associated with transcriptional silencing. Silencing of the SHP1 gene by promoter methylation has been detected in various kinds of leukaemia, lymphomas and Myeloma. Mutations in SHP2 (PTPN11) occur in approximately 50% of individuals with Noonan's Syndrome. Some of these patients develop a myeloproliferative disease which usually resolves but can develop into leukaemia. [20] It is not known whether each JAK is dephosphorylated by a different tyrosine phosphatase or whether one enzyme carries out this task for the whole family.

CD45 is a transmembrane phosphatase that negatively regulates JAK/STAT signalling stimulated by IL-3, IL-4 and EPO. [12] CD45 is restricted to haematopoietic

cells and appears to regulate all four JAKs. Mice deficient in CD45 show hyperactivation of JAK1 and JAK3, associated with a loss of antigen responses in T and B lymphocytes. However CD45 has no major effect on cytokine signalling. Neither SHPs nor CD45 associate with the JAK kinase domain suggesting that other tyrosine phosphatases may deactivate JAKs.[20]

The PTP1B and T cell protein tyrosine phosphatase (TC-PTP) have a high level of homology in their catalytic domain. PTP1B is expressed in many tissues and is located on the cytosolic face of the endoplasmic reticulum due to a hydrophobic sequence in its C-terminal end. [20] JAK2 and Tyk 2 have been shown to be physiological substrates of PTP1B and fibroblasts deficient in this phosphatase show changes in phosphorylation including hyperphosphorylation of JAK2 suggesting a role in regulation of cytokine signalling. [4]TC-PTP is widely expressed but has a particularly high expression in haematopoietic cells. [21] Both TC-PTP and PTP1B selectively recognise a motif centred on two tyrosine residues present in the JAK activation loop, but each one exhibits a different specificity for surrounding residues. PTP1B interacts with the D/E-pYpY-K/R sequence present in JAK2 and Tyk2 while TC-PTP interacts with the D/E-pYpY-T/V sequence present in JAK1 and JAK3.This allows for selective JAK dephosphorylation and subsequent deactivation. [20]

The mammalian SOCS family contains eight members which include CIS and SOCS1-7. They all share a central SH2 domain, a conserved C-terminal motif called the SOCS Box and an amino terminal region which is highly variable in length and

nucleotide sequence. The SH2 domain allows for protein-protein interactions with the cytokine receptor and with other signalling components. SOCS 2, 3 and CIS bind phosphotyrosines on the receptor whereas SOCS1 binds to phosphotyrosine residues on JAK. [20] Both the SH2 domain and the N terminal domain are required for inhibition of cytokine signalling. [22] The first SOCS family member to be isolated was CIS which was cloned as an immediate early gene induced by IL-2, IL-3 and erythropoietin. CIS is able to associate with phosphorylated tyrosines on the receptor. It binds to phosphorylated Y 401 of the EPO receptor, the binding site for STAT 5, thereby suppressing STAT 5 mediated signalling. [4,18,20] The SOCS family can suppress cytokine signalling either by inhibiting the activity of JAKs, by competition with STATs for phosphorylated docking sites on the receptors or by targeting bound signalling proteins to the ubiquitin proteasome pathway through the SOCS box which is part of an E3 ubiquitin ligase. [20] Thus protein turnover is regulated by targeting of proteins for polyubiquitination and proteasome mediated degradation. This is brought about by the SOCS box acting as a link between SH2 interacting proteins and an E3 ubiquitin ligase. In unstimulated cells most SOCS genes are expressed at very low levels. Many cytokines that signal via the JAK/STAT pathway induce the expression of SOCS genes to a variable extent in different cell types and tissues.

Overexpression of SOCS1 can inhibit virtually any JAK signal. SOCS1 interacts with the JAK kinase domain and and suppresses IL6 signal transduction pathways. [23] There is evidence that SOCS1 binds directly to the activation loop of JAK2

specifically binding Tyrosine 1007 which as previously stated is required for JAK kinase activity. SOCS may function as a tumour suppressor gene and its down regulation may contribute to tumour progression. The growth of cells transformed by an oncogenic form of KIT or by the TEL-JAK2 fusion protein can be suppressed by over expression of SOCS1. It requires the presence of the SH2 domain for inhibition of TEL-JAK2 but not for KIT implicating different functions of the SOCS1 proteins. [20] The SOCS gene can be down regulated by transcriptional repression by proto-oncoproteins or by silencing via hypermethylation. Methylation of CpG islands in the region of the tumour suppressor induces a block in transcriptional initiation. Hypermethylation of the SOCS1 gene has been reported in different solid tumours and haematopoietic malignancies and is associated with activation of the JAK/STAT pathway and of expression of downstream target genes. [20] Recently amplification of the JAK2 gene with resultant constitutive phosphorylation of JAK2 has been described in primary mediastinal large B cell lymphoma (MedB1 cell line). [24] The constitutive activation was found to be due to delayed protein degradation caused by a biallelic mutation of SOCS1 abrogating the SOCS box function of the protein. [25]

The STAT proteins can be modulated directly. Carboxy terminus truncated isoforms can act as dominant negative inhibitors and the STATs can be directly dephosphorylated. The PIAS (protein inhibitor of activated STATs) family appear to bind to activated STAT dimers blocking their ability to bind DNA. The PIAS family consists of PIAS1, PIAS 3, PIAS x and PIAS y. PIAS 1 and 3 have been proposed to block the DNA binding activity of STAT 1 and STAT 3 respectively. In contrast

PIASx and PIASy repress the transcriptional activity of STAT 1 and 4 by recruiting co-repressor molecules such as histone deacetylases. [20] Cells derived from patients with anaplastic lymphoma expressing NPM-ALK have constitutive activation of STAT3 which is thought to be due to loss of PIAS3. There is also some evidence to suggest that PIASy may contribute to the growth of MDS blasts and disease progression. [20]

In summary several different steps of the signal transduction pathway appear to be targeted by negative regulators, including the receptor/ligand complex, JAK kinases, and STAT transcription factors. This negative regulation can be achieved by dephosphorylation of signalling intermediates by protein tyrosine phosphatases such as SHP-1, interruption of the pathways and negative feedback loops (CIS family) and by proteolytic degradation.

1.4 PI 3-Kinase Pathway

The class 1 phosphoinositide 3 kinases are a ubiquitously expressed family of proteins which are central to cell survival and proliferation. They are subdivided into 2 groups-the class Ia and class Ib PI3-Kinases.The class Ia PI3-Kinases signal downstream of tyrosine kinases and Ras. They are heterodimeric proteins consisting of a 110 kda catalytic unit (p110α, p110β or p110δ) and a regulatory unit (p85α, p85β or p55γ) and are responsible for phosphorylating inositol lipids on the 3'hydroxyl position, in response to growth factor signalling. Each of the catalytic

units can associate with all of the regulatory units. [26] The class Ib PI3-Kinase, p110γ is activated by G-protein coupled receptors. There is only one catalytic unit and one regulatory unit known as p110γ and p101 respectively. The expression of p110δ and p110γ subunits is mainly restricted to leukocytes whereas p110α and p110β are more widely expressed. [26]

Akt, a serine/threonine kinase is a key mediator of PI3-Kinase signalling. [27] Stimulation by cytokines leads to the binding of the p85 regulatory unit, via its SH2 domains, to phosphotyrosine residues on the receptor or associated signalling proteins. This activates PI3-Kinase and stabilises the active complex at the cell membrane where it's major physiological substrate (phosphatidylinositol (4,5) P2) resides. PI3-Kinases can also be directly activated by GTP bound Ras. PI3-kinase phosphorylates PI(4,5)P2 creating PI (3,4,5) P3 (PIP3)- this recruits Akt via its Pleckstrin Homology (PH) domain to the plasma membrane where it is phosphorylated by another PH domain containing serine/threonine kinase, 3'phosphoinositide dependent kinase 1 (PDK1). PDK1 phosphorylates Akt on threonine 308 leading to its activation. Maximal activation requires further phosphorylation on serine 473 carried out by PDK2 an enzyme which is uncharacterised. [28] So far approximately 10 kinases have been proposed to function as PDK2 including mTOR, integrin linked kinase and DNA dependent protein kinase. [29] Activated Akt is then responsible for the phosphorylation of numerous downstream targets, regulating apoptosis and survival, cell growth and the cell cycle. [28]

The PI3-Kinase/Akt pathway is tightly controlled by the phosphatases, phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and SH2 containing phosphatase 1 and 2 (SHIP 1/2), which are responsible for dephosphorylating PIP3 on its 3' and 5' phosphates respectively.

There is now increasing evidence for PI3-Kinase/Akt dysregulation in human malignancy. [28] The p110α subunit is encoded for by the PIK3CA gene. Heterozygous point mutations in PIK3CA have been reported to occur in 30% of all breast and colon cancers but are less frequent in cancers of the brain, stomach, liver and ovary. The mutations are non randomly distributed over the primary structure of p110α and cluster to regions in the p85 binding domain, the C2 domain, the helical domain and the C terminus of the catalytic domain. The cancer specific point mutations of p110α confer a gain of function resulting in increased lipid kinase activity. [30] In AML, we have screened 92 samples and found no PIK3CA mutations (unpublished data). Lee et al found 1 mutation in 88 acute leukaemias screened. [31] The molecular mechanisms by which the mutants gain enzymatic function and hence oncogenic potential are not known.

PTEN is considered a tumour suppressor gene and loss of PTEN or SHIP activity can lead to over activity of PI3-Kinase. PTEN mutations have been described in a variety of solid tumours at high frequency and in addition in acute leukaemia and non Hodgkin's lymphoma (NHL). Sakai et al looked at a series of lymphoid cell lines and primary lymphoid tumours and found PTEN mutations in 22% of the cell

lines but only 4.6% of the primary lymphoid tumours. [32] Gronbaek et al found 5% of their diffuse large B cell lymphoma samples had PTEN mutations (2/39) [33] and Dahia et al found mutations in 10% of NHL samples but only 1.35% of acute leukaemias. [34] Therefore it can be seen that although PTEN inactivation is a possible mechanism for PI3-Kinase overactivity, so far mutations in PTEN have only been found in a small proportion of primary haematopoietic malignancies.

Dysregulation of upstream protein tyrosine kinases (PTK) may also lead to activation of the PI3-Kinase pathway. This loss of control of PTK activity may be due to chromosomal translocations such as t(9:22)(Bcr-Abl), gain of function mutations e.g. FLT3 internal tandem duplication (ITD) in AML and PTK gene overexpression e.g. neu/ ErbB2 and EGF receptor in breast and lung cancer.[35] The final common pathway in these genetic changes is of constitutive kinase activation and of quantitative and qualitative changes in downstream signalling.

Activating Ras mutations (20-30% across all malignancies) may also lead to PI3-Kinase overactivity. [28] Akt2 mutations have been described in some solid tumours, as a rare occurrence. [28] Staal et al described Akt1 gene amplification in an adenocarcinoma of the stomach out of 225 samples of a diverse range of malignancies screened. [36] Cheng et al found Akt2 gene amplification in 2/15 ovarian primary tumours and 1/10 pancreatic carcinoma samples. [37,38] Akt overexpression has also been linked to tumour progression. [39]

The role of dysregulation of PI3-Kinase in AML is less clear and is investigated further in Chapter 5.

1.5 MAP-Kinase Pathway

The MAP-Kinases are widely expressed serine/threonine kinases which have important roles in cell proliferation and survival. Aberrant activation of this pathway is common in malignantly transformed cells. Three different groups of MAP-Kinases exist, P38 MAPKinase family, the extracellular signal regulate kinase (ERK) family and the JNK family. I will be concentrating here on the Ras/Raf/MEK/ERK signaling pathway.

Ras is a small GTP binding protein which is a common upstream molecule in several signaling pathways including Ras/Raf/MEK/ERK and the PI3-Kinase pathway. Three different Ras proteins exist, H-Ras, N-Ras and K-Ras. Ras proteins are small membrane associated proteins with intrinsic GTPase activity allowing them to switch between active and inactive states. This switch depends upon the binding of GTP and GDP respectively. In order for Ras to function in the active state it is recruited to the membrane which is highly dependent on lipid modification of the Ras protein called prenylation. Prenylation is accomplished by two enzymes, farnesyl and geranylgeranyl transferases which add 15 and 20mer isoprenoids to the Ras protein. [40] Binding of ligand to the receptor leads to dimerisation of the receptor tyrosine kinase and activation of the tyrosine kinase domains. The signal is

transmitted to the GRB2/SOS complex which then promotes Ras activation. Although Ras has some intrinsic GTPase activity proteins such as p120GAP and NF-1 promote inactivation of Ras changing Ras-GTP to Ras-GDP. [40]

Raf is a serine/threonine kinase which can be activated by a number of different mechanisms. It can be activated by recruitment to the plasma membrane by interaction with Ras, by dimerisation of two Raf molecules and by phosphorylation on different domains. [39] Raf activity can be modulated by adapter proteins such as Bag-1, Hsp70 and 14-3-3. There are three members of the mammalian Raf family, Raf1, A-Raf and B-Raf. All three family members are able to phosphorylate and activate MEK although B-Raf is more potent than Raf-1 which itself is more potent than A-Raf.

MAPKs are activated by dual phosphorylation of conserved threonine and tyrosine residues within the activation loop (denoted T-X-Y) and phosphorylate targets on serine and threonine residues within a consensus PXT/SP motif (X can depend on the MAPK).

MEK a dual specificity kinase phosphorylates ERK on threonine and tyrosine residues which are required for full activation. ERK is then able to directly phosphorylate a set of transcription factors; Ets1, cJun and cMyc. ERK may also phosphorylate RSK which translocates to the nucleus and phosphorylates other transcription factors including CREB. CREB activation results in transcription of

Bcl-2 an antiapoptotic factor. Thus the overall effect of activation of the Ras/Raf/MEK/ERK pathway is of increased DNA synthesis, promotion of proliferation and increased survival.

Deregulation of the RAS pathway in cancer can occur via several methods; either directly through activating point mutations or indirectly through mutations of other oncogenes e.g receptor tyrosine kinases such as FLT3 and c-KIT or tumour suppressor genes e.g Neurofibromin (NF-1). Ras mutations are frequently observed in AML (20-30%), juvenile myelomonocytic leukaemia (30%), CMML (50%) and MDS (15-20%). N-RAS is mutated in the majority of cases and RAS proteins are typically activated by point mutations at critical RAS regulatory sites such as codons 12, 13, and 61. These mutations increase the half life of RAS-GTP through interruption of the normal intrinsic or GAP-stimulated GTPase activity. Inactivation of the RAS-GAP AF9Q34 through the t(9;11)(q34;q23) or inactivation of NF-1 can also cause deregulation of RAS activity by increasing the half life of activated GTP bound RAS. [41]

Juvenile myelomonocytic leukemia (JMML) is a rare, clonal, mixed myeloproliferative and myelodysplastic disorder afflicting young children. The pathogenesis of JMML arises from dysregulation of signal transduction through the Ras pathway. Potential causative mutations or other genetic abnormalities in three genes (RAS, NF1, and PTPN11), all of which are positioned in the GM-CSF/Ras signal transduction pathway, account for up to 75% of cases of JMML.

Alterations in the RAS signaling cascade are very common in melanoma. RAS itself is not frequently mutated although signaling molecules downstream of RAS such as B-Raf are often altered. B-Raf, has been found to be mutated in approximately 60% to 70% of superficial spreading melanomas. [42]

Activation of these signaling cascades results in changes in proliferation, differentiation and apoptosis.

1.6 Protein Kinase inhibitors

Small molecule inhibitors are useful agents in the investigation of cell signaling. They are rapid and simple to use and can be used both in transformed cell lines and normal cells and tissues. They inhibit endogenous kinases and there is no need for exogenous overexpression of dominant-inhibitory molecules leaving the cells little time to adapt and develop resistance to the agent. Early workers felt that as the majority of small molecule inhibitors are targeted to the ATP binding site which is highly conserved across protein kinases it would be difficult to achieve specificity. The kinase domains of all tyrosine kinases have a bilobar structure, with an N terminal lobe that binds ATP and magnesium, a C terminal lobe containing the activation loop and a cleft between the lobes to which polypeptide substrates bind. However it is possible to gain some selectivity as the development of agents such as the Abl inhibitor STI571 (Imatinib mesylate) illustrate. This is likely to be due to some key diversity around the proximal region of the ATP binding domain together with conformational changes between active and inactive kinases. The activation

loop controls catalytic activity by switching between states in a phosphorylation dependent manner, in fully active kinases the loop is held in an open conformation by phosphorylation on serine, threonine or tyrosine residues within the loop and in this conformation a β strand loop provides a base for substrate binding. This active conformation is very similar in all known structures of protein kinases. [43] In the inactive state, however, there is much greater diversity and the activation loop often occludes substrate binding.

The potency of these inhibitors is compared using the IC50 i.e. the drug concentration required for 50% inhibition. Of course, for ATP competitive inhibitors this will be dependent on the ATP concentration within the assay and this is also important to remember when comparing in vitro (ATP concentrations in micromolar range) and in vivo (ATP intracellular concentration in millimolar range) results.

As well as being important for the investigation of the physiological role of kinases within the cell these agents are now being developed for therapeutic use in many malignancies. This is because several protein kinases have been found to be dysregulated in a number of cancers. Protein tyrosine kinases can be activated by several mechanisms including chromosomal translocation as in BCR-Abl in chronic myeloid leukaemia; point mutations, in-frame deletions or insertions as in FLT3 in AML and over expression for example epidermal growth factor receptor and HER2 in various carcinomas. Increased activity can also occur as a result of reduced negative regulation e.g. reduced tyrosine phosphatase activity or decreased expression of inhibitor proteins. Aberrant PTK activation can lead to increased

survival and proliferation in tumour cells, resistance to cytotoxic therapy and in some tumours an increase in angiogenesis, invasiveness and metastatic potential.

The issue of selectivity is of course paramount when considering transferring these drugs to clinical practice. These protein kinases are widely expressed and one could expect many unwanted consequences associated with inhibition of tyrosine kinases in normal tissues. Selectivity can be achieved depending on the mode of binding of the tyrosine kinase inhibitor - for instance does it bind to the wild type or mutated kinase, to the activation loop in the active or inactive conformation? This can be illustrated by the findings of Schnittger et al. They investigated the activity of 3 inhibitors; Imatinib, SU5614 and PKC412 on BAF3 cells expressing the KIT D816V mutation. This mutation leads to constitutive activation of the kinase. They found that both Imatinib (known to inhibit wild-type KIT) and SU5614 were inactive in these cells but they were fully sensitive to the effects of PKC412. The profound differences in the sensitivity of the KIT D816V mutant to different PTK inhibitors probably relates to the binding mode of these agents. Structural studies have shown that Imatinib binds the kinase domain of KIT as well as ABL in the inactive state. A point mutation within the activation loop like Asp 816 valine in KIT confers constitutive activation and thus resistance to Imatinib. In contrast PKC412 is thought to bind within the ATP binding pocket of the active conformation of PDGFRA, which is probably also the case for its binding to the KIT receptor. [44] One of the new Abl kinase inhibitors Dasatinib (BMS-354825) is also a Src inhibitor. In vitro this drug is more potent than imatinib.[45]Whereas Imatinib binds to the inactive

conformation of the kinase loop which is distinct between Abl and Src, Dasatinib binds to the loop whether open or closed therefore inhibiting both kinases. In addition it is a smaller molecule than Imatinib and thus the P-loop must undergo major conformational changes to inhibit its binding and it is therefore able to inhibit many of the tyrosine kinase mutations that lead to imatinib resistance. Nilotinib (AMN107) is an ABL kinase inhibitor which was specifically developed to be more selective for BCR ABL and yet maintain clinical efficacy against some of the commoner mutations asssociated with imatinib resistance. Like imatinib Nilotinib also binds to the inactive conformation of ABL but gains in potency due to an improved topological fit [46]. Selectivity can also be achieved by targeting cancer specific proteins especially if the protein is restricted to cancer tissue, if it has specific enzymatic activity that shows a gain of function compared with wild type enzyme and if the mutant protein plays a causative role in the disease process. This is the situation for BCR-Abl in CML. In addition CML cells display 'oncogene addiction' and the cells cannot survive with out BCR-Abl. This can be explained by the fact that BCR-Abl blocks downstream apoptotic pathways and so pro-apoptotic proteins accumulate upstream. When the apoptotic block is removed by inhibition of BCR-Abl, apoptosis occurs. Non small cell lung cancer cells expressing upregulated EGFR also demonstrate 'oncogene addiction' and are susceptible to EGFR inhibition with Gefitinib. It has been suggested that cells with multiple genetic aberrations, and thus alteration of a number of signalling pathways are less likely to become addicted to a single abnormal kinase. [47]

Experience with Imatinib has demonstrated that resistance to tyrosine kinase inhibitors can occur through a number of mechanisms likely to be applicable to other tyrosine kinase inhibitors. Decreased intracellular drug levels may occur because of excess binding by α-1-acid glycoprotein or by increased drug efflux from P-glycoprotein over expression. [48]Influx proteins are less well characterised in the area of multi drug resistance than efflux proteins. The organic cation transporter proteins (OCT) are involved in the absorption, distribution and elimination of drugs in vivo[49]. White et al demonstrated that the interpatient variability in IC50 of imatinib, defined as 50% reduction in phosphorylated CrKL, was mainly due to differences in intracellular uptake and retention of imatinib. Prazosin, an OCT-1 transporter blocker, eliminated this interpatient variability suggesting that differential expression or function of OCT-1 is a significant determinant of imatinib response.[49]Gene amplification of the BCR-Abl kinase has been associated with the development of resistance to Imatinib as has clonal evolution. This evolution has been observed in paired cytogenetic analyses from the beginning of Imatinib therapy and at the development of resistance. These chromosomal abnormalities include second Philadelphia chromosome, the development of trisomy 8, loss of a p53 allele via alteration of the short arm of chromosome 17 or the development of new reciprocal translocations. The best documented mechanism of resistance to Imatinib in CML is the occurrence of gene mutations in the Abl tyrosine kinase domain. These single nucleotide substitutions result in replacement of individual amino acids that have varying effects on the conformation of the Abl portion of the BCR-Abl and it's binding to drugs or substrates. These mutations are distributed at multiple sites

throughout the BCR-Abl kinase, including within the nucleotide binding or P-loop, within the active site where Imatinib binds and within the activation loop and carboxy terminal. [48] It is clear that CML stem cells are relatively more resistant to imatinib than more differentiated cells[50] leading to disease persistence even in optimally responding patients.[51]Copland et al investigated possible underlying mechanisms for this resitance and were able to exclude gene amplification as a possible cause. They found that these more primitive cells expressed higher BCR ABL transcript levels and in addition found increased protein expression of BCR ABL, Phosphotyrosine and phospho-CrKL confirming increased BCR ABL activity in this fraction.[52] Methods of overcoming resistance include increasing the dose of Imatinib, the development of new inhibitors and the use of combination therapy. Dasatinib is very much more potent than imatinib and inhibits BCR ABL + cells further back in the stem cell compartment; however the quiescent fraction of stem cells appears to be inherently resistant to imatinib and dasatinib.[52]There is some in vitro evidence that suggests that using imatinib in combination with the farnesyl transferase inhibitor, Lonafarnib, may reduce resistance of this primitive quiescent cell fraction.[53]

The success of Imatinib in CML has provided encouragement that kinase inhibitors may be valid therapeutic modalities; however in chronic phase CML, the BCR-Abl translocation is thought to be the sole abnormality driving the disease; in diseases where several genetic abnormalities exist, such as in AML, a combined therapeutic approach is likely to be necessary. The phenomenon of 'oncogene addiction' discussed earlier may allow for a greater therapeutic window.

1.7 JAK 2 Inhibitors

JAK2, a member of the Janus kinase (JAK) family of protein tyrosine kinases (PTKs), is an important intracellular mediator of cytokine signaling. Mutations of the JAK2 gene are associated with haematologic cancers (discussed in detail in chapter 4), and aberrant JAK activity is also associated with a number of immune diseases, including rheumatoid arthritis. Therefore, the development of JAK2-specific inhibitors could have many clinical applications. However as a corollary of its widespread involvement in haematopoietic cytokine signaling, predictable side effects of JAK2 inhibition would include anaemia, thrombocytopenia and neutropenia.

1.7.1 AG490

AG490 is a tyrphostin inhibitor containing a cyano group which gives it its yellow colour. It inhibits JAK2 and JAK3 at micromolar concentrations and is also known to inhibit the EGFR tyrosine kinase. It has negligible effects on JAK1 and Lck. AG490 was found to induce death in Pre B ALL cells which constitutively express phosphorylated JAK2 and was also found to eradicate the recurrent form of Pre B ALL in SCID mice into which the disease had been engrafted. [54] De Vos et al found that AG490 suppressed cell proliferation and induced apoptosis in IL-6-dependent MM cell lines. They found JAK2 kinase activity, ERK2 and STAT3 phosphorylation were all inhibited. [55] Another group combined therapy with AG-490

and IL-12 and found that this combination induced greater antitumour effects than either agent alone in a murine myeloma tumor model. [56] AG490 was also found to inhibit JAK3 mediated IL-2 induced T cell proliferation in T cell lines. [57] Levitzki also reported that AG490 triggers apoptosis in prostate cancer cells by inhibition of autocrine/paracrine IL-6. [58] On the face of it AG490 would appear to be a useful JAK2/3 inhibitor; however Kleinberger-Doron et al report that AG490 inhibits the activation of the enzyme CDK2 without affecting its levels or its intrinsic kinase activity, leading to cell cycle arrest at G1/S. [59] Therefore it is possible that some of the effects of AG490 on cell survival and proliferation may have been mediated by CDK2 inhibition and cell cycle arrest rather than being directly attributable to JAK inhibition. Without the use of other JAK inhibitors with a different structure, a dominant negative JAK protein or even RNA interference these results may be regarded as being flawed.

1.7.2 JAK Inhibitor 1

JAK inhibitor 1 (JI1) is a Pyridone containing tetracycle that inhibits JAK family members. It is a reversible inhibitor competitive with respect to ATP and non competitive with respect to substrate. It shows selectivity for JAK family members and within the family inhibits Tyk2 and JAK2 (IC50 1nM) more potently than JAK1 and JAK3 (IC50 5and 15nM respectively). [60] Lucet et al use JI1 to examine the crystal structure of the active catalytic domain of JAK2. They found that JI1 was buried deep within a constricted ATP-binding site, in which extensive interactions,

including residues that are unique to JAK2 and the JAK family, are made with the inhibitor. [61] JI1 is able to inhibit IL-2 and IL-4 driven proliferation of a murine T cell lymphoma line, mediated through JAK1 and JAK3 inhibition (IC50 100nM). Simultaneously inhibition of STAT 5ab phosphorylation occurs. Non specific effects of JI1 are evident with its ability to inhibit PMA induced proliferation which does not require signaling through JAK family members. [60]

1.7.3 JAK3 Inhibitors

Unlike other JAKs, JAK3 expression is restricted to haematopoietic tissues and its use is limited to cytokines signalling through the common γ chain including IL-2, 4, 7 and 15 and 21. These cytokines modulate lymphoid development and function and JAK3 inhibitors are being developed as immunosuppressant and anticancer agents. A highly specific JAK3 inhibitor should have limited and precise effects but because of the homology between JAKs this selectivity is particularly challenging. JAK2 is essential for many haematopoietic cytokines including EPO and GMCSF and TPO. By inference pharmacological inhibition of JAK2 could lead to anaemia, thrombocytopenia and neutropenia. JAK1 inhibition could lead to an interruption in IFN signaling and may lead to an increased susceptibility to viral infections. Therefore concurrent inhibition of these kinases could lead to an intolerable side effect profile. However despite this some selective agents have been discovered and are discussed below.

CP690550 is an orally active drug which is a potent inhibitor of JAK3 in the nanomolar range in vitro and is ~ 30 fold and ~ 100 fold less potent for JAK 2 and JAK1 respectively. This drug is effective at preventing transplant rejection in 2 models: a murine heterotopic heart transplant and a non human primate renal transplant model. In both cases the drug prolonged graft survival. It was found to block IL-2 signalling and IL-2 dependent gene expression without having an effect on T cell receptor signaling. This would allow it to be used synergistically with calcineurin inhibitors. The side effect profile of this drug is acceptable with no reported granulocytopenia or thrombocytopenia suggesting that in vivo JAK2 inhibition is not a great problem. [62]

The dimethyl quinazolines inhibitors, WHI-P154 and WHI-P131 are reported to inhibit JAK3 with an IC50 for WHI-P154 of 28μM but it did not inhibit JAK1 and JAK2. The reported IC50 for WHI-P131 was 9μM. WHI-P131, did not inhibit JAK1, JAK2, SYK, BTK, LYN, or IRK at concentrations as high as 350 microM. WHI-P131 and WHI-P154 inhibited JAK3 and induced apoptosis in leukaemia cell lines, NALM6 and LC1;19 but not in JAK3 negative melanoma or squamous carcinoma cells. [63]

PNU 156804, an undecylprodigiosin antibiotic has been shown to inhibit IL-2 induced cell proliferation, IL-2 induced JAK3 autophosphorylation and lead to a reduction of STAT 5a/b and ERK phosphorylation. Stepkowski et al report that PNU 156804 was able to prolong the survival of murine heart allografts in a dose

dependent manner. It also acted synergistically with cyclosporine and additively with rapamycin to block allograft rejection. [64]

Because of JAK3's limited expression and the fact that its only consequential biological function is restricted to immune cells, a selective JAK3 antagonist is not associated with widespread effects on other organs and as such a selective JAK3 antagonist would differentiate itself from other currently available immunosuppressants. These drugs are heading towards clinical trials and may also find uses in the treatment of cancer, autoimmunity and allergy.

Figure 1.1

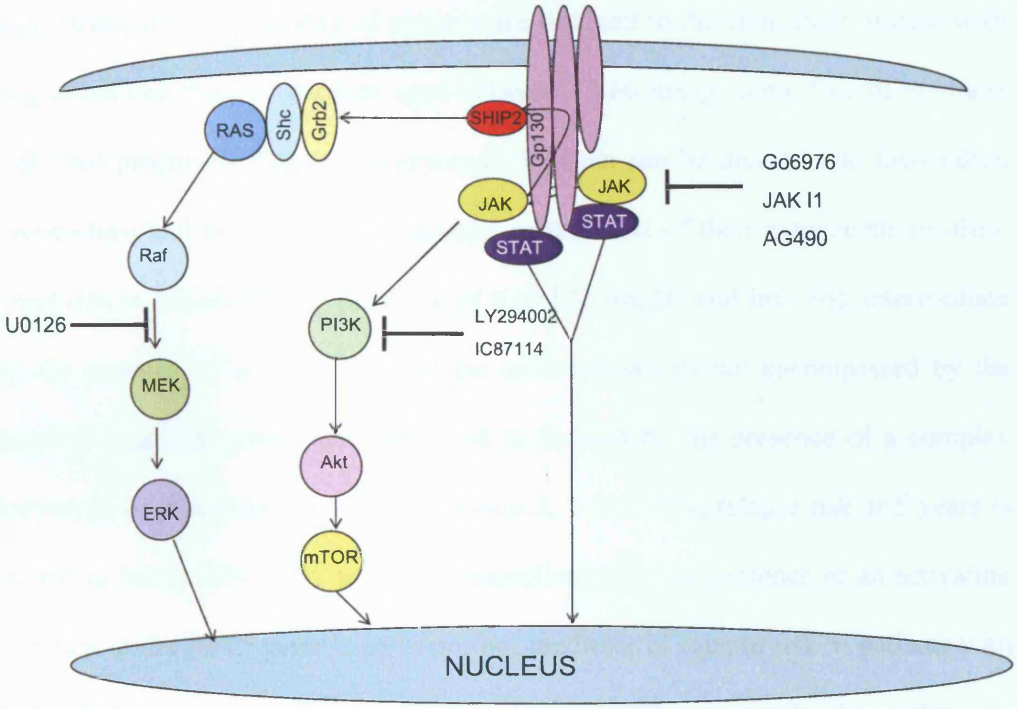


Figure 1. 1. A diagram illustrating the 3 main signalling pathways downstream of a class 1 cytokine receptor and the level of blockade of relevant kinase inhibitors.

1.8 Acute Myeloid Leukaemia

Acute myeloid leukaemia is a clonal haematopoietic stem cell disorder encompassing a group of heterogeneous diseases which are characterized by uncontrolled proliferation of the malignant clone and impaired normal haematopoiesis leading to neutropenia, thrombocytopenia and anaemia. Untreated patients die of infection or bleeding in a matter of weeks. The diagnosis is made

morphologically with immunophenotyping, cytogenetics and molecular techniques all adding to the biological picture. Despite improvements in cytotoxic therapy and supportive care, the majority of patients are destined to die from their disease with only about one third of patients aged between 18-60 being cured. One of the most important prognostic factors is karyotype. Patients can be divided into favourable, intermediate and poor prognostic groups on the basis of their cytogenetic profiles. Good risk is defined by the presence of t(15;17), t(8;21) and inv (16), intermediate by the presence of a normal karyotype or abnormalities not encompassed by the good or poor risk groups and poor risk is defined by the presence of a complex karyotype or abnormalities of chromosome 3, 5 or 7. The relapse risk at 5 years is quoted as being 35%, 51% and 76% respectively. [65] The presence of an activating mutation in the FLT3 gene is an important predictor of relapse risk in patients with AML. [66] An internal tandem duplication of the FLT3 gene can be detected in 20-30% of adults with AML and appears to be associated with an increased relapse risk rather than a failure to enter remission. The presence of the FLT3 mutation has aided prognostication in the intermediate cytogenetic risk group. Other predictors of relapse are increasing patient age, high white cell count and a history of preceding myelodysplasia. The increasing relapse risk with age is likely to reflect the increase in adverse cytogenetic abnormalities and/or an increased incidence of drug resistant phenotypes e.g. P-gp positivity. There is an increased frequency of FLT3 gene mutations in patients with a high white count and this may account for the higher relapse rate in this group.

AML has been proposed to arise from the collaboration of two broad classes of mutation, a class I or proliferative mutation and a class II or blocking mutation. Thus dysregulated proliferation and impaired differentiation bestows a survival advantage on the leukaemic cell. A very large number of mutations associated with AML have been discovered with over 300 chromosomal translocations having been reported to occur.

Deregulated proliferation may arise as a result of mutations affecting proliferative signaling pathways for instance in class III tyrosine kinase receptors. The FLT3 receptor is present on early haematopoietic progenitors and AML blasts. Mutations in the FLT3 receptor juxtamembrane region result in a gain of function of the receptor. The exact stretch of DNA duplicated can vary from approximately 16 to 108 nucleotides but is always in frame. Mutation can also occur in the activation loop of the kinase again leading to presumed constitutive activation, the prognostic impact of these mutations is less clear.

C-KIT is a class III receptor tyrosine kinase for stem cell factor, stem cell factor binding promotes dimerisation and transphosphorylation and downstream signaling events leading to cell growth. Constitutively activated forms of the c-KIT have been associated with systemic mast cell disease, acute myeloid leukemia, and gastrointestinal stromal tumors. The most common mutations in systemic mast cell disease and AML are the D816V and D816Y mutations in the activation loop. [67]

JAK2 is activated by the V617F point mutation in the overwhelming majority of polycythaemia vera and a significant proportion of essential thrombocythaemia and myelofibrosis, all these disorders can evolve into AML. It has also been reported in 5% of patients with myelodysplastic syndrome [68] and so would be thought likely to be found in those patients who transform to AML, although most reports so far have not found this to be the case.

Activated tyrosine kinases can transmit proliferative signals by the engagement of the Ras family of G proteins and mutations of genes encoding these proteins may mimic the effects of receptor tyrosine kinase mutations. There are 3 functional Ras genes, N-(neuroblastoma line), K-(Kirsten) and H-(Harvey) Ras. Bowen et al screened a large number of AML patients less than 60 years and found N-Ras mutations in 11% and K-ras mutations in 5% of patients, no H-Ras mutations were found. They found these mutations rarely coexisted with FLT3 mutations and unlike FLT3 ITD, they did not appear to influence clinical outcome. [69]

Usually the RTK pathways in AML are activated by gain of function mutations one exception to this would be a mutation in NF-1 leading to loss of function. NF-1 inactivates RAS by enhancing its GTPase activity and thus this mutation leads to activation of RAS. An activating mutation in the PTPN11gene encoding SHP-2, leads to removal of phosphates from key substrates which paradoxically leads to increased signalling through receptor tyrosine kinase pathways.

Transcription factors are commonly disrupted in AML usually as a result of chromosomal translocations but also as a result of point mutations. Transcription factors commonly involved in chromosomal rearrangements include the core binding factor complex, the retinoic acid receptor, the MLL protein and Hox proteins. Point mutations in myeloid transcription factors including C/EBPα and PU1, may also lead to loss of normal myeloid differentiation in AML.

The core binding factor complex is a heterodimer of CBFβ and one of 3 alpha units; CBFα1, AML1 (CBFα2) and CBFα3. CBFβ enhances DNA binding by stabilization of the protein's conformation. CBFβ markedly augments the metabolic stability of AML1, which by itself is highly susceptible to proteasome-mediated degradation. CBFβ does not bind DNA directly but increases the affinity of the alpha subunits for consensus DNA binding motifs. The core binding factor regulates a number of haematopoietic genes critical for myeloid development. CBFβ and AML1 are reported to be disrupted by chromosomal translocations in 30% of cases of AML leading to chimeric fusion proteins such as AML-ETO, AML1-EVI1 and CBFβ-MYH11. These chimeric fusion proteins disrupt normal AML1 function by acting as dominant negative transcription factors. The characteristic differentiation block occurring in CBF leukaemias may also occur as a result of point mutations in CCAAT/enhancer binding protein, a myeloid transcription factor required for granulocytic differentiation. Interestingly C/EBPα point mutations are known to act as a class II mutation in 7-11% of cases of AML.

Acute promyelocytic leukaemia is always associated with rearrangement of the retinoic acid receptor alpha (RARα). In over 98% of cases of APL this is secondary to the t(15;17) which generates the PML-RARα fusion protein. Other fusion partners include PLZF, STAT 5b and nucleophosmin. RARα acts as a regulator of myeloid development. In normal cells RARα would heterodimerise with a member of the retinoid-X receptor (RXR) family to allow high affinity DNA binding. [70] The configuration of the heterodimer binding together with the presence of corepressor or coactivator binding determines the pattern of response. In the absence of ligand, retinoic acid, corepressors bind to the ligand binding domain of RAR and silence gene expression by the recruitment of histone deacetylases and SIN 3. PML-RARα or the other chimeric proteins bind corepressors and recruit HDAC and SIN3 and thus lead to gene silencing. PML-RARα also disrupts the normal PML nuclear bodies. [70] FLT3 mutations are a common cooperating mutation in APL occurring in approximately 35% of cases.[66]

The mainstays of treatment in APL are ATRA and Arsenic which overcome the differentiation block by reactivation of the RAR target genes and degradation of the PML-RARα, leading to terminal differentiation of the promyelocytes.

Mutations in AML may also affect self renewal an example of this is the MLL rearrangement. The mixed lineage leukaemia gene is located on chromosome 11q23 and rearrangements of this gene are common in therapy related leukaemias particularly where topisomerase II inhibitors have been previously used. The MLL gene may be rearranged with a number of fusion partners. The MLL-fusion protein

is able to dimerise with itself and wild type MLL and these complexes are recruited to Hox genes to activate their expression. Hox gene expression is associated with increased self renewal in haematopoietic cells. [71]

In many cases of AML the clinical outcome may be correlated with expression of pro apoptotic and pro survival molecules. These molecules may be activated by protein tyrosine kinase activation via pathways such as PI3-Kinase. Akt phosphorylates BAD leading to the release of the anti-apoptotic molecule Bcl-2.
P53 is a focal point in the regulation of apoptosis and cell cycle. Mutations within P53 are associated with adverse response to chemotherapy in AML. The function of this protein may be affected in many ways including repression of p14 ^{ARF} by AML1-ETO or by mutations in the nucleophosmin (NPM) gene.[72] [71] Mutations in nucleophosmin occur in approximately one third of de novo AML cases, the expression of this cytoplasmic variant is associated with the expression of genes thought to maintain a stem cell phenotype. [71] Disruption of the normal PML body function and failure of NPM to sequester MDM2 can also lead to decreased P53 and loss of G1 checkpoint control.[71,73,74]

It can be seen that the molecular biology of AML is complex with many defects and biological processes intertwining. It is envisaged that through a more clear understanding of these processes more precise and specific therapies for AML will be developed.

1.9 Molecularly targeted treatment in AML

NPM1, FLT3 and Ras mutations are the most frequently mutated genes in haematological malignancies.[75]Intensive research has been focused on the development of specific inhibitors that target these enzymes.

The FLT3 receptor is a membrane bound receptor crucial for the maintenance, proliferation and differentiation of haematopoiesis.[76] It is expressed by normal myeloid and lymphoid progenitors as well as by leukaemic cells in 70-90% of AML patients.[77] Mutations are commonly found in AML (25-40%) [41] and less commonly in myelodysplasia (2.5-8.8%). There are two main types of FLT3 mutations-internal tandem duplications (ITD) of the juxtamembrane domain and point mutations in the highly conserved tyrosine kinase domain (TKD)-both of which cause constitutive activation of FLT3 and concomitant activation of downstream signalling pathways (e.g Ras and PI3-kinase/Akt).[78] Interestingly these two types of activating mutations show differences in their signaling properties. FLT3 ITDs activate STAT 5 and repress c/EBPα and Pu-1 whereas FLT3 TKDs do not. [79]Several small molecule tyrosine kinase inhibitors with varying specificity for FLT3 have been developed including CEP701, SU5416, SU5614 and PKC412. Some of these compounds have demonstrated promising clinical results. CEP701 is an orally available indolocarbazole derivative that preferentially inhibits autophosphorylation of wild type and mutant FLT3 with only limited inhibition of c-KIT, c-FMS and PDGFRβ. In a phase I/II trial in relapsed and refractory AML, CEP701 was well tolerated and

demonstrated some clinical activity in 5 out of 14 patients. [41] However responses were short lived (2 weeks to 3months).

SU5416 is a potent inhibitor of several tyrosine kinase receptors, including both wild type and mutant FLT3 (IC50 0.1-0.25μM), vascular endothelial growth factor receptor (VEGFR)-1 and VEGFR-2 and c-KIT. Several phase II trials of this agent in refractory AML have been carried out. Objective responses have been few. A phase II trial of SU5416 in c-KIT positive refractory AML was carried out in 42 patients. One patient had a complete remission lasting 2 months and 7 patients had partial responses lasting 1-5 months. None of the patients with FLT3 ITD responded to SU5416. [41]

PKC 412 is an inhibitor of protein kinase C, kinase insert domain containing receptor, c-KIT, fibroblast growth factor receptor, PDGFRα and β. In a phase II study in 20 relapse/refractory AML or high grade MDS patients with FLT3 mutations, 14 patients achieved more than a 50% reduction in peripheral blast counts and 7 of these 14 had a more than 2 log reduction in peripheral blast count that lasted more than 4 weeks. FLT3 autophosphorylation was inhibited in most patients demonstrating in vivo target inhibition. [41] Trials are underway to investigate FLT3 inhibitors in combination with chemotherapy.

As discussed in section 1.5 dysregulation of Ras in AML can occur directly through point mutations or indirectly through mutations of other oncogenes. Proper

intracellular localization of Ras proteins is critical for the successful transduction of mitogenic stimuli. To properly bind to the plasma membrane and gain full biological activity Ras proteins must undergo several post translational modifications including prenylation, proteolysis, carboxymethylation and palmitoylation. These steps are catalysed by specific enzymes including farnesyltransferase, human Ras converting enzyme-1, isoprenylcysteine carboxyl methyltransferase and palmitoyl acyl transferase. Inhibitors of each of these steps are being developed but the development of farnesyltransferase inhibitors is the most advanced. FTI's elicit a number of cellular effects including alteration of cell cycle progression, induction of apoptosis, effects on actin stress fibres, changes in cell morphology and inhibition of anchorage dependent growth. [41] R115777 (tipifarnib/Zarnestra) has been the most extensively studied. Several clinical trials have been reported in AML/MDS and overall response rates of 20-30% have been observed. Responses have included complete remissions. R115777 is currently under investigation in combination with classical chemotherapeutic agents such as etoposide, idarubicin and cytarabine.

Mammalian target of rapamycin (mTOR) is an essential mediator of growth signals that originate from PI3-kinase. As discussed earlier dysregulation of the PI3-kinase pathway has been reported in a number of malignancies including AML. mTOR was originally identified as the target of the macrolide antibiotic rapamycin. mTOR is a serine/threonine kinase involved in the regulation of cell growth and proliferation by translational control of key proteins such as the cyclin-dependent kinase (CDK) inhibitor p27kip1, retinoblastoma protein, cyclin D1, c-myc, or STAT 3. mTOR is

activated by different stimuli including nutrients or growth factors. Once activated, mTOR can phosphorylate its downstream targets, the ribosomal p70S6 kinase (p70S6K) and the 4E-binding protein 1 (4E-BP1) [80]. mTOR inhibitors (rapamycin, or analogues such as CCI-779, RAD001, AP23573), have been shown to have a potent anti-neoplastic effect in many solid tumor models. Recher et al found that 4 out of 9 patients with relapsed/refractory AML had a significant clinical response after treatment with Rapamycin[81]. These agents are currently being further evaluated.

An example of molecularly targeted therapy which is now in common use is that of ATRA in acute promyelocytic leukaemia. In 100% of APL cases the RARα gene is involved in reciprocal translocations which generate RAR fusion proteins. The most common translocation is the t(15;17)(q22;q21) which results in the PML-RARα and is found in 95% of all APL cases. This translocation results in the abnormal recruitment of NCoR/SMRT through oligomerisation of RARα by the coiled coil domain of PML. PML-RARα oligomers are able to associate with more than one NCoR/SMRT complex simultaneously while wild type binds to only one. This results in a local increase in concentration of HDAC complexes on the DNA of target genes and enhanced transcriptional repression in the presence of physiological concentrations of retinoic acid. Higher than physiological doses of retinoic acid are required for the dissociation of the NCoR/SMRT/HDAC complex and thus to allow recruitment of the coactivators and for transcriptional activation. Treatment with

ATRA has revolutionized the management of APL leading to a good prognosis and overall survival rates of 60-70%.

AML is a heterogeneous disease with aberrations in multiple signaling pathways often within the same clone. As has been discussed above, the cooperation of at least two mutations is thought to be necessary for the onset of AML one leading to a proliferative advantage such as FLT3-ITD and another leading to a block in differentiation such as CBFβ. It seems unlikely that inhibition of a single pathway would be sufficient in AML to bring about long term responses and this seems to be the case with clinical trials with FLT3 inhibitors so far. However a combinatorial approach either using two or more kinase inhibitors or with a kinase inhibitor together with classical chemotherapeutic agents may be more successful.

1.10 Aims

The aim of the following research was to investigate the activity of the protein kinase inhibitor Gö6976 on JAK family members and to take this information and further our knowledge of the role of the JAK/STAT pathway in AML. In particular the effect of JAK kinase inhibition on survival and proliferation in haematopoietic cell lines and primary AML cells was investigated. As PI3-Kinase/Akt and Ras/MAPK are involved in signaling downstream of a number of tyrosine kinases including JAKs and as they may be affected directly by mutation, the activity of PI3-kinase and MAPK inhibitors in AML was also examined.

CHAPTER 2-MATERIALS AND METHODS

2.1 General Cell Culture

2.1.1 Reagents

Cell culture media

Gibco,Invitrogen LifeTechnologies Paisley,UK

RPMI 1640 medium (21875-034)

DMEM medium (31885-023)

Foetal Calf Serum (FCS) (10106-169) heat inactivated at 56°C for 30mins

Dulbecco's phosphate buffered saline (PBS) (14190-094)

Dulbecco's phosphate buffered saline containing Ca++ and Mg ++ (14040-091)

Foetal Bovine serum Hyclone, USA (SH30071)

Tissue culture plastics were from Costar NY 14831 USA and BD Biosciences, San Jose, CA, USA

2.1.2 Growth factors

Murine IL-3	Peprotech EC Ltd, London, UK
Human stem cell factor	Peprotech EC Ltd, London, UK
Recombinant human GMCSF	Behringwerke-Hoechst, Marburg, Germany
Human Erythropoietin	POM 'Eprex'
Human IL-3	Sandoz, Frimley Park, UK
Human IL-6	Peprotech EC Ltd London UK
Interferon α	Peprotech EC Ltd London UK
Interferon γ	Peprotech EC Ltd London UK

The following products were purchased from SIGMA-Aldrich, UK.

Phorbol 12 Myristate 13 Acetate (PMA)	(P-1680)
Penicillin and Streptomycin	(P-4458)
Trypsin-EDTA	(T-4174)

2.1.3 Cell lines

Name	Description	Mutations/Fusion protein
32D	murine myeloid line	
TF-1	human erythroleukaemia line	
Mo 7E	human megakaryoblastic leukaemia	
HL60	human acute myeloid leukaemia M2	N Ras
KG-1a	human acute myeloid leukaemia	
K562	human chronic myeloid leukaemia in blast crisis	BCR-ABL
Jurkat	human T cell Leukaemia	PTEN deletion
U937	human histiocytic lymphoma/monocytic markers	
U266	}	B-RAF
MM1S	} Cell lines derived from human myeloma patients	
KMS-BM	}	
KMS-PE	}	
KARPAS 299	human anaplastic lymphoma	NPM-ALK
SUDHL1	human anaplastic lymphoma	NPM-ALK
KMH2	human Hodgkin's lymphoma	
293T	human embryonal kidney	
HELA	human cervical carcinoma line	

293T and HELA cells were cultured in DMEM with 10% FCS. The human myeloma lines were grown in RPMI 1640 with 10% foetal bovine serum; all other lines were cultured in RPMI 1640 with 10% FCS. TF-1 and Mo7E cells were supplemented

with 20ng/ml of rhGMCSF whilst 32D cells were either supplemented with 10% WEHI 3B conditioned medium or with 10ng/ml murine IL-3.

2.1.4 Primary cells

Patient samples were obtained from patients presenting to University College Hospital, London, at presentation or relapse of AML. Informed consent was obtained from all patients prior to obtaining the sample. All patients had circulating leukaemic blasts in the peripheral blood and these were isolated by ficoll gradient centrifugation. All samples tested had more than 90% blasts by morphology and/or immunophenotyping. Mononuclear cells were frozen in RPMI 1640 with L Glutamine (30%), foetal calf serum (50%) and dimethyl sulfoxide (DMSO) (20%) and stored in liquid nitrogen.

2.1.5 Inhibitors

Inhibitor	**Stock concentration**	**Cat N°**	**Supplied by**
Gö6976	10mM	G-6203	LClabs,Woburn,MA01801
Gö6983	5mM	365251	Calbiochem,California,USA
AG490	100mM	658401	Calbiochem California,USA
LY294002	50mM	L-7962	LClabs,Woburn,MA01801
U0126	10mM	U-6770	LClabs,Woburn,MA01801
Staurosporine	1mM	S4400	Sigma Aldrich, UK

All inhibitors were dissolved in DMSO and stored at-20°C or -80°C for prolonged periods.

2.1.6 Chemotherapeutic agents

Cytosine Arabinoside (Ara C) David Bull Laboratories, Victoria, Australia..

Etoposide (VP16) Medac, Stirling University, UK.

2.1.7 Ficoll gradient Centrifugation

Peripheral blood or bone marrow was diluted 1:2 with PBS with no additives and layered on to an equal volume of Ficoll-Paque ^{TM} Plus (Amersham, Bucks, UK (17-1440-03)). The cells were then spun at 1800rpm for 20mins. Cells from the interface were then removed, placed in a clean tube, and washed once in PBS. The resulting pellet was then resuspended in RPMI 1640 and incubated at 37° 5% CO_2.

2.2 SDS-PAGE and immunoblotting

2.2.1 Reagents

Pefabloc Boehringer-Mannheim, Germany

Aprotinin (A4529) SIGMA, Poole UK

Leupeptin SIGMA

PepstatinA SIGMA

Microcystin LR SIGMA (M2193)

Protease inhibitor cocktail SIGMA (P8340)

dl-Dithiothreitol (DTT) SIGMA (D9163)

Acrylamide/N'N'-bis-methylene 30%:0.8% National Diagnostics, Hull,UK

Ammonium persulphate (APS) BDH, UK

N'N'N'N-tetra-methylethylenediamine (TEMED) Biorad, CA,USA

Prestained molecular weight markers, Gibco (10748-010)

Hybond-C-Extra nitrocellulose membrane, Amersham Life Sciences, Bucks, UK

Non-fat dried milk (MARVEL)

Peroxidase-conjugated anti-sera DAKO Ltd, High Wycombe, Bucks,UK

Enhance chemoluminescence kits (ECL and ECL-plus) Amersham Life sciences

Hyperfilm^{TM} ,high performance autoradiography film, Amersham Life Sciences

2.2.2 SDS-PAGE buffers, stains and gels

Lysis Buffer (made in ddH_20)-50mM HEPES pH7.5, 100mM NaCl, 1% Triton X100, 1mM EDTA, 1mM EGTA, 20mM NaF, 1mM Na orthovanadate, Aprotinin 10 μg/ml, Pepstatin 10 μg/ml, Leupeptin 10 μg/ml, Microcystin 5μM and Pefabloc 1mM added to lysis buffer immediately prior to use.

1 litre of 10x Gel running buffer-Tris base 30.3g, Glycine 144.2g, SDS 10g

1 litre of 10x transfer buffer-Tris base 30.3g, Glycine 144.2g, Methanol 100mls

Gel sample buffer-50mls 4x made in ddH_20, 1M Tris pH 6.8 17.5ml, SDS 4g, DTT 2.315g, Glycerol 20mls, Bromophenol blue 50mg

Coomassie blue stain-Methanol 500mls, Water 400mls, Acetic acid 100mls, Coomassie R-250 2.5g

Destain-Methanol 250mls, Water 680mls, Acetic acid 70mls

Acrylamide Gels

	7.5% separating gel	*10% separating gel*	*Stacking gel*
H_20	3.66mls	2.86mls	3.49mls
1MTris	3.74mls (pH 8.8)	3.74mls (pH 8.8)	625µl (pH6.8)
Acrylamide	2.54mls	3.34mls	835µl
10% SDS	100µl	100µl	50µl
10% APS (v/v)	75µl	75µl	38µl
TEMED	9µl	9µl	7.5µl

2.2.3 Antibodies

Phospho-p42/44 (Thr 202/Tyr204)(9211)	New England Biolabs
Phospho-FKHRL1 (Thr 32)(06-952)	Upstate Biotechnology
Phospho-Akt (Ser 473) (9271)	Cell Signalling Technology
Phospho-Akt (Thr 308)	Cell Signalling Technology
Phospho-STAT 5(Y694) (9351)	Cell Signalling Technology
Phospho-STAT 3 (Tyr 705) (9131)	Cell Signalling Technology
Phospho-STAT 1 (Tyr 701) (9171)	Cell Signalling Technology
Phospho-p70S6Kinase (Thr 389) (9205)	Cell Signalling Technology

Phospho-GSK 3β(Ser 9)(9331)	Cell Signalling Technology
p27 (C-19) (sc-528)	Santa Cruz Biotechnology Inc
PTEN (N-19) (sc-6818)	Santa Cruz Biotechnology Inc
Stat 5b (C-17)	Santa Cruz Biotechnology Inc
Bcl-X (610211)	BD Transduction Labs
Bax (N-20) (sc-493)	Santa Cruz Biotechnology Inc
Bcl-2 (610538)	BD Transduction labs
JAK 2 PYPY(44-426)	Biosource Int,CA93012.USA
JAK 2(C-20) (sc-294)	Santa Cruz Biotechnology
JAK 1 PYPY(44-422)	Biosource Int,CA93012.USA
Anti-phosphotyrosine 4G10(05-321)	Upstate Biotechnology Inc, NY
USA	
Anti-HA probe (F-7) (sc-7392)	Santa-Cruz Biotechnology

2.2.4 Preparation of denaturing polyacrylamide gel.

Gels were prepared on the day of use. The separating gel was prepared and poured into a Mighty-Small Hoefer gel caster (Hoefer scientific instruments, San Francisco, USA), isobutanol was layered on top and the gel allowed to polymerise at room temperature.

The isobutanol layer was removed and the gel washed with ddH_20. The stacking gel was then prepared and layered on top and a 10 well comb inserted making sure that

there were no air bubbles. The gel was allowed to polymerise and once set, the comb removed. The wells were then washed 3 times with running buffer to remove unpolymerised acrylamide. The gels were then transferred to the running apparatus.

2.2.5 Cell lysate preparation

The factor dependent cell lines were washed free of growth factors starved for a minimum of 4 hours. They were then incubated with the indicated concentrations of inhibitor for 30mins before stimulation with either IL-3 10ng/ml (32D) or rhGMCSF 20ng/ml (M07E and TF-1). Lysates were made after 10minutes stimulation.

Primary cells were cultured in RPMI 1640 and 10% FCS. The inhibitor under investigation was added at the indicated concentration and the cells incubated at 37° 5% CO_2. Lysates were made after a minimum of 4 hours incubation. Factor independent cell lines were treated as primary cells but were incubated with inhibitor for a minimum of 6 hours.

Samples were pelleted by centrifugation at 2000 rpm for 3mins at 4°C, the supernatant was aspirated and the cells washed with 1 ml of PBS. The pellet was aspirated to dryness and resuspended in lysis buffer. The cells were lysed on ice for 10mins. Samples were then clarified by centrifugation at 14000rpm for 10 mins at 4°C, and the supernatant removed to a clean tube. 4x sample buffer was then added,

the samples boiled for 5mins and then either loaded immediately on to the gel or stored at -20°C.

For the suspension cell lines, both factor dependent and independent, $1x10^6$ cells were lysed in 45µl of lysis buffer and 15 µl of sample buffer added and $4x10^5$ cell equivalents were loaded per well. 1 million primary cells were lysed in 15µl of lysis buffer and 5 µl of sample buffer added, $1x10^6$ cell equivalents were loaded per well.

2.2.6 Preparation of lysates from adherent cells

The media was aspirated from plate and placed in a labelled 15ml tube. The plate was washed twice in cold PBS and the wash added to the tube. The cells were then trypsinised from the plate using 1x trypsin-EDTA and added to the same tube. The cells were centrifuged at 1500rpm for 4mins and the supernatant removed. 1ml of PBS was then added and the cells resuspended and transferred to a 1.5ml centrifugation tube. The cells were again centrifuged at 1500rpm for 4 minutes and the supernatant aspirated. 1ml of lysis buffer was then added to the cells and the cells lysed on ice for 30mins. The nuclear debris was then removed by centrifugation at 14000rpm for 10mins and the supernatant removed to a clean tube. If samples were to be used for western blotting 45µl of this was removed and 15µl of 4x sample buffer added, for immunoprecipitation method 2.7.2 was followed.

2.2.7 Polyacrylamide gel electrophoresis and immunoblotting

Acrylamide gels were prepared as described in section 2.2.4. Prestained molecular size markers and equal volumes of samples were loaded on to 10% or 7.5% SDS-PAGE gels. They were run using Hoefer gel apparatus at 150V until adequate separation had occurred. Protein samples were transferred on to Hybond-C-Extra nitrocellulose membrane using the Owl Panther Semidry Electroblotter (HEP 1) (Owl separation systems) at a constant current of 0.75 AMPs for 1 hour. The membrane was then blocked for 1 hour in 5% dried non-fat milk (w/v) in PBS/0.1% Tween-20 to prevent non-specific protein binding. The membrane was then washed 3 times for 5mins each wash in 0.1% PBS/Tween-20. Membranes were then incubated in the indicated primary antibody, at the manufacturer's recommended concentration, made up in 3% BSA (w/v) 0.1% PBS/0.1% Tween-20. All phospho-specific antibodies were incubated overnight at 4°C, most other antibodies were incubated at room temperature for 1 hour. Primary antibody was then removed and the membrane washed 3 times, 5mins per wash, in 0.1% PBS/Tween-20. The membrane was then incubated with the appropriate horseradish peroxidase linked secondary antibody 1:10 000 in 2% (w/v) milk /PBS 0.1%/Tween-20, for 1hour. The membrane was then washed 3 times in PBS.0.1%/Tween-20 and protein bands detected with enhanced chemiluminescence (ECL or ECL-plus for phospho-specific antibodies) according to the manufacturer's guidelines. The nitrocellulose membrane was then exposed to autoradiography film.

Where appropriate, gels were stained with Coomassie blue for 10 minutes and then destained until only the protein bands were stained blue. The gel was then vacuum dried for 1 hour in a Bio-Rad Model 583-Gel dryer, before exposure to autoradiography film.

2.3 Cell Proliferation Assay (MTS)

The cells under investigation were plated at $2x10^5$/ml for cell lines and $1x10^6$/ml or greater for primary cells. For factor dependent cell lines, the cells were starved for a minimum of 4 hours before pre-incubation with the inhibitor under investigation for 30 mins and stimulation with appropriate growth factor. They were then incubated at 37° 5% CO_2 for 48 hours. Factor independent cell lines and primary cells were incubated with the inhibitor under investigation or DMSO control for 48 hours. 20µl of CellTiter aqueous 96® one solution cell proliferation assay, Promega, (Wisconsin, USA) was added to 100 µl of cells, in duplicate and analysed according to the manufacturer's guidelines. Results were expressed as a percentage of control (cells without inhibitor).

2.4 Annexin V Binding Assay

2.4.1 Reagents

Annexin-V Binding Buffer

10mM HEPES pH 7.4

140mM $NaCl_2$

5mM $CaCl_2$

2.4.2 Methods

Cells were stained with FITC-Annexin V((1828681) Roche Biochemicals, Lewes UK) according to the manufacturer's guidelines; briefly, cells were pelleted by centrifugation and washed with 1ml of Annexin-V binding buffer. To the cell pellet 100µl of Annexin-V binding buffer with 10µl/ml of FITC-Annexin-V was added and incubated for 10 mins at room temperature. A further 250µl of Annexin-V binding buffer was added and the samples placed on ice prior to analysis by flow cytometry (Epics Elite, Beckman Coulter, High Wycombe, UK).

2.5 JAK 2 Kinase Assay

Storage Buffer

150mM NaCl

50mM Tris-HCL pH 8.0

10% glycerol

0.1mM EDTA

0.1mM sodium orthovanadate

50mM NaF

0.5% NP40

Kinase Assay Buffer

50mM NaCl

10mM HEPES pH7.4

5mM $MgCl_2$

5mM $MnCl_2$

0.1mM sodium orthovanadate

2.5.1 Method

10-20µl of settled JAK 2 immune complex agarose (CAT 14-134 Upstate, Lake Placid, NY 12946) beads were taken per assay point and washed twice with 1 ml of kinase buffer then aspirated to dryness. The agarose beads were then suspended in 10µl of kinase buffer. Five µl of inhibitor dilution were then added to the appropriate tube and 5µl of kinase buffer to the control tube. γ-^{32}PATP was added to a final concentration of 1µCi/µl. The tubes were then incubated at 30° for 30mins with agitation. The reaction was stopped by washing the JAK 2 agarose 3 times in storage buffer, 1ml per wash. 50µl of SDS-PAGE sample buffer was then added and the sample boiled for 5 minutes. 25µl of sample was then run on a 7.5% SDS-PAGE

gel. The gel was fixed with Coomassie Blue stain, dried and visualised by standard methods of autoradiography.

2.6 Formation of stable cell lines

2.6.1 Plasmids

Tel-JAK2, Tel-JAK3 in pBabeNeo vector were kindly donated by Dr Virginie Lacronique (Paris, France)

HA-JAK2 and 3 in pEF-BOS vector kindly donated by Dr Jane McGlade, Toronto, Canada.

mAKT-ER , mA2-ER myristolated Akt Δ4-129 and the control A2myrAkt Δ4-129 are fused to an oestrogen receptor in a PWZL retrovirus vector containing a neomycin resistance gene, kindly donated by Dr R. Roth, Stanford University[82]

2.6.2 Bacterial transformation

A vial of DH5α competent cells (donated by Dr G Morley, University College London) was thawed slowly on ice. 100 μl of cells was placed in a 1.5ml centrifugation tube and returned to the ice. 1-10ng of plasmid DNA was added to the

cells and incubated on ice for 1hour. The cells were then heat shocked at 42°C for 45 seconds. The cells were cooled on ice briefly before being added to 500µl of LB medium containing no antibiotics; this was placed at 37°C with constant shaking (300rpm) for 30mins-1hour to allow the bacterial cells to express the appropriate antibiotic resistance. After this time a proportion of cells were spread on to a selective agar plate (1.5% agar) and placed at 37°C in a bacterial incubator.

2.6.3 Plasmid Preparation

A single colony was picked from a selective plate and 2mls of LB medium containing the selective antibiotic inoculated. The starter culture was incubated for approximately 8 hours at 37°C with vigorous shaking (300rpm) and then used to inoculate 250mls of LB medium. This was then grown at 37°C overnight with vigorous shaking as before. The following morning the bacterial cells were harvested by centrifugation at 5000rpm in a Beckman JA-10 rotor at 4°C. DNA was extracted using Qiagen Maxi-Prep kit according to the manufacturer's protocol.

2.6.4 Electroporation

32D cells in culture were split 1:3 with RPMI/10F the night prior to electroporation to ensure that they were in log phase growth. The following day 10-20x10^6cells/point were taken, centrifuged and washed once in PBS. They were then

resuspended in 500µl phenol red free media, SIGMA (R-7509) containing 10% FCS and 10% WEHI 3B conditioned medium and 20µg of plasmid were added. The cells were transferred to a gene pulser cuvette (0.4cm) Bio-Rad (1652088) and electroporated at 250V and 960µF in a Bio-Rad Gene Pulser™. Following transfection the cells were left to recover for 5-10mins and then transferred to 15-20mls of RPMI 1640 10% FCS 10% WEHI conditioned medium for 6 hours. The cells were then ficolled to remove any dead cells and suspended in RPMI 10% FCS and 10% WEHI conditioned medium. The cells were allowed to grow for 24-48 hours before selecting out the transfected cells with the appropriate antibiotic.

2.6.5 Calcium phosphate transfection of 293T cells

Promega Profection mammalian transduction kit #E120040, Promega,Southampton UK

293T cells were grown in DMEM 10% FCS until near confluence. The night before the transfection the cells were trypsinised in 5mls of 1x Trypsin/EDTA, 1ml transferred to one 10cm plate per vector and 10mls of media added. The cells were allowed to adhere overnight at 37°C 5% CO_2. Two sterile tubes were taken per vector to be transfected, to the first (A) 416µl of H_2O, 20µg of vector DNA and 64µl of 2M $CaCl_2$ were added and mixed well. 500µl of 2xHEPES buffered saline was added to the second tube (B). The contents of tube A were added gradually to tube B with constant agitation and the mixture added drop-wise to cover the whole area of

the appropriate 10cm plate. The following morning the plates were washed 3 times gently with 5mls of PBS added to the side of the plate so as not to disturb the cells. The media was replaced with fresh media and the cells left to express the plasmid for 24-48hours.

2.7 Immunoprecipitation

2.7.1 Reagents

Protein G immobilised on Sepharose 4B fast flow (P 3296) Sigma

Protein A immobilised on Sepharose 4B fast flow (P 3391) Sigma

Species	**Protein A**	**Protein G**
Rabbit	++++	+++
Goat	-	++
Rat	+/-	++
Sheep	+/-	++
Mouse		
IgG1	+	++++
IgG2a	++++	++++
IgG2b	+++	+++
IgG3	++	+++

2.7.2 Method

Cell lysates were prepared according to method 2.2.5. The immunoprecipitating antibody was added according to manufacturer's protocol usually between 1-5µg. The tubes were mixed thoroughly and placed on a mixer wheel at 4°C overnight. 25µl of protein A or Protein G 50% slurry was added and placed on a mixer wheel for 90mins at 4°C. The beads were then washed 3 times with cold PBS/0.1% Triton-X-100 using micro bio-Spin chromatography columns, according to manufacturer's guidelines. Briefly the seal on the column is broken and the column placed on ice. The sample is then added to the column and the aqueous phase drains through. 1ml of PBS/0.1% Triton-X-100 was then added and when all the fluid has drained through this step is repeated for 2 further washes. The tubes were then placed in a 1.5ml centrifugation tube and spun at 1000rpm for 2mins to dry the column. The column is then removed and sealed using seals provided and placed in a clean 1.5ml centrifugation tube. 30µl of 1x boiling sample buffer was then added to the column for 3mins, before removing the seal and spinning the tube at 5000rpm for 5mins. The eluate is then boiled for an additional 1 minute.

2.8 JAK3 Kinase Assay

2.8.1 Reagents

Kinase Buffer

Na Cl 50mM

HEPES pH 7.6 10mM

$MgCl_2$ 5mM

$MnCl_2$ 5mM

Na_3 $V0_4$ 0.1mM

2.8.2 Method

293T cells were transfected with HA-JAK3 according to method in 2.6.5, one 10cm plate per point. The cells were lysed in 1ml of lysis buffer on ice as previously described. The lysates were clarified of nuclear debris by spinning at 14000rpm for 10mins, at this point the lysates from different plates were pooled and divided into 1ml aliquots. 10µl of anti-HA antibody was added and the sample placed on a mixer wheel at 4°C overnight. 25µl of Protein G agarose beads 50:50 slurry were added and mixed at 4°C for 90mins. The immobilised JAK 3 was then washed twice in PBS 0.1%Triton-X 2mM EDTA and the samples were again pooled. One further

wash was carried out in Kinase buffer and the agarose beads were aspirated to dryness. 45µl of kinase buffer were then added to each tube and 5µl of indicated inhibitor concentration for 10mins at room temperature. 10µCi of γ ^{32}PATP were then added and the tubes incubated for 30mins at room temperature with regular agitation. The reaction was stopped by the addition of 1ml of cold PBS/0.1% Triton X-100/2mM EDTA and the beads washed twice further using micro bio-spin chromatography columns Bio-Rad (732-6204) according to the manufacturer's protocol. 25µl of sample was then loaded on to a 7.5% PAGE gel and run. The gel was then fixed with Coomassie and vacuum dried according to manufacturer's guidelines. The JAK 3 was then visualised by standard autoradiography methods.

2.9 Cell Sorting

Leukaemic blasts were either labelled with CD38 FITC clone AT13/5, (DAKO), CD34 Phycoerythrin (PE) BD (Biosciences Pharmingen) or the appropriate FITC and PE controls, according to manufacturer's guidelines. They were then electronically sorted on an EPICS, Beckman-Coulter flow cytometer.

2.10 Luciferase Reporter Assay

The NF-kB and P53 luciferase reporter plasmids and the renilla luciferase plasmid (pRL-CMV) were obtained from Promega. Between 1 and 5x10^6 AML blasts/

CD34+cells were nucleofected (Amaxa, CD34 kit, using the manufacturer's instructions) with 2 μg of either NF-kB or P53 reporter plus 0.5 μg of pRL-CMV. Transfected cells were incubated overnight (with or without LY294002) in RPMI/10% FCS. Cells were lysed and NF-kB or p53 reporter activity was corrected for the constitutive renilla luciferase expression using a Dual Luciferase Kit (Promega). Results were compared to the background reading obtained with the backbone pGL2 luciferase vector (Promega) that lacks eukaryotic promoter and enhancer sequences. Readings above background were considered positive.

2.11 DNA Extraction

2.11.1 Reagents

Lysis Buffer

DTAB 8%	20g
Na Cl 1.5M	22g
Tris Cl 100mM	25mls of 1M Tris
dd H_20	to 250mls

Chloroform	
Ethanol	100% and 70%

2.11.2 Method

The cells were suspended in PBS at a concentration of $1x10^6$ cells per 100µl and DTAB was added at a ratio of 2:1. The sample was mixed well and placed in a water bath at 68°C for 5minutes and then allowed to cool down. A volume of chloroform equal to the total sample volume was added and mixed well. This was then centrifuged at 3000rpm for 20mins. The upper layer was then carefully removed to a clean tube and an equal volume of 100% ethanol was added and the sample gently mixed until the DNA could be seen precipitating. When fully precipitated the DNA was removed with a yellow tip to a 1.5ml centrifugation tube containing 200µl of 70% ethanol in order to wash the pellet. The tube was centrifuged at 14000rpm for 5mins to pellet the DNA, the ethanol was removed and the pellet allowed to air dry. The pellet was dissolved in dd H_20 and Optical Density at 260nm was measured using the Gene Quant pro (Amersham-Pharmacia), to measure the DNA concentration.

2.12 Mutational analysis

2.12.1 Reagents

Primers

FLT3/ITD

11F (5'-GCAATTTAGGTATGAAAGCCAGC-3')

12R (5'-CTTTCAGCATTTTGACGGCAACC-3').

FLT3/D835

F 5'-CCGCCAGGAACGTGCTTG-3'

R 5'-GCAGCCTCACATTGCCCC-3'

NRAS/Exon 1

1F 5'-GCTCGCCAATTAACCCTGATTAC-3',

1R 5'-TGGGTAAAGATGATCCGACAAGTGA-3'

NRAS/Exon 2

2F 5'-ACACCCCCAGGATTCTTACAGA-3'

2R 5'-TCTTCCCTAGTGTGGTAACCTC-3'

BIOTAQ DNA polymerase-Bioline, London, UK

10x DNA polymerase buffer-Bioline, London, UK

dNTP-Bioline, London, UK

EcoRV-New England Biolabs, Ipswich, UK

2.12.2 FLT3/ITD Mutations

Internal tandem duplications (ITDs) in the juxtamembrane region of the FLT3 gene were detected using polymerase chain reaction (PCR) amplification of exons 14 and 15 (previously 11 and 12) and the intervening intron.

Approximately 100 ng DNA was added to a reaction mix containing:-

1 x buffer (16 mM $(NH_4)2SO_4$, 67 mMTris HCl pH 8.8, 0.01% Tween 20), 1.0 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate (dNTPs) and 10 pmol of each primer in a total volume of 19 μL. The mixture was heated to 95°C for 5 minutes and held at 85°C while 1 μL containing 0.5 U BIOTAQ DNA polymerase was added; then 30 cycles each of 95°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds were performed, followed by 5 minutes at 72°C. Amplified products were electrophoresed through 2% agarose gels and visualized under UV light with ethidium bromide staining. A fragment of 328 base pair (bp) was produced from WT alleles.

2.12.3 FLT3/D835 Mutations

Mutations in the activation loop of FLT3 (D835) were detected by PCR and restriction enzyme digestion of exon 20. Approximately 100 ng DNA was added to a reaction mix containing 1x buffer (16 mM $(NH_4)2SO_4$, 67 mM Tris HCl pH 8.8, 0.01% Tween 20), 1.0 mM MgCl2, 200 μM dNTPs, and 10 pmols of each primer in a total volume of 19 μL. The mixture was heated to 95°C for 5 minutes and held at 85°C while 1 μL containing 0.5U BIOTAQ DNA polymerase was added; then 35 cycles each of 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 30 seconds were performed, followed by 5 minutes at 72°C. Amplified products were digested with *Eco*RV then electrophoresed through 4% agarose gels and visualized under ultraviolet light with ethidium bromide staining. In alleles containing a D835 mutation, the 114-bp PCR fragment remained uncut, but in WT alleles it was digested to fragments of 68 and 46 bps.

2.12.4 N-Ras Mutations

N-Ras mutations were detected using heteroduplex analysis. DNA was amplified using Optimase Polymerase™ (Transgenomic Limited, Crewe, UK) according to manufacturer's specifications with 32 cycles of amplification, each 30 secs at 95°C, 30 secs at 63°C and 30 secs at 72°C. Primers for N-Ras exon 1 covering codons 12 and 13 gave a product of 228bps, and for exon 2 covering codon 61 a product of

274bps. PCR products were denatured and analysed using denaturing HPLC on a Transgenomic WAVE™ DNA fragment analysis system at 60.5°C for exon 1, 59.2°C for exon 2. Patterns from patient samples were compared with those from DNA of the HL60 myeloid cell line (exon 2 mutation, wild type exon 1). PCR products from samples with altered patterns were directly sequenced using the CEQ™ DTCS Quick Start kit and a CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Inc, Fullerton, USA).

Figure 2.1

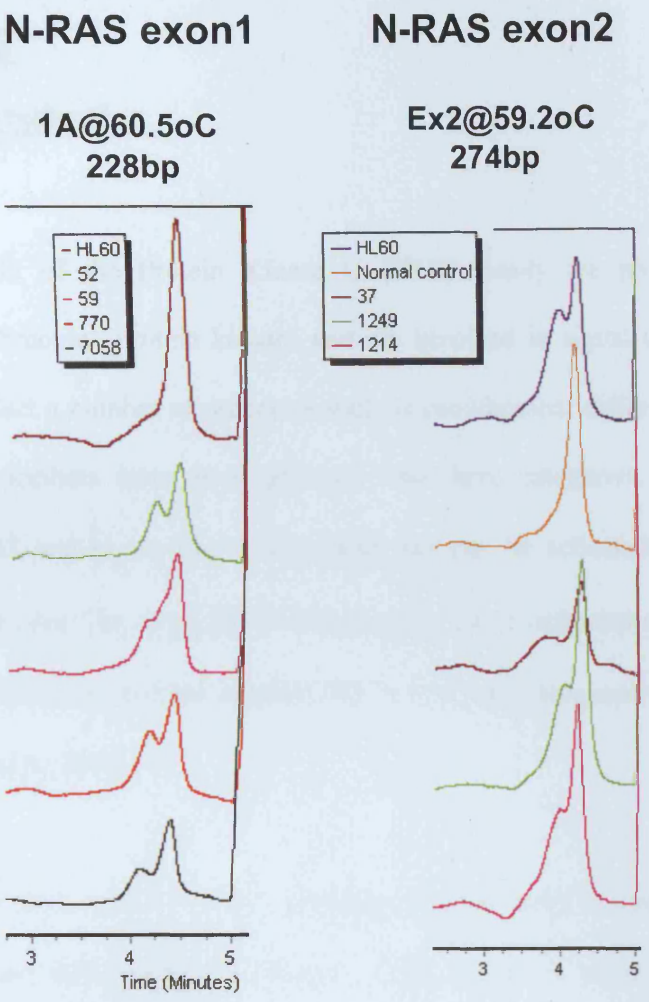


Figure 2.1 Wave sequence showing Ras mutations in exon 1 and 2.
HL60 myeloid cell line is shown as a normal control for exon1 and a positive control for exon 2.

CHAPTER 3-GÖ6976 IS A POTENT INHIBITOR OF JAK 2

3.1 Introduction

Members of the Protein Kinase C (PKC) family are phospholipid dependent serine/threonine protein kinases and are involved in signal transduction pathways that affect a number of processes such as proliferation, differentiation and motility. PKC members have been grouped into three categories. The classical PKCs (α,β1,β2 and γ) are Ca++ dependent and can be activated by diacylglycerol or phorbol ester The novel PKC's (δ,ε,θ,η) are Ca++ independent but can be activated by phorbol ester and the atypical PKC's (ζ,λ) are unresponsive to both and can be activated by PIP3. [83] [84]

Gö6976 is a selective PKC inhibitor derived from staurosporine. It is a non glycosidic indolocarbazole **(Figure 3.1a)** which is active against the calcium dependent isozymes (α and β1) in nanomolar concentrations but even micromolar concentrations are ineffective against the calcium independent PKC subtypes (δ,ε and ζ). Kinetic analysis reveals that Gö6976 inhibits PKC in a competitive manner with respect to ATP, non competitive with respect to substrate and mixed type with respect to phosphatidyl-serine. The IC50 for PKC inhibition is 7.9nM. [85] Gö6976 also inhibits PKC μ /PKD. Gö6983, a bisindolylmaleimide, **(Figure 3.1b)** is a potent

inhibitor of PKC that inhibits most PKC isozymes (IC_{50} = 7 nM for PKC_α and PKC_β; 6 nM for PKC_γ; 10 nM for PKC_δ; and 60 nM for PKC_ζ)[85].

Figure 3.1(a) **b)**

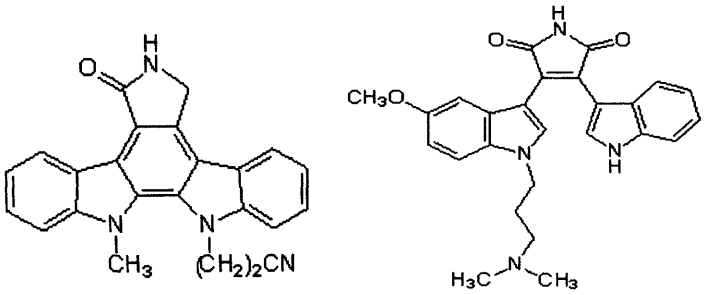


Figure 3.1 The chemical structure of Gö6976 and Gö6983

(a) illustrates the chemical structure of Gö6976 an indolocarbazole PKC inhibitor. (b) shows the bisindolylmaleimide structure of Gö6983.

Preliminary experiments in our lab investigating the role of Protein kinase C (PKC) in human growth factor signalling found that Gö6976 had biological effects out of keeping with an effect solely on PKC. Incubation of IL-3, GMCSF but not SCF dependent cell lines with Gö6976 led to an increase in apoptosis and reduction in proliferation. A panel of PKC inhibitors with a broader spectrum of anti PKC activity (Gö6983, GF109203X, Gö7874[85]) had no effect on cell viability. Together these results suggested that Gö6976 may be exerting its effects via another kinase. The differential effect between inhibition of IL-3/GMCSF signalling (receptors which utilise JAK 2) and the lack of abrogation of stem cell factor signalling (a receptor with intrinsic tyrosine kinase activity) suggested that Gö6976 may be inhibiting JAK kinases[4]. The following chapter identifies the targets of Gö6976 as

JAK kinases and further delineates the effects of Gö6976 on cell survival and proliferation.

3.2 Gö6976 inhibits signalling downstream of the cytokine receptor super-family but not C-Kit.

3.2.1 Gö6976 inhibits IL-3 survival signals in 32D cells, a factor dependent cell line

To assess the effect of Gö6976 on IL-3 signalling, 32D cells were starved of growth factor for a minimum of 4 hours, incubated with or without Gö6976 (10nM-10μM) or Gö6983 (1μM), the pan-PKC inhibitor for 30 minutes and then stimulated with IL-3 10ng/ml. Total cell lysates were made after 10 minutes (method 2.2.5) stimulation and examined by Western blotting utilising phospho-specific antibodies against pSTAT 5 (Tyr 694), phospho ERK-p42/44 (Thr 202/Tyr204), pAKT (ser 473) and the phosphotyrosine antibody 4G10 (method 2.2.7). **Figure 3.2a** shows quiescence of the cells with starvation. Stimulation with IL-3 at 10 minutes led to an increase in phosphorylation of STAT5, AKT and ERK. Incubation with increasing doses of Gö6976 completely abrogated these effects; however no effect on signalling was seen after incubation with 1μM Gö6983. In **Figure 3.2b** total tyrosine phosphorylation was reduced by growth factor starvation of the cells and markedly increased on IL-3 stimulation. Incubation with Gö6976 led to a reduction in phosphorylation of multiple proteins on tyrosine residues whereas Gö6983 had no

effect. AG490, a member of the tyrphostin family, which has been reported to decrease constitutive JAK 2 activity in B cell Acute lymphoblastic leukaemia and has widely been used as a JAK2 inhibitor [54] also reduced tyrosine phosphorylation but to a lesser extent than Gö6976.

Figure 3.2a **b**

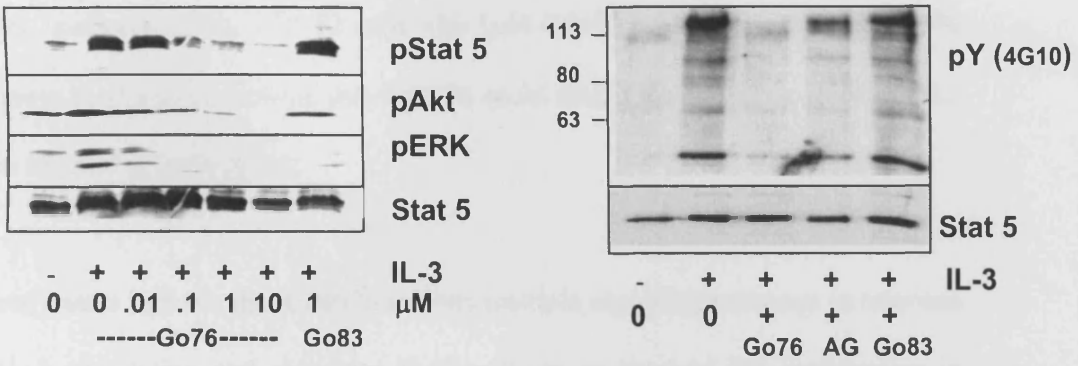


Figure 3.2 Gö6976 reduces signalling downstream of IL-3.
32D cells were quiesced overnight, pre-incubated for 30mins with the indicated concentration of Gö6976 (Go76), and then stimulated with IL-3 (10ng/ml) for 10 minutes. Immunoblot analysis was carried out with the indicated antibodies (phosphorylated STAT 5, Akt and ERK in the left panel and phosphotyrosine in the right panel). Comparison was made with a control PKC inhibitor, Gö6983 (Go83) and the JAK2 inhibitor AG490 (AG). Total Stat 5 is shown as a protein loading control.

To examine how the above results on signalling translated into biological effects, proliferation (MTS) and apoptosis (Annexin V) were examined in two IL-3 dependent cell lines, 32D and FDCP-1. Cells were starved of growth factors for a minimum of 4 hours, pre incubated with increasing concentrations of Gö6976 or

Gö6983 (1μM) and then stimulated with IL-3 (10ng/ml). Annexin V staining was carried out at 24 hours (method 2.4.2) and an MTS assay at 48 hours (method 2.3). Incubation with Gö6976 blocked the proliferative effects of IL-3 on 32D cells in a dose dependent manner with an average IC50 of 93+/-50nM (n=4) with complete abrogation at 0.5 μM **(Figure 3.3a).** Withdrawal of IL-3 from FDCP-1 cells led to a 34+/-9% increase in apoptosis above control cells (growing with IL-3) after 24 hours. Incubation of the FDCP1 cells with 1μM Gö6976 plus IL-3 led to a 22+/-6% increase in apoptosis showing that Gö6976 could inhibit the protective effects of IL-3 on survival **(Figure 3.3b).**

These results indicate that Gö6976 inhibits multiple signalling pathways in response to IL-3 stimulation and abrogates IL-3's effects on survival and proliferation in factor dependent cells. These effects appear not to be related to its PKC inhibitory activity.

To substantiate these findings, further cytokine signalling pathways were examined.

Figure 3.3

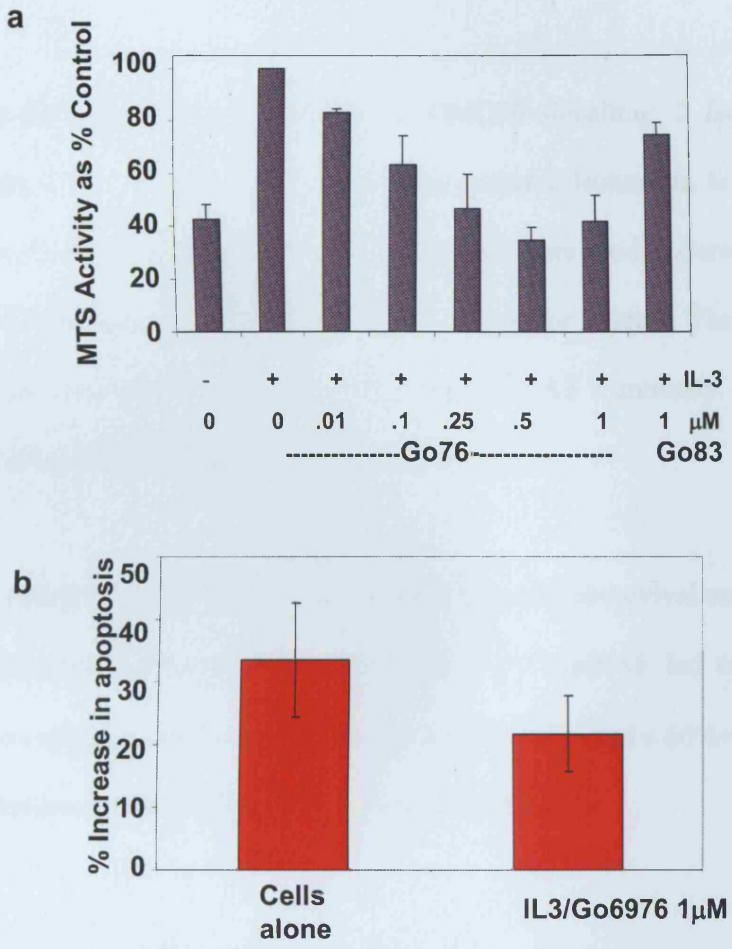


Figure 3.3 Gö6976 reduces proliferation and increases apoptosis in factor dependent myeloid cells

32D or FDCP-1 cells were incubated with IL-3 plus Gö6976 or Gö6983 and apoptosis measured by Annexin V staining at 24hours and proliferation assessed by MTS activity at 48 hours. Incubation with Gö6976 blocked the proliferative effects of IL-3 on 32D cells in a dose dependent manner and incubation with 1μM Gö6976 together with IL-3 led to a 22+/-6% increase in apoptosis above cells incubated with IL-3 alone.

3.2.2 Gö6976 abrogates the effect of GMCSF signalling in factor dependent cell lines

To study the consequence of Gö6976 on GMCSF signalling, 2 factor dependent lines were used - Mo7E, a human megakaryoblastic leukaemia line and TF-1 a human erythroleukaemia line. After starvation cells were pre-incubated with Gö6976 or Gö6983 and stimulated with GMCSF (20ng/ml) for 10mins. The resultant total cell lysates were probed with a phospho-specific STAT 5 antibody. Gö6976 led to an inhibition of STAT 5 phosphorylation **(Figure 3.4)**

Similar effects of Gö6976 were seen on GMCSF induced survival and proliferation. Incubation with 1μM Gö6976 in the presence of GMCSF led to a 29%+/-9% increase in apoptosis of TF-1 cells (0% for Gö6983 n=3) and a 60%+/-7% reduction in proliferation of Mo7E cells (2%+/-7% for Gö6983 n=3).

3.2.3 Gö6976 inhibits STAT 5 phosphorylation in response to other cytokines utilising JAK2.

Other cytokines which utilise JAK2 include Thrombopoietin (TPO), Erythropoietin (EPO) and IL-6. Mo7E cells are responsive to TPO as well as GMCSF and stem cell factor (SCF). To examine the effect of Gö6976 on Thrombopoietin signalling, Mo7e cells were first starved of growth factor overnight, incubated with increasing concentrations of Gö6976 and 1μM Gö6983 prior to stimulation with TPO

(45ng/ml) for 10 minutes. Total cell lysates were made and examined by Western Blotting **(Fig 3.5a).** Growth factor withdrawal led to a reduction in phospho-STAT5, the levels of which were increased by TPO stimulation. Incubation with 1μM Gö6976 led to complete inhibition of STAT 5 phosphorylation.

The same experiment was repeated with TF-1 cells stimulated with EPO (10iu/ml). There was a marked reduction in pSTAT 5 signalling after incubation with Gö6976 with complete inhibition at 100nM **(Figure 3.5b).**

Figure 3.4

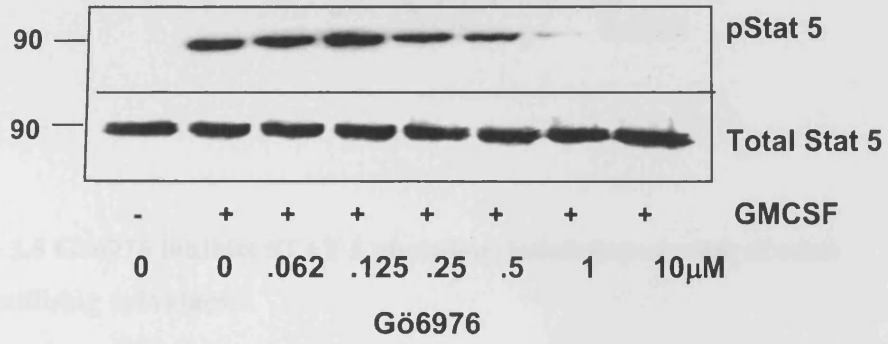


Figure 3.4 Gö6976 abrogates the effect of GMCSF signalling in Mo7E cells.

Mo7E cells were quiesced overnight, pre-incubated with increasing concentrations of Gö6976 for 30mins and stimulated with the indicated cytokine for 10 minutes.

Figure 3.5

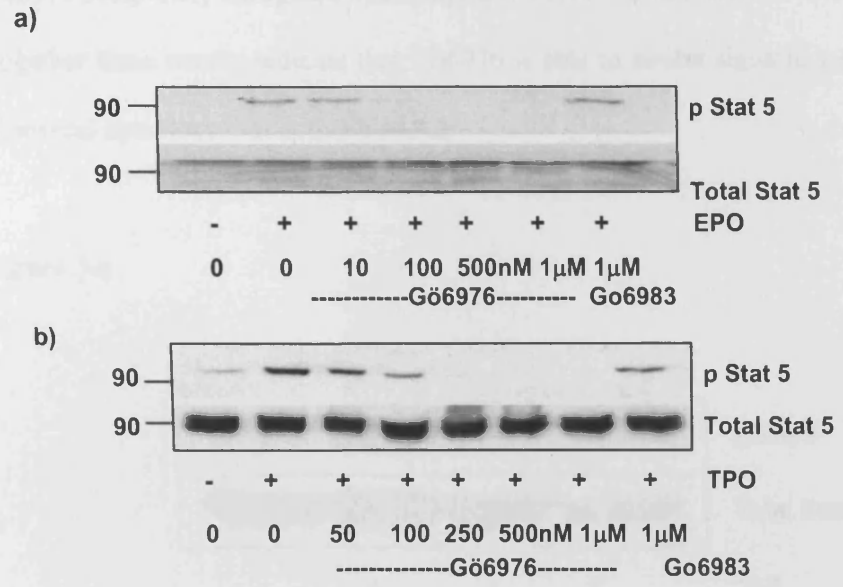


Figure 3.5 Gö6976 inhibits STAT 5 phosphorylation downstream of other JAK2 utilising cytokines.

Factor dependent cells lines were quiesced overnight, pre-incubated with increasing concentrations of Gö6976 for 30mins and stimulated with the indicated cytokine for 10 minutes. Mo7E cells were stimulated with thrombopoietin (TPO) and TF-1 cells with erythropoietin (EPO). Immunoblot analysis was carried out with the indicated antibodies.

IL-6 signalling was investigated in a myeloma cell line, U266 which although factor independent responds to signals through IL-6 and IFNα and γ **(Figure 3.6).** IL-6

stimulation led to an increase in STAT 5 activation and incubation with 1μM Gö6976 completely abrogated this bringing p STAT 5 levels back to baseline.

Together these results indicate that Gö6976 is able to inhibit signalling downstream of several cytokines that activate JAK2.

Figure 3.6

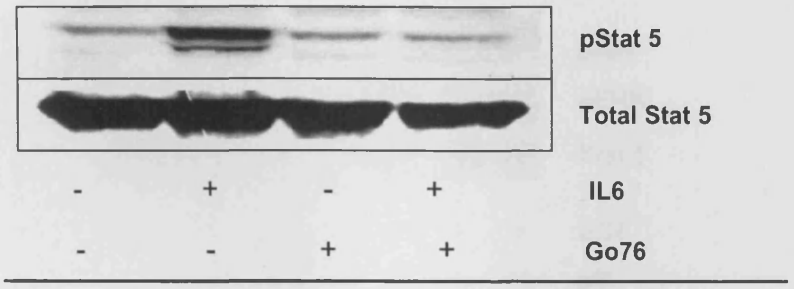


Figure 3.6 Gö6976 inhibits STAT 5 phosphorylation in response to IL-6 stimulation in U266 cells.

U266 myeloma cells were pre incubated with Gö6976 for 1hour and then stimulated with IL-6 (20ng/ml) for 10mins. Immunoblot analysis was carried out with the indicated antibodies.

3.2.4 Gö6976 has no effect on signalling after stimulation with stem cell factor

Mo7E cells were used to study the effect of Gö6976 on stem cell factor signalling. Stem cell factor stimulation rapidly brought about phosphorylation of Akt and ERK above quiescent levels. Incubation with Gö6976 or Gö6983 had no effect on this phosphorylation **(Figure 3.7).**

Figure 3.7

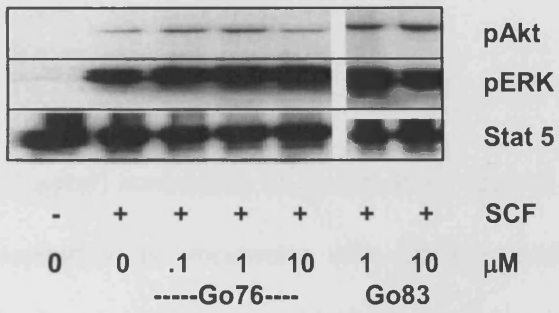


Figure 3.7 Gö6976 has no effect on signalling after stimulation with stem cell factor.

Quiescent Mo7E cells were pre-incubated with the indicated concentrations of Gö6976 (Go76) or Gö6983 (Go83), then stimulated with Stem Cell Factor (SCF) (20ng/ml, 10 minutes) and immunoblot analysis carried out with the indicated antibodies.

3.3 Gö6983 reduces ERK phosphorylation in response to phorbol ester

These experiments show that Gö6976 has profound effects on signalling in response to several growth factors whilst Gö6983 does not. This indicates that these effects are likely to be independent of PKC. To confirm that Gö6983 is biologically active, its effect on Phorbol Ester induced activation of ERK, a PKC dependent mechanism, was examined.

Phorbol Ester (TPA) (1μg/ml) stimulation of growth factor starved cells led to an increase in ERK phosphorylation. Incubation with Gö6983 resulted in a dose dependent decrease in phospho-ERK signal **(Fig3.8).** These results indicate that Gö6983 is an active PKC inhibitor at the concentrations used in these experiments.

Figure 3.8

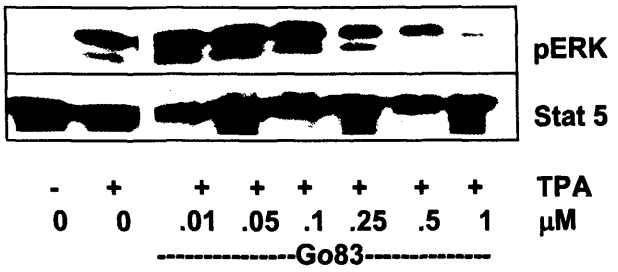


Figure 3.8 Gö6983 reduces ERK phosphorylation in response to phorbol ester.

Growth factor starved Mo7E cells were pre-incubated with the indicated concentration of Gö6983 and stimulated with Phorbol Ester (TPA) (1μg/ml) for 10 minutes. Immunoblot analysis was carried out with the indicated antibodies.

3.4 Gö6976 has a direct effect on JAK2 in vitro kinase activity.

These results suggest that Gö6976 exerts its effects through a kinase other than PKC. Identical results were obtained using Gö6976 from 3 separate sources (LC Labs, Biomol, Calbiochem, data not shown). The fact that signalling downstream of several members of the cytokine receptor superfamily but not that of SCF was affected led us to postulate an effect on JAK2. In order to assess the direct effect of Gö6976 on JAK2 activity, in vitro kinase assays were carried out. Recombinant JAK 2 was utilised according to the method in 2.5.1. In vitro kinase activity using γ^{32}P-ATP was detected by autoradiography following SDS-PAGE. As can be seen in figure 3.9a, incubation of recombinant JAK2 with 1 μM Gö6976 led to a marked reduction in JAK2 kinase activity compared to the control (no inhibitor) whereas

incubation with the control PKC inhibitor Gö6983 had no effect. Using scanning densitometry, incubation with 1 μM Gö6976 reduced JAK 2 activity to 18+/-5% of control (n=5).

The above experiment was repeated with varying concentrations of Gö6976 **(Figure 3.9b)** which resulted in a dose dependent reduction in JAK2 kinase activity with an IC50 of 130nM (by densitometry).

We compared the effect of AG490 on JAK 2 kinase activity to that of Gö6976 using the above method. Incubation with AG490 led to a dose dependent reduction of JAK2 kinase activity with an in vitro IC50 of 35μM (by densitometry) **(Figure 3.9c)**. Incubation with 100μM AG490 led to a 70% reduction in JAK2 kinase activity whereas 1μM Gö6976 by comparison led to a 74% reduction in the same experiment suggesting that these two doses have similar efficacy **(Figure 3.9d).**

Figure 3.9

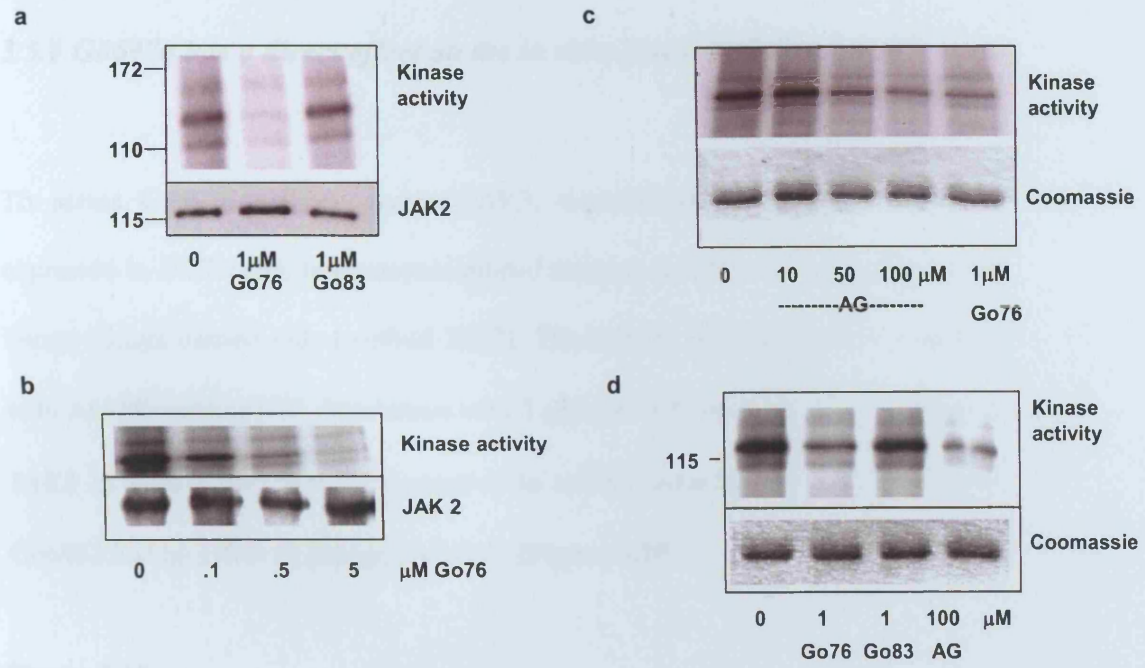


Figure 3.9 Gö6976 has a direct effect on JAK2 in vitro kinase activity

a) Recombinant JAK2 was used in a kinase assay with ^{32}P labelled ATP in the absence or presence of Gö6976 (Go76) or Gö6983 (Go83) and phosphorylation of JAK2 (125kD) detected by autoradiography (upper panel). Equal loading was measured by immunoblotting with anti-JAK2 (lower panel)

b) Autokinase activity was measured in the presence of increasing concentrations of Gö6976. Figure 3.9c Autokinase activity was measured in the presence of increasing concentrations of AG490. Figure 3.9d Autokinase activity was measured in the presence of Gö6976, Gö6983 and the known JAK2 inhibitor, AG490 (AG).

3.5 To examine the effect of Gö6976 on other JAK kinases

3.5.1 Gö6976 has a direct effect on the in vitro kinase activity of JAK3

To assess Gö6976 activity against JAK3, recombinant HA tagged JAK3 was expressed in 293T cells, immunoprecipitated using an anti HA antibody and in vitro kinase assays carried out. (method 2.8.2). The activity of Gö6976 was compared with AG490 and Gö6983. Incubation with 1 μM Gö6976 led to an 81% reduction in JAK3 in vitro kinase activity compared to a 64% reduction for 100μM AG490. Gö6983 had no effect on phosphorylation. **(Figure 3.10)**

Figure 3.10

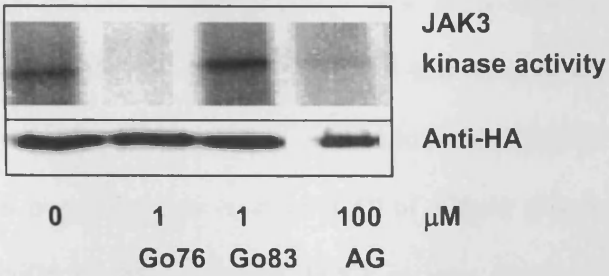


Figure 3.10 Gö6976 has a direct effect on JAK 3 kinase activity.

HA-tagged JAK 3 was transfected into 293T cells, immunoprecipitated with an anti-HA antibody and an *in vitro* kinase assay with ^{32}P labelled ATP was carried out. The upper panel is an autoradiograph showing JAK3 autophosphorylation and the lower panel is an immunoblot with anti HA antibody as a loading control. The activity of Gö6976 was compared with AG490 and Gö6983.

3.5.2 Gö6976 inhibits JAK3 mediated IL-2 signalling in peripheral blood derived lymphocytes.

IL-2 signalling is thought to be primarily mediated by JAK3. To further investigate Gö6976 inhibition of JAK3, we examined the effect of Gö6976 on IL-2 stimulation of peripheral blood derived T-lymphocytes. Isolated lymphocytes were incubated with 10μg/ml of phytohaemaglutinin (PHA), a plant mitogen with specificity for T cells, for 72 hours prior to incubation with the indicated concentration of inhibitor and stimulated with IL-2 10iu/ml. Phosphorylated proteins were examined by Western blotting.

STAT 5 phosphorylation in the presence of IL-2 was abrogated by Gö6976 in a dose dependent manner (**Figure 3.11a**). ERK phosphorylation did not appear to be increased by IL-2. The above experiment was repeated and a MTS assay carried out at 72 hours after IL-2 addition. Incubation with Gö6976 led to a dose dependent reduction in proliferation with an IC50 of 370nM **(Fig 3.11b).** These results show that Gö6976 is able to inhibit JAK3 in vitro kinase activity and abrogate IL-2 induced STAT 5 phosphorylation in intact cells.

Figure 3.11

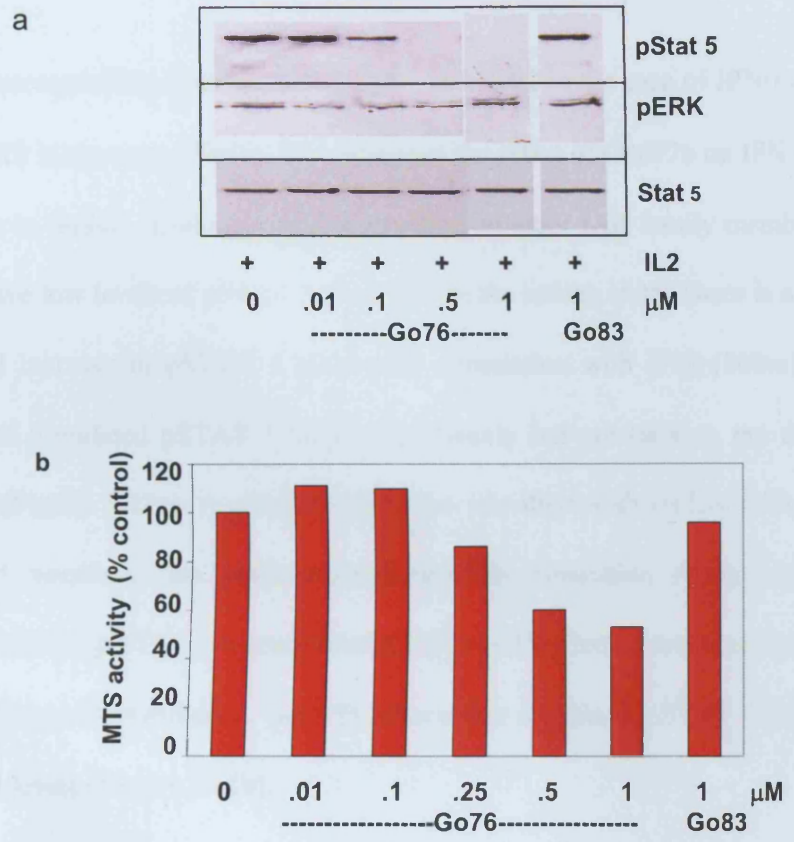


Figure 3.11 Gö6976 inhibits JAK3 mediated IL-2 signalling in peripheral blood derived lymphocytes.

Peripheral blood derived lymphocytes were cultured in 10μg/ml phytohaemaglutinin (PHA) for 72 hours, incubated with Gö6976 for 30mins and stimulated with IL-2 (10iu/ml). Immunoblot analysis was carried out with the indicated antibodies. Figure 3.11b The above experiment was repeated and proliferation assessed by an MTS assay carried out at 72 hours after IL-2 stimulation.

3.5.3 Gö6976 partially abrogates the effect of IFN signalling in U266 and HELA cells

Interferon signalling is mediated by JAK1 and Tyk2 in the case of IFNα and JAK1 and JAK2 in the case of IFNγ. We examined the effect of Gö6976 on IFN signalling in order to further explore its inhibitory effect on other JAK family members. U266 cells have low levels of pSTAT 1 (Tyr 701) in the resting state. There is a rapid and marked increase in pSTAT 1 level after stimulation with IFNγ (500iu). Gö6976 reduced stimulated pSTAT 1 levels significantly but not back to pre stimulation levels **(Figure 3.12a).** A similar experiment was done with HELA cells, a human cervical carcinoma line which responds to αIFN stimulation. Again there are low basal levels of p STAT1 in unstimulated HELA cells which increase substantially on αIFN (20ng/ml) stimulation. Gö6976 reduces the stimulated pSTAT 1 but not back to basal levels **(Figure 3.12b).**

Therefore in whole cell assays Gö6976 1μM is sufficient to completely inhibit JAK2 and JAK3 mediated STAT 5 phosphorylation but has only a partial effect on signalling presumed to be via JAK1 and TYK2.

Figure 3.12

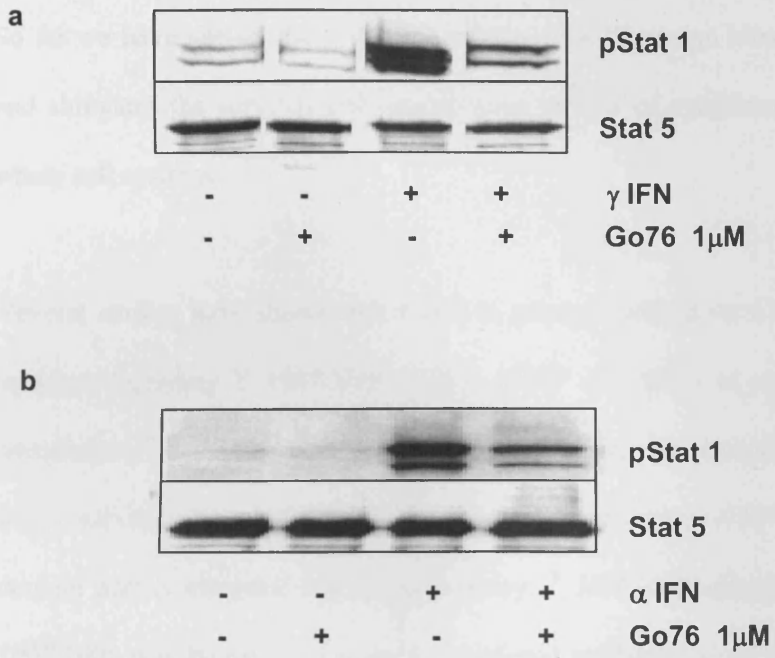


Figure 3.12 Gö6976 partially abrogates the effect of IFN signalling.

a) U266 myeloma cells were incubated with Gö6976 for 2 hours prior to stimulation with IFNγ (500iu/ml) for 10 minutes. Immunoblot analysis was carried out with the indicated antibodies. b) HELA cells, a human cervical carcinoma line, were incubated with Gö6976 for 1 hour prior to stimulation with IFNα (20ng/ml).

3.6 Gö6976 does not inhibit JAK2 Phosphorylation at Y1007 and Y1008 despite a reduction in down stream signalling

So far we have shown that Gö6976 inhibits JAK2 tyrosine kinase activity in vitro and abrogates the survival and proliferative effects of cytokines utilising JAK2 in whole cell systems.

Several studies have shown that JAK2 is phosphorylated on a number of tyrosine residues including Y 1007/1008, Y221, Y570 and Y813 in response to cytokine stimulation. [8,11,86] JAK2 mutant studies have shown that phosphorylation of Y1007 in the activation loop allows access of the catalytic loop to ATP in the ATP binding domain and is essential for kinase activity. [8] JAK2 phosphorylation at tyrosines 1007/1008 was investigated using a polyclonal antibody raised against a chemically synthesized phosphopeptide derived from a region of JAK2 containing PY1007and 1008.

Mo7E cells were quiesced for 6 hours prior to being incubated with increasing concentrations of Gö6976 for 30mins and stimulated with GMCSF 20ng/ml for 10mins. Cell lysates were probed with phospho JAK2, p Akt and p ERK. GMCSF induced JAK2 phosphorylation at tyrosines 1007/1008 but Gö6976 did not affect this phosphorylation despite clearly abrogating downstream signaling **(Figure 3.13).**

Figure 3.13

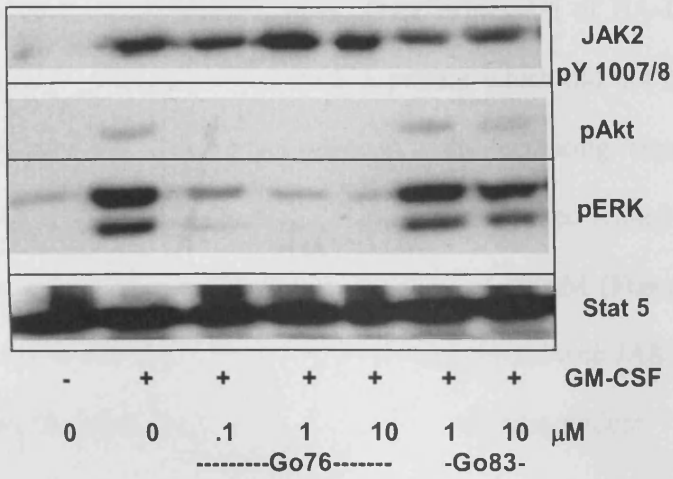


Figure 3.13 Gö6976 does not inhibit JAK2 phosphorylation at Y1007

Quiescent Mo7E cells were preincubated with increasing concentrations of Gö6976 or Gö6983, stimulated with GMCSF(20ng/ml, 10 minutes) and cell lysates analysed by immunoblot with antibodies to phosphorylated JAK2 (tyrosines 1007/1008), p Akt and pERK.

3.7 Overexpression of HA-JAK2 in 293T cells leads to activation of p STAT 1; Gö6976 abrogates this activation

Overexpression of JAK2 is known to result in constitutive activation of JAK2 kinase activity. In order to investigate the effect of Gö6976 on this activation, 293T cells were transfected with an HA tagged JAK2 plasmid using the calcium phosphate method (method 2.6.5). The cells were trypsinised and split into a 6 well plate,

allowed to adhere and then incubated overnight with or without Gö6976. Total cell lysates were made and probed with an antibody to pSTAT 1. Non transfected 293T cells were treated in an identical manner. Over expression of HA-JAK2 in 293T cells led to phosphorylation of the STAT 1 protein which did not occur in mock transfected cells **(Figure 3.14a)**. Incubation with increasing concentrations of Gö6976 led to a reduction in this phosphorylation. A marked reduction in STAT 1 phosphorylation was seen at concentrations as low as 100nM **(Figure 3.14b).** The samples from the above experiment were re run and probed with JAK2 pYpY1007/8 **(Figure 3.14b)**. No reduction in JAK2 phosphorylation was evident.

Figure 3.14

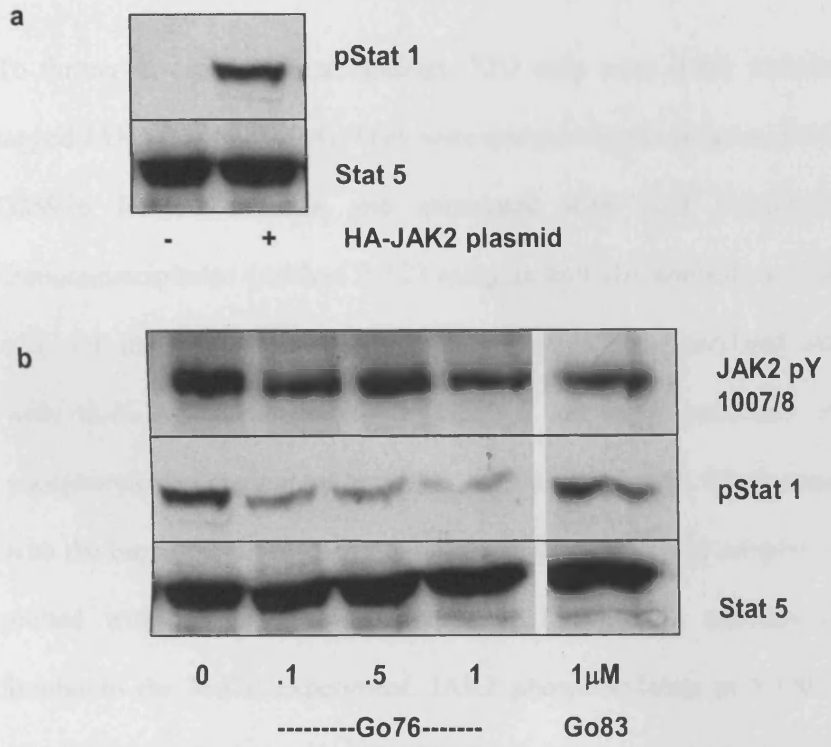


Figure 3.14 Gö6976 abrogates STAT 1 phosphorylation secondary to overexpression of JAK2 in 293T cells

a) 293T cells transfected with or without HA-JAK2 were lysed and immunoblot analysis performed with specified antibodies. Transfection of HA-JAK2 alone led to increased phosphorylation of STAT 1. b) 293T cells were transfected with HA-JAK2. The cells were then incubated over night with increasing concentrations of indicated inhibitor. Total cell lysates were made and immunoblot analysis was carried out. STAT 1 phosphorylation was reduced by incubation with Gö6976 but not by the control inhibitor Gö6983. No effect on JAK2 phosphorylation at tyrosines 1007/8 was observed with either inhibitor.

3.8 Gö6976 does not reduce phosphorylation at Y1007/1008 in JAK2 expressing 32D cells.

To further investigate these findings, 32D cells were stably transfected with HA tagged JAK2 (method 2.6.4). They were starved of growth factor, pre incubated with Gö6976 for 30 minutes and stimulated with IL-3 (10ng/ml). JAK2 was immunoprecipitated (method 2.7.2) using an anti HA antibody and samples probed with anti-phosphotyrosine. JAK 2 was tyrosine phosphorylated after stimulation with IL-3 and incubation with Gö6976 led to a reduction in total JAK2 phosphotyrosine content but not back to quiescent levels. Similar results were seen with the broad spectrum kinase inhibitor staurosporine. The samples were re run and probed with the phospho-specific JAK2 (1007/1008) antibody (**Figure 3.15**). Similar to the Mo7E experiment, JAK2 phosphorylation at Y1007/1008 was not reduced by incubation with Gö6976. Total cell lysates from the same experiment were probed with p STAT 5, Akt and ERK and results show that downstream signalling was inhibited (data not shown). Staurosporine appeared to behave in the same way as Gö6976 whereas AG490 reduced JAK2 phosphorylation at Y 1007/1008. These results indicate that unlike downstream signalling, JAK2 phosphorylation at Y1007/1008 is not inhibited by Gö6976 and suggests that Gö6976 binds to and inhibits JAK2 that is already phosphorylated at Y1007/1008. The binding of Gö6976 inhibits JAK2 kinase activity and prevents further auto/trans phosphorylation of JAK2 on other tyrosine residues and also inhibits

phosphorylation of the receptor, adapter proteins and downstream signalling molecules.

Figure 3.15

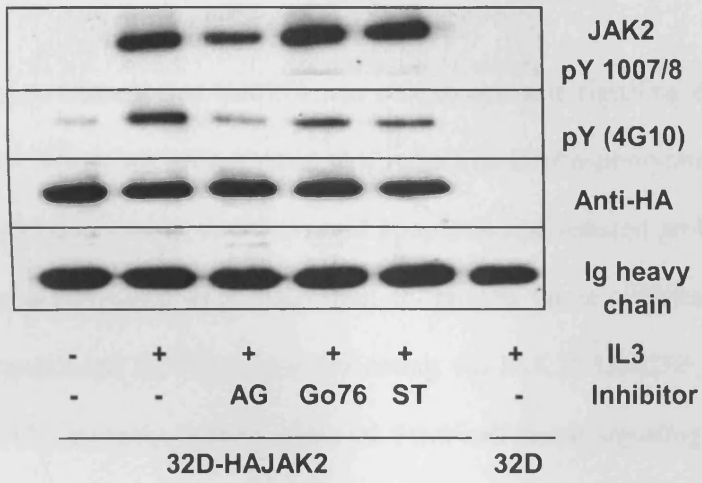


Figure 3.15 Gö6976 does not reduce phosphorylation at Y1007/1008 in JAK2 expressing cells.

Quiescent 32D cells stably expressing HA tagged JAK2 were pre incubated with 1μM Gö6976 (Go76), 100μM AG490 (AG) or 1μM Staurosporine (ST) and stimulated with IL-3 (10ng/ml, 10 minutes). JAK2 was immunoprecipitated using an anti-HA antibody and samples probed by immunoblotting with anti phosphotyrosine and phospho-specific JAK2 (1007/1008) antibodies. The last lane labelled 32D is an immunoprecipitate from parental 32D cells.

3.9 Discussion

The aim of this chapter was to identify the mechanism by which Gö6976 exerted its biological effects.

Initial experiments showed that Gö6976 was able to abrogate signaling downstream of IL-3 in factor dependent cells leading to a reduction in phosphorylated STAT 5, Akt and ERK. This translated into increased apoptosis and reduced proliferation in these cells to levels which were equivalent to growth factor withdrawal. These results were reproduced for other cytokines acting via JAK 2: GMCSF, EPO, TPO and IL-6. Gö6976, however, had no effect on Stem cell factor signaling which acts on the KIT receptor which has intrinsic tyrosine kinase activity and therefore does not utilize the cytosolic JAK tyrosine kinases. Throughout all these experiments the control inhibitor Gö6983 had no effect indicating that these results were not mediated through PKC inhibition. Although in vitro kinase assays do not reproduce the situation within the whole cell it was necessary to use this method to investigate the direct effect of Gö6976 on JAK 2. We found that Gö6976 inhibited JAK2 autophosphorylation with an IC50 of 130nM (compared to its anti-PKCα IC50 7.9nM). Under the same conditions AG490 was found to have an IC50 of 35μM for JAK2 inhibition (quoted AG490 IC50 for JAK2=0.1μM). [87]

We have also shown that Gö6976 has an inhibitory effect on JAK3 kinase activity in vitro and inhibits the IL-2 stimulatory effect in peripheral blood derived T cells

which is thought to be mediated predominantly by JAK3. [88] These results together indicate that Gö6976 is also a potent inhibitor of JAK3. The effects of Gö6976 on JAK1 and Tyk 2 are more difficult to define. Interferon signalling is mediated by JAK1 and Tyk2 in the case of IFNα and JAK1 and JAK2 in the case of IFNγ.[88] For both interferons, Gö6976 was able to decrease stimulated levels of STAT 1 significantly but not back to baseline levels. This may suggest that Gö6976 is not a potent inhibitor of both JAK family members implicated in IFN signaling or other pathways are involved in STAT 1 activation. Future work should include individual kinase assays for JAK1 and Tyk 2.

Typically, protein kinase inhibitors are directed to the highly conserved ATP binding pocket of the catalytic domain. A structure based approach looking at protein kinases bound to ATP-analogues or kinase inhibitors has confirmed that although the ATP-binding pocket is highly conserved across protein kinases, the proximal region does afford some key diversity. [89] Specific inhibitors take advantage of this limited sequence variation surrounding the ATP binding pocket as well as conformational differences between inactive and active forms of the kinases. The activation loop controls catalytic activity in most kinases by switching between different states in a phosphorylation dependent manner. In fully active kinases the loop is stabilised in an open conformation and the active conformation is similar in all known structures of active kinases. There is however great diversity in the loop conformation in inactive kinases. [43] Several groups have investigated which of the 49 tyrosines in JAK2 are autophosphorylated. In many kinases regulation of

catalytic activity by phosphorylation occurs on residues within the activation loop of the kinase domain. Feng et al demonstrated that Y 1007 and Y 1008 are sites of trans- or autophosphorylation in vivo and in in vitro kinase reactions. [8] They found mutation of Y1007 or both Y 1007/1008, to phenylalanine essentially eliminated kinase activity, whereas mutation of Y 1008 alone had no detectable effect on kinase activity. Other tyrosine residues known to be autophosphorylated include tyrosine 221, 570 and 813. Argetsinger et al found that when tyrosine 221 was mutated it markedly decreased autophosphorylation of JAK2, more than would be expected from the loss of just one tyrosine. When tyrosine 570 was mutated the phosphorylation associated with 1007/1008 was increased compared to wild type. They felt that tyrosine 570 may serve as a binding site for an inhibitor or have some steric effect that decreases JAK2 activity. [11] Because receptors that bind with JAK2 bind to the FERM domain, the FERM domain presumably assumes different conformations to transmit signals from the receptor to the JH1 domain. It seems likely that conformational changes in the region between Y221 and Y240 (e.g. as a result of phosphorylation of Y221 or changes in receptor conformation due to ligand binding) would play an important role in the regulation of kinase activity.

Fig 3.16

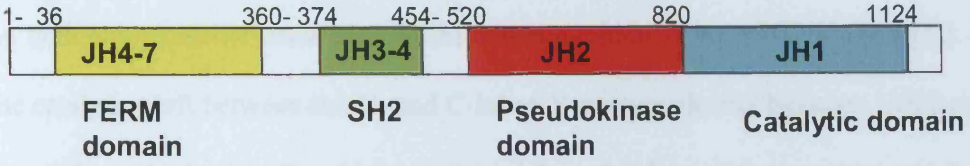


Fig 3. 16 The domain structure of JAK kinases. JAKs comprise FERM, SH2, pseudokinase and kinase domains. The FERM domain mediates receptor interactions. Both the FERM and pseudokinase domains regulate catalytic activity[14].

Although we have demonstrated that Gö6976 is a JAK2 inhibitor, we found that JAK2 phosphorylation at Y1007/1008 as assessed by Western blot with a phosphospecific antibody was not reduced by incubation with Gö6976 despite a reduction in total JAK2 phosphorylated tyrosine content and clear inhibition of downstream signalling. We obtained similar findings with staurosporine and JAK inhibitor 1 [60] (data not shown) but not with the tyrphostin AG490. These findings also have implications for the screening of JAK2 inhibitory compounds - if autophosphorylation at Y1007/1008 is used as the endpoint, useful inhibitors could be scored as inactive.

Our findings indicate that Gö6976 preferentially binds to and inhibits JAK2 which is already phosphorylated at Y1007/1008. This prevents phosphorylation of other tyrosine residues in JAK2 as well as phosphorylation of various substrates, preventing activation of downstream signalling. In support of this hypothesis Boggon et al have recently reported on the crystal structure of JAK3 in complex

with AFN941, a staurosporine analogue. They found that the JAK3-AFN941 complex is crystallized in a catalytically active state and is tyrosine phosphorylated on both autophosphorylated sites in the activation loop. [90] AFN 941 binds JAK3 in the catalytic cleft between the N- and C-lobes. Sequence identity between JAK2 and 3 is 62% with almost all residues in the inhibitor-binding cleft conserved and it is likely that staurosporine and by implication Gö6976, binds to JAK2 that is phosphorylated at Y1007/1008 and is in an active conformation. Kinase inhibitors have been shown to bind distinct conformations of their target molecules - for example imatinib binds to and inhibits an inactive conformation of Abl whereas the compound PD173955 binds to and inhibits Abl in multiple, including active, conformations.[91]

3.10 Conclusions

Preliminary experiments suggested that Gö6976 had biological effects out of keeping with its action on PKC. Gö6976 inhibited signaling downstream of IL-3 and reduced levels of pSTAT 5, pAkt and pERK, components of the JAK-STAT, PI3-Kinase and MAP-Kinase pathways respectively. Incubation with Gö6976 abrogated the survival signals of IL-3 and led to a fall in proliferation and an increase in apoptosis to levels equivalent to growth factor starvation with an IC50 of 93+/-50nM. Similar results were obtained when signaling and biological effect were examined downstream of other JAK2 utilizing cytokines, GM CSF, IL-6, EPO and TPO. These effects were not seen however with signaling downstream of stem cell

factor which acts via the KIT receptor tyrosine kinase nor was any effect seen on signaling or apoptosis/proliferation with the control PKC inhibitor Gö6983. In vitro assays utilizing recombinant JAK2 showed that Gö6976 directly inhibits JAK2 kinase activity (IC50 130nM). Gö6976 was significantly more potent than the known JAK2 inhibitor AG490 (IC50 35micromolar). In spite of clear evidence, both in whole cell systems and in in vitro kinase assays, that Gö6976 inhibits JAK2 kinase activity, it does not inhibit phosphorylation of JAK2 at Y1007/1008 in the catalytic loop using a phosphospecific antibody against these sites. This is probably due to preferential binding of Gö6976 to pre phosphorylated JAK2 preventing subsequent phosphorylation of the receptor and adapter molecules and thus propagation of downstream signaling.

CHAPTER 4-THE EFFECT OF Gö6976 ON HAEMATOPOIETIC CELL LINES AND PRIMARY AML CELLS

4.1 Introduction

Until recently the most direct evidence implicating dysregulation of the JAK/STAT pathway in haematopoietic malignancies was the identification of the TEL-JAK2 fusion. This fusion occurs as a result of t(9;12) or t(9;15;12) and has been reported to occur in myeloid and lymphoid leukaemias. [92,93] JAK2 is also likely to be activated by fusion of JAK2 with PCM1 resulting from a novel t(8;9) which has been described in a variety of haematopoietic malignancies[93] and JAK2 gene amplification has been shown to be a common finding in Hodgkin's and primary mediastinal B-cell lymphomas. [24,94] Recently a mutation at position 617, resulting in a conversion from valine to phenylalanine, in the JAK homology 2 (JH2) domain of JAK2 has been reported to be present in myeloproliferative disorders including the majority of cases of polycythaemia vera and also in essential thrombocythaemia and idiopathic myelofibrosis. This mutation in the pseudokinase domain of JAK2 is thought to lead to loss of autoregulation and increased kinase activity. [68,95]

In addition to these well characterised JAK2 abnormalities constitutive STAT activation has been reported in many malignancies including multiple myeloma, acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), non Hodgkins

lymphoma and anaplastic cell lymphoma (ALCL). [96,97] STAT activation can occur as a result of increased tyrosine kinase activity – for example, by chromosomal translocation and formation of a novel fusion product such as BCR-ABL, by acquired mutations such as internal tandem duplications in the Flt3 gene (FLT3 ITD) or secondary to autocrine/paracrine growth factor secretion. The frequency of constitutive STAT activation reported in AML varies between studies. Biethahn et al reported constitutive STAT 3 activation in all of 25 patients examined and constitutive STAT 5 activation in 21 out of 25 patients examined. [98] This is similar to the findings of Hayakawa et al who found STAT 5 activation in 80% and STAT 3 activation in 74% of samples. [99] Benekli et al, however, only found constitutive STAT 3 phosphorylation in 28 out of 63 (44%) patients[100] and Xia et al found STAT 3 and STAT 5 activation in 28% and 22% of patients respectively. [101] The reasons for the difference in reported frequencies is not clear but may relate to different methods of detection i.e. tyrosine phosphorylation versus DNA binding or may be due to differences in sample handling.The observation that STATs are activated in most cases of AML suggests that selective pharmacologic inhibition of activation of these proteins could be a valid therapeutic strategy in this disease.

In this chapter we examine the effect of Gö6976 on survival and proliferation of haematopoietic cell lines expressing activated forms of JAK2 as well as of other tumour-derived cell lines We also investigate the frequency of constitutive STAT activation in primary AML cells and the effect of JAK2 inhibition by Gö6976 on this activation and on survival and proliferation.

4.2 Gö6976 reduces constitutive Tel-JAK2 signalling and signalling downstream of the JAK2 V617F mutant.

The data so far detailed in chapter 3 show that Gö6976 can inhibit cytokine induced changes in signalling, survival and proliferation mediated by JAK2 and 3.

The Tel-JAK 2 fusion protein, consisting of the amino terminal 336 amino acids of the oligomerisation domain of Tel and the tyrosine kinase domain of JAK2 (amino acids 814-1130) was stably expressed in 32D cells. (method 2.6.4) This resulted in constitutive activation of tyrosine kinase activity and growth factor independence on these cells, which normally require IL-3 for survival.

32D cells stably expressing Tel-JAK2 were incubated with increasing concentrations of Gö6976 and 1μM Gö6983 for 6 hours. Phosphorylated proteins were examined by Western blotting.

Tyrosine phosphorylation of a number of proteins was markedly increased when compared to the parental 32D line. Constitutive phosphorylation of PI3-Kinase/Akt, MAPK and of STAT 3 and STAT 5 was detected. Incubation with increasing concentrations of Gö6976 showed a marked reduction in global tyrosine phosphorylation as well as phosphorylation of STAT 3, 5, ERK and Akt. **(Figure 4.1a/b)**

Figure 4.1

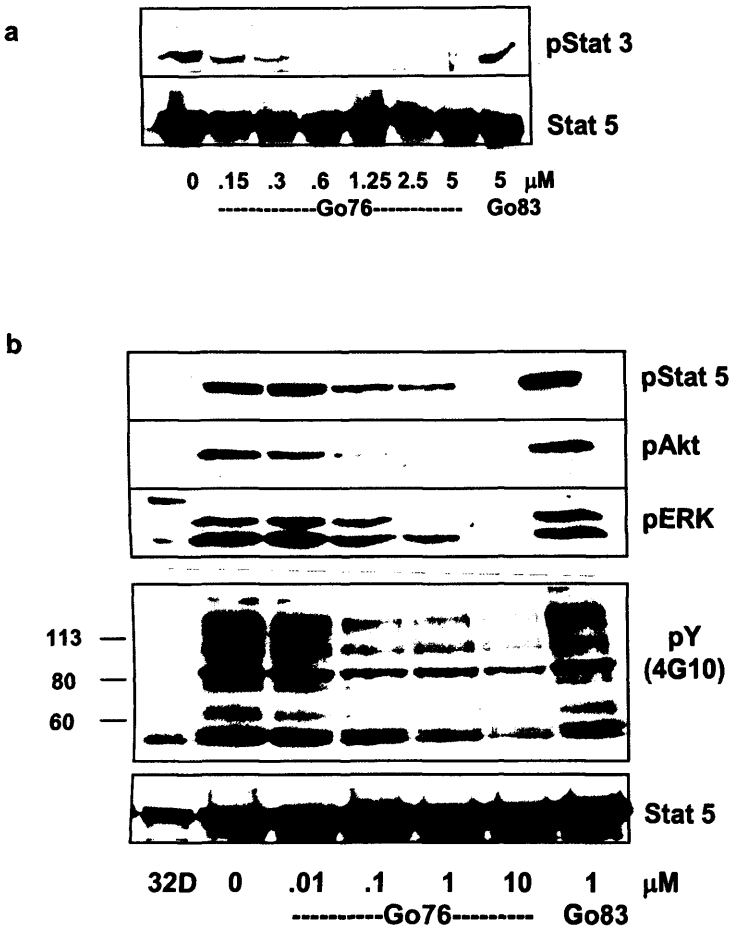


Figure 4.1 Gö6976 reduces constitutive Tel-JAK2 signalling

32D cells stably expressing the leukaemia associated Tel-JAK2 fusion were incubated with Gö6976 for 6hours and immunoblot analysis carried out with indicated antibodies. In 4.1b, lane 1 (marked 32D) contains lysate from parental 32D cells.

Tel-JAK2 cells were incubated with Gö6976 and apoptosis (Annexin V staining) at 24 hours and proliferation (MTS assay) at 48 hours were examined. Apoptosis was

increased by an average of 20+/-3% by incubation with 1μM Gö6976. The IC50 for apoptosis was 242+/-15nM (range 210-300nM n=5).

Proliferation fell to an average of 37+/-8% of control cells (no inhibitor) on incubation with 1μM Gö6976. The average IC50 was 291+/-44nM Gö6976 (range 130-445nM n=7). **(Figure 4.2a/b)** In contrast, Gö6983 had no significant effect on apoptosis or proliferation.

To confirm these results cell number and viability (trypan blue) were assessed after 48 hours incubation with Gö6976. The control cell number increased from $1x10^5$ to $4.5x10^5$ viable cells at 48 hours. Incubation with 600nM Gö6976 reduced viable cell number from $1x10^5$ to $6x10^4$ at the same time point. There was no significant effect of the Gö6983 on viable cell number ($3.4x10^5$). Gö6976 is therefore able to inhibit signaling downstream of the Tel-JAK2 fusion protein and negate its effects on survival and proliferation.

Figure 4.2

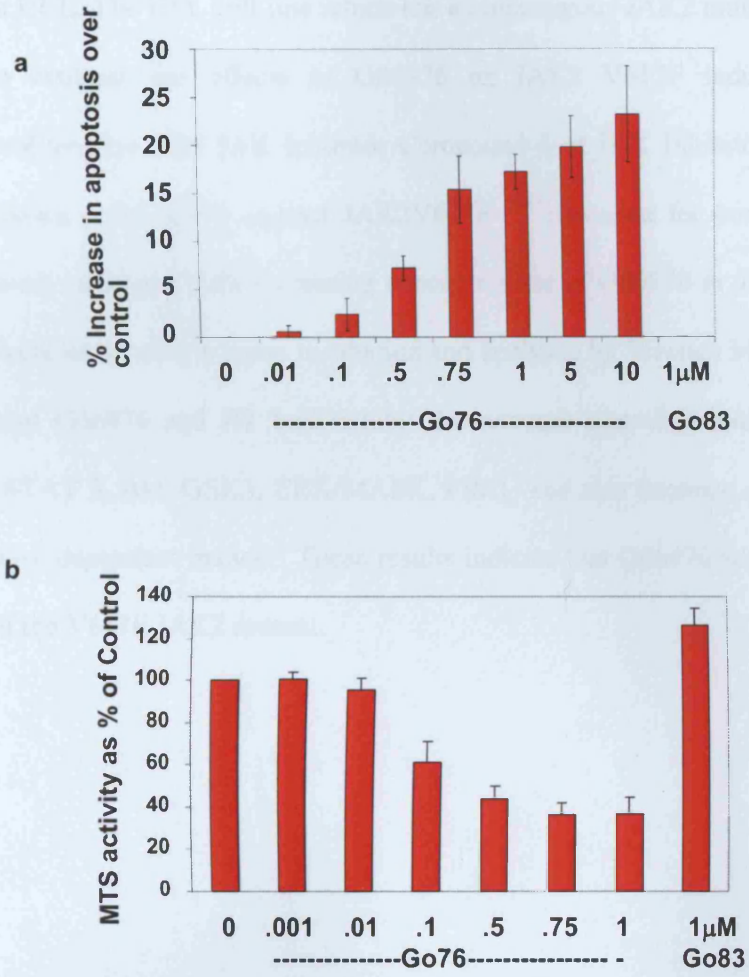


Figure 4.2 Gö6976 reduces proliferation and increases apoptosis in Tel-JAK2 expressing cells.

Tel-JAK2 expressing 32D cells were incubated with the indicated concentration of Gö6976 and apoptosis assessed at 24hours (Annexin V (n=5)) and proliferation at 48hours (MTS assay (n=7)).

The JAK2 V617F mutation is present in myeloproliferative disorders including polycythemia vera and results in its constitutive activation. [102,103] The following experiment was carried out by Dr T Everington in the Department of Haematology at UCL. The HEL cell line which has a homozygous JAK2 mutation [104] was utilized to evaluate the effects of Gö6976 on JAK2 V617F induced signalling and proliferation. The JAK inhibitor Compound 6 or JAK Inhibitor 1 (JI1) previously shown to be active against JAK2V617F [104] was used for comparison. HEL cells were incubated with increasing concentrations of Gö6976 or JI1. Total cell lysates were made after 6 hours incubation and analysed by Western blot. Figure 4.3 shows that Gö6976 and JI1 both inhibit downstream signalling pathways in HEL cells (STAT 5, Akt, GSK3, ERK/MAPK, PIM1) and also decrease cell proliferation in a dose-dependent manner. These results indicate that Gö6976 is an effective inhibitor of the V617F JAK2 mutant.

Figure 4.3

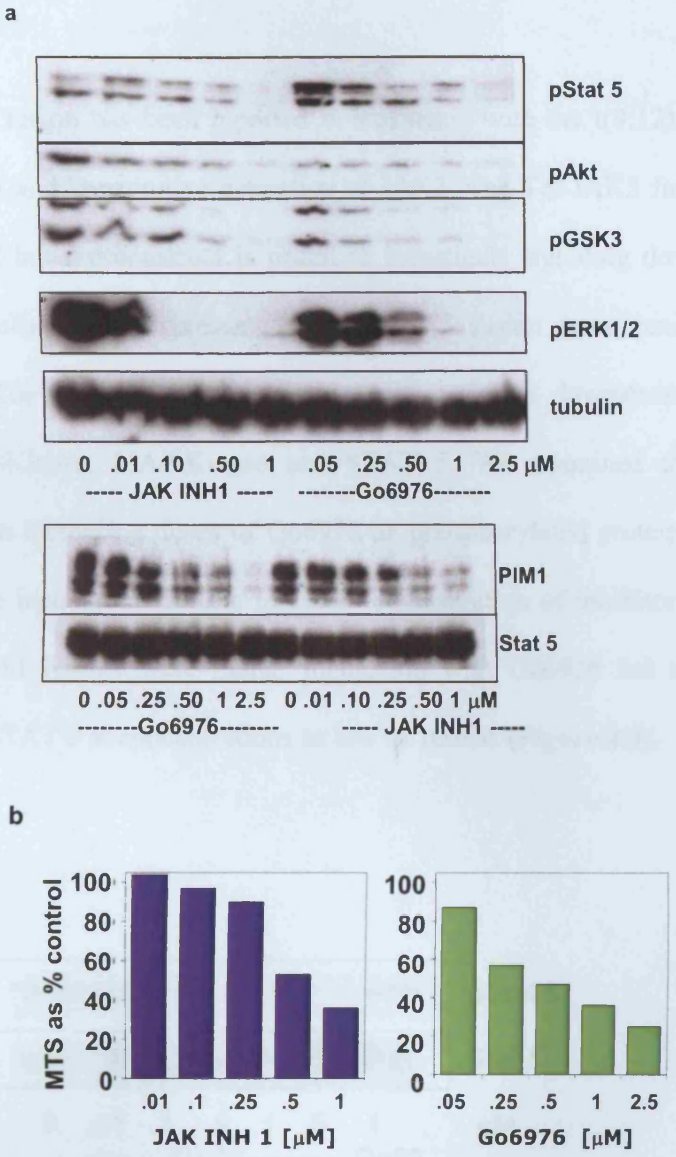


Figure 4.3 Gö6976 inhibits signalling downstream of the JAK2 V617F mutation.

HEL cells which have a homozygous JAK2 V617F mutation were incubated with increasing concentrations of Gö6976 or JAK inhibitor 1 (Compound 6). a) Cell lysates were made after 6 hours and analysed by immunoblot with the indicated antibodies. b) samples were analysed by MTS assay after 48 hours and are expressed as a % of control value (no inhibitor).

4.3 Tel-JAK3 cells are also sensitive to the effects of Gö6976

The Tel-JAK2 fusion has been reported in leukaemia with the t(9;12) resulting in oligomerisation and constitutive activation of JAK2. The Tel-JAK3 fusion has not been described but the construct is useful to investigate signaling downstream of JAK3. 32D cells stably expressing the Tel-JAK3 fusion were rendered factor independent. The constitutively activated JAK3 activates downstream pathways including PI3-Kinase, MAP-Kinase and STAT 5. We examined the effect of incubation with increasing doses of Gö6976 on phosphorylated protein expression. The cells were incubated with the indicated concentration of inhibitor for 6 hours before total cell lysates were made. Incubation with Gö6976 led to a marked reduction in pSTAT 5 at concentrations as low as 100nM **(Figure 4.4).**

Figure 4.4

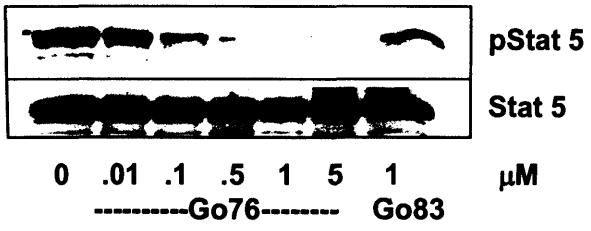


Figure 4.4 Gö6976 inhibits signalling downstream of cells expressing Tel-JAK3. Tel-JAK3 expressing 32D cells were incubated with increasing concentrations of Gö6976 for 6 hours prior to immunoblot analysis with indicated antibodies.

To examine the biological effect of inhibition of the Tel-JAK3 fusion protein by Gö6976, cells were incubated with increasing doses of Gö6976; apoptosis at 24 hours and proliferation at 48 hours were assessed. Incubation with 1μM Gö6976 increased the level of apoptosis by 19%. The percentage of apoptotic cells remained at basal levels for the control inhibitor, Gö6983 **(Figure 4.5a).**

Gö6976 reduced proliferation in Tel-JAK3 expressing cells. Incubation with 1μM Gö6976 led to a 75% reduction compared to control cells. The average IC50 was 144+/-50nM (n=5 range 75-340nM) **(Figure 4.5b).**

Cell number and viability were also assessed by trypan blue staining after 48 hour incubation with Gö6976. Initially $3x10^5$ cells were seeded per point. At 48hours the control viable cell number had increased to $1x10^6$ -however the cells incubated with 1μM Gö6976 remained at $4x10^5$viable cells. Gö6983 had no significant effect on viable cell number ($1.1x10^6$ cells).

Figure 4.5

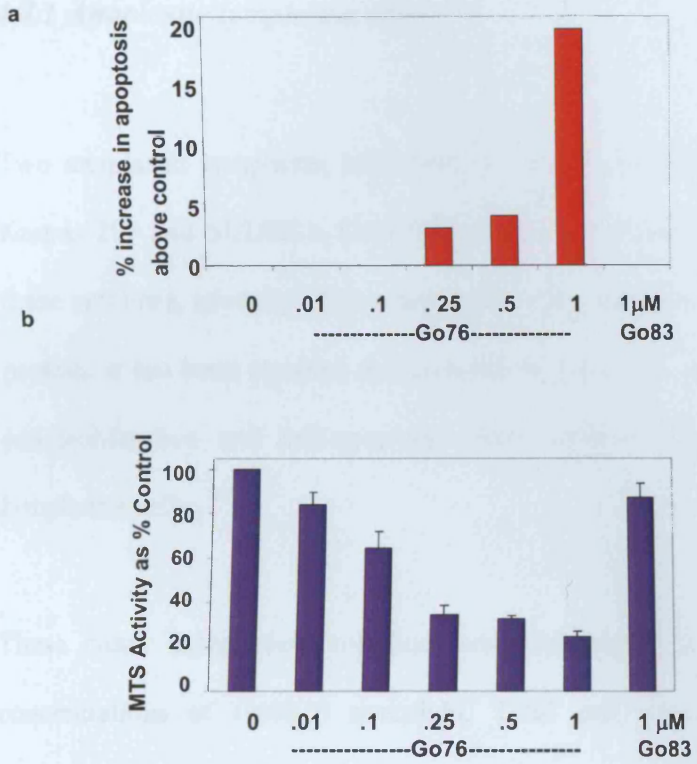


Figure 4.5 Gö6976 reduces proliferation and increases apoptosis in Tel-JAK3 expressing cells.

a) Tel-JAK3 expressing 32D cells were incubated with increasing concentrations of Gö6976 and apoptosis assessed at 24 hours (Annexin V).

b) Tel-JAK3 expressing 32D cells were incubated with the indicated concentration of Gö6976 and proliferation at 48 hours assessed by MTS assay (n=5).

4.4 The effect of Gö6976 on Haematopoietic tumour-derived cell lines

4.4.1 Anaplastic lymphoma lines

Two anaplastic lymphoma lines were examined for their sensitivity to Gö6976, Karpas 299 and SUDHL1. Over 50% of cases of Anaplastic lymphoma, and both these cell lines, have the t(2;5) which leads to the formation of the NPM-ALK fusion protein. It has been reported that constitutive activation of JAK2 contributes to the pro-proliferative and anti-apoptotic effect of this fusion protein in anaplastic lymphoma cells. [105]

These factor independent cell lines were cultured in the presence of increasing concentrations of Gö6976 overnight. Total cell lysates were then made and examined by Western blotting. Both cell lines show constitutive activation of STATs 3 and 5 which was decreased in a dose dependent manner by incubation with Gö6976 (**Figure 4.6a/b**).

The effect of Gö6976 on proliferation at 48 hours was also assessed (**Figure 4.7**). Karpas 299 and SUDHL1 were both sensitive to Gö6976 with IC50's 108+/-26nM (n=3) and 235+/-87nM (n=3) respectively.

Figure 4.6

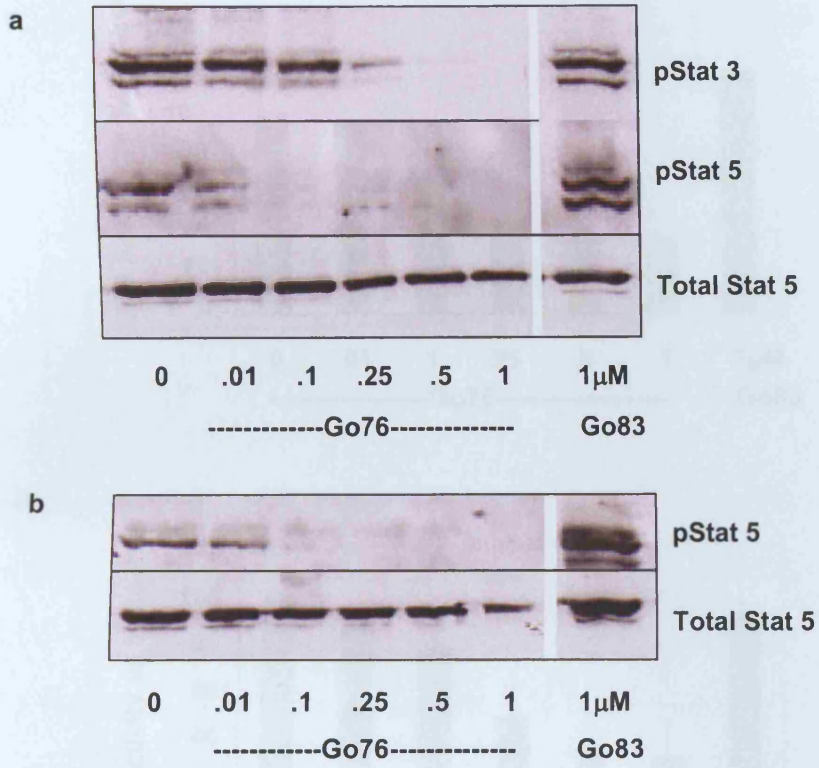


Figure 4.6 Gö6976 reduces STAT 5 phosphorylation in anaplastic lymphoma cell lines.

Karpas 299 (panel a) and SUDHL1 (panel b) anaplastic cell lines were cultured overnight in the indicated concentration of inhibitor. Immunoblot analysis was performed with specified antibody.

Figure 4.7

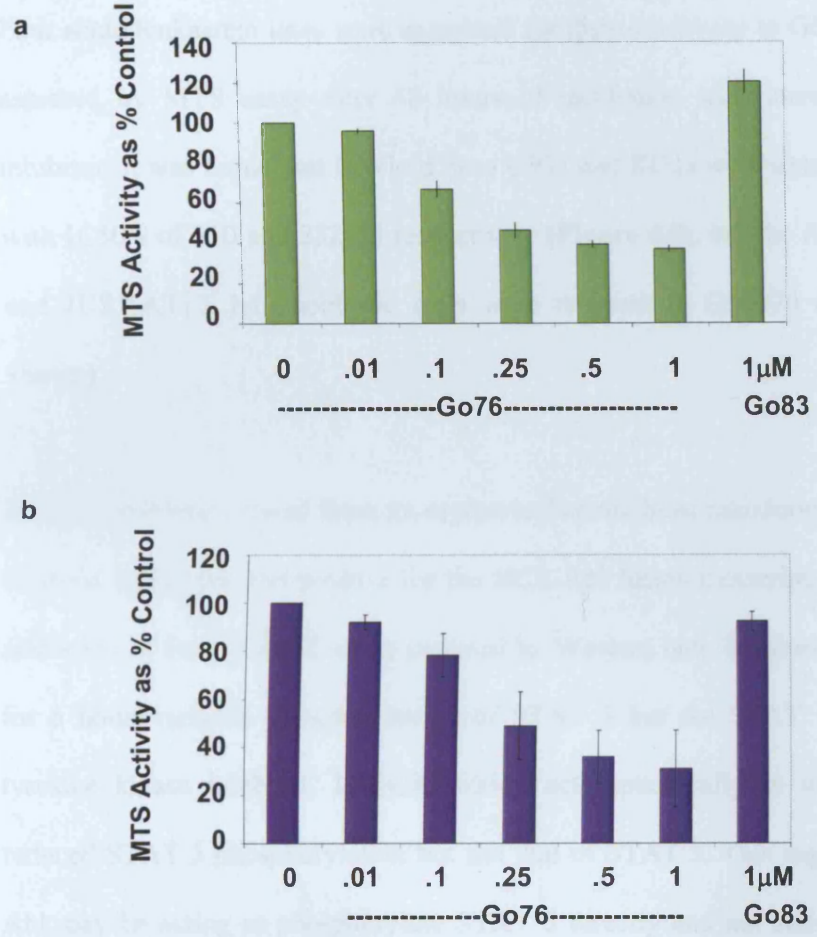


Figure 4.7 Gö6976 reduces proliferation in anaplastic lymphoma cell lines.

Karpas 299 (a) and SUDHL1 (b) anaplastic lymphoma cell lines were incubated with the indicated concentration of inhibitor. Proliferation was assessed at 48hours by MTS assay. Proliferation is expressed as a percentage of the proliferation of the control cells (no inhibitor).

4.4.2 Gö6976 effect on acute leukaemia cell lines

Four acute leukaemia lines were examined for their sensitivity to Gö6976. This was assessed by MTS assay after 48 hours of incubation with increasing doses of inhibitor. It was found that Myeloid lines U937 and KG1a were sensitive to Gö6976 with IC50's of 310 and 232nM respectively **(Figure 4.8)**, but the AML HL60 cells and JURKAT T lymphoblastic cells were resistant to Gö6976 effect (data not shown).

K562, a cell line derived from an erythroleukaemic blast transformation of chronic myeloid leukaemia and positive for the BCR-Abl fusion transcript, has constitutive activation of Stats 3 and 5 when assessed by Western blot. Incubation with Gö6976 for 6 hours reduced phosphorylation of STAT 3 but not STAT 5. However, the tyrosine kinase inhibitor, Imatinib which acts specifically to inhibit BCR-Abl, reduced STAT 5 phosphorylation but not that of STAT 3. This suggests that BCR-Abl may be acting to phosphorylate STAT 5 directly and not acting via JAK2. In spite of this, Gö6976 reduced proliferation in a dose dependent manner with an IC50 of 240+/-46nM **(Figure 4.9).**

Figure 4.9

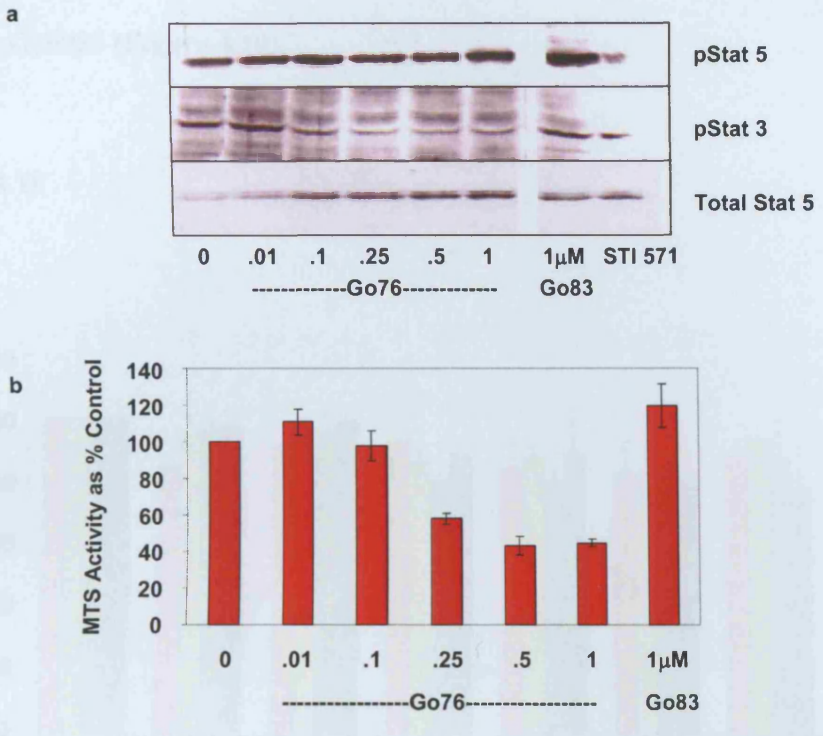


Figure 4.9 Gö6976 reduces STAT phosphorylation and proliferation in K562 cells

a) K562 cells were cultured with the indicated concentration of inhibitor for 6 hours after which total cell lysates were made. Immunoblot analysis was carried out with the specified antibody. b) Proliferation was assessed after 48 hours of culture by MTS assay.

4.4.3 The effect of Gö6976 on proliferation in Multiple Myeloma cell lines

Autocrine IL-6 production has been reported in multiple myeloma cell lines. Four myeloma lines; U266, MM1S, KMS BM and KMS PE, were investigated.

Proliferation was assessed at 48 hours after incubation with increasing doses of Gö6976. All four lines were found to be insensitive to Gö6976 as well as the control inhibitor Gö6983 **(Figure 4.10).**

Figure 4.10

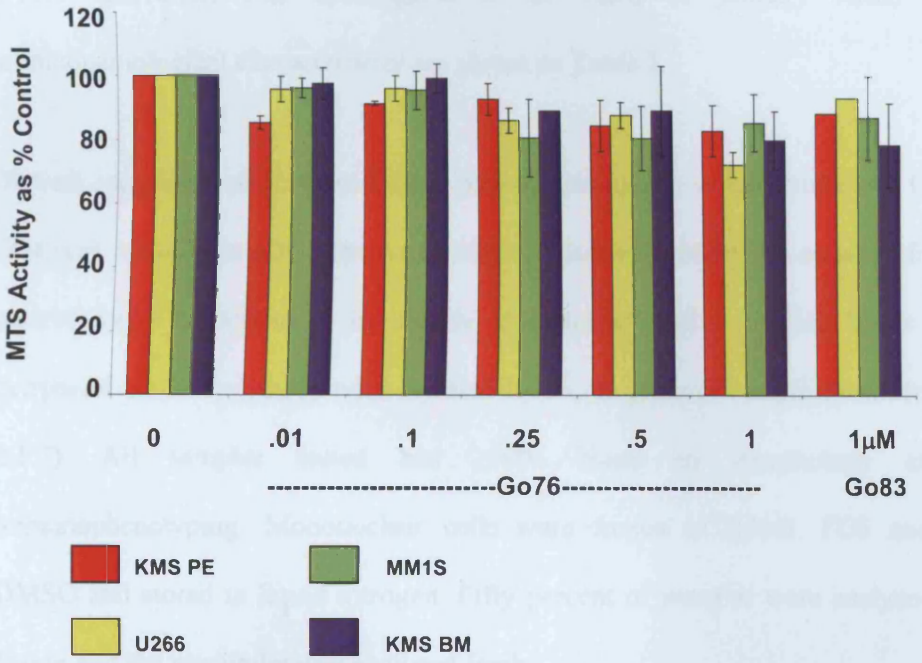


Figure 4.10 Gö6976 has no effect on proliferation in multiple myeloma cell lines.

Four multiple myeloma cell lines were cultured with indicated concentration of inhibitor. Proliferation was assessed at 48 hours by MTS assay and results expressed as percentage of control inhibitor (no inhibitor).

4.5 The effect of Gö6976 in Primary AML cells

4.5.1 The effect of Gö6976 on signalling in Primary AML cells

Constitutive STAT activation has been reported to occur in a number of haematopoietic malignancies including AML. [106] The frequency of constitutive STAT activation was investigated in 26 cases of primary AML whose clinicopathological characteristics are shown in **Table 2.**

Patient samples were obtained from patients presenting to the University College Hospital, London, at presentation or relapse. Informed consent was obtained from all patients prior to obtaining the sample. All patients had circulating blasts in the peripheral blood and these were isolated by Ficoll gradient centrifugation (method 2.1.7). All samples tested had ≥90% blasts by morphology and or immunophenotyping. Mononuclear cells were frozen in RPMI, FCS and 10% DMSO and stored in liquid nitrogen. Fifty percent of samples were analyzed from frozen and the remainder was analyzed fresh.

Primary cells were cultured with 1μM Gö6976, 1μM Gö6983 or no inhibitor for a minimum of 6 hours. Total cell lysates were made and samples probed with phospho specific antibodies for STATs 3 and 5, Akt and ERK. Twenty-four out of 26 patient samples were found to have constitutive STAT activation: 9 out of 26 had activation

of both STAT 3 and STAT 5, 14 out of 26 of STAT 3 only and 1 patient had activation of STAT 5 alone.

All patients had activation of the MAPK pathway with constitutive phosphorylation of ERK and 24/25 assessable patients had activation of the PI3-Kinase pathway.
When the primary AML cells were cultured in the presence of Gö6976, STAT phosphorylation was reduced in 23 out of the 24 cases examined, suggesting that this activation was due at least in part to dysregulation of JAK kinase activity. Of the 24 patients with constitutive STAT activation, incubation with Gö6976 in 12 cases showed a reduction in all 3 pathways (STATs, Akt, ERK) and 8 showed a reduction in STATs alone. The 2 patients without STAT activation both had activation of the MAPK and PI3-Kinase pathways and in both cases this activation was reduced after incubation with Gö6976.The remainder of patients showed complex patterns of pathway inhibition.

Table 2 shows the biological details of the primary AML samples examined.

N/A-Not available, WT-wild type, ITD-Internal tandem duplication *-MTS assay performed,+-AnnexinV assay performed.

Patient	STAT 3	STAT5	FAB Type/Rel/2°	Pres WCC $x10^9/l$	Cytogenetics	Flt 3 Status	N-Ras
1 *+	N/A	N/A	M1	14	46xx	N/A	N/A
2 *	+	-	M2	35	46xy	WT	EX1
3 *	+	-	M1	401	46xx	ITD	WT
4 *	+	+	M5b	52	46xx	WT	WT
5 *+	+	+	M2	117	Del(9) q12q34	WT	EX2
6	+	+	M5	257	46xy	ITD	WT
7*+	+	+	M4	233	Inv16(p13q22)	WT	EX2
8 *	+	-	2° ET	146	complex	WT	WT
9 *+	N/A	N/A	M5/ Relapse	3	N/A	N/A	N/A
10 *+	+	-	M0	131	N/A	N/A	N/A
11 *	-	-	M2	29	t(8;21) -y	WT	N/A
12 *	+	-	M5	69	failed	WT	WT
13 *+	+	+	M1	140	46xy	ITD	WT
14 *+	+	-	M5a	89	46xx	ITD	EX1
15 +	-	N/A	relapsed	36	46xx,r(7)(?p?q)t(9;22)(q34;q11)	WT	WT
16	+	-	M5a	162	46xy del(10)	WT	EX1/2
17 *	+	-	M2	47	Trisomy 21	ITD/D835	WT
18	+	-	M1	80	46xx	WT	EX1
19 *	+	+	2°MDS	38.1	N/A	WT	WT
20 +	+	-	M1	34	N/A	WT	WT
21*	N/A		M2	10	Trisomy 11	ITD	WT
22 *	+	-	M5b	188	46xx	WT	WT
23 +	+	+	M1	33	46xy	ITD	WT
24	N/A	+				WT	WT
25 *+	N/A		M2	42		WT	WT
26 *+	+	-			46xx	WT	WT
27 *	+	-	M2	8	45xyadd(8)(q22)add(15)(p1)add(21)(p1)	N/A	EX2
28	+	+	M3			ITD	N/A
29 +	+	-	M4	50	Trisomy 21	N/A	N/A
30 *	+	-	M4	541	T(10;11) Progressed with 7q-	N/A	N/A
31 +*	N/A	N/A	M4	120	46xy	N/A	N/A
32 +*	N/A	N/A	M4	100	46xx	N/A	N/A
33 *	N/A	N/A	AML M1	43	Abn haplo t(3;13)	N/A	N/A
34 *	N/A	N/A	N/A	N/A	N/A	N/A	N/A
35 *	N/A	N/A	M2	10	t(8;21)	N/A	N/A
36 *	N/A	N/A	M3			N/A	N/A
37 *	N/A	N/A	M3			N/A	N/A
38 *	N/A	N/A	M2	10	7q-	N/A	N/A

Figure 4.11

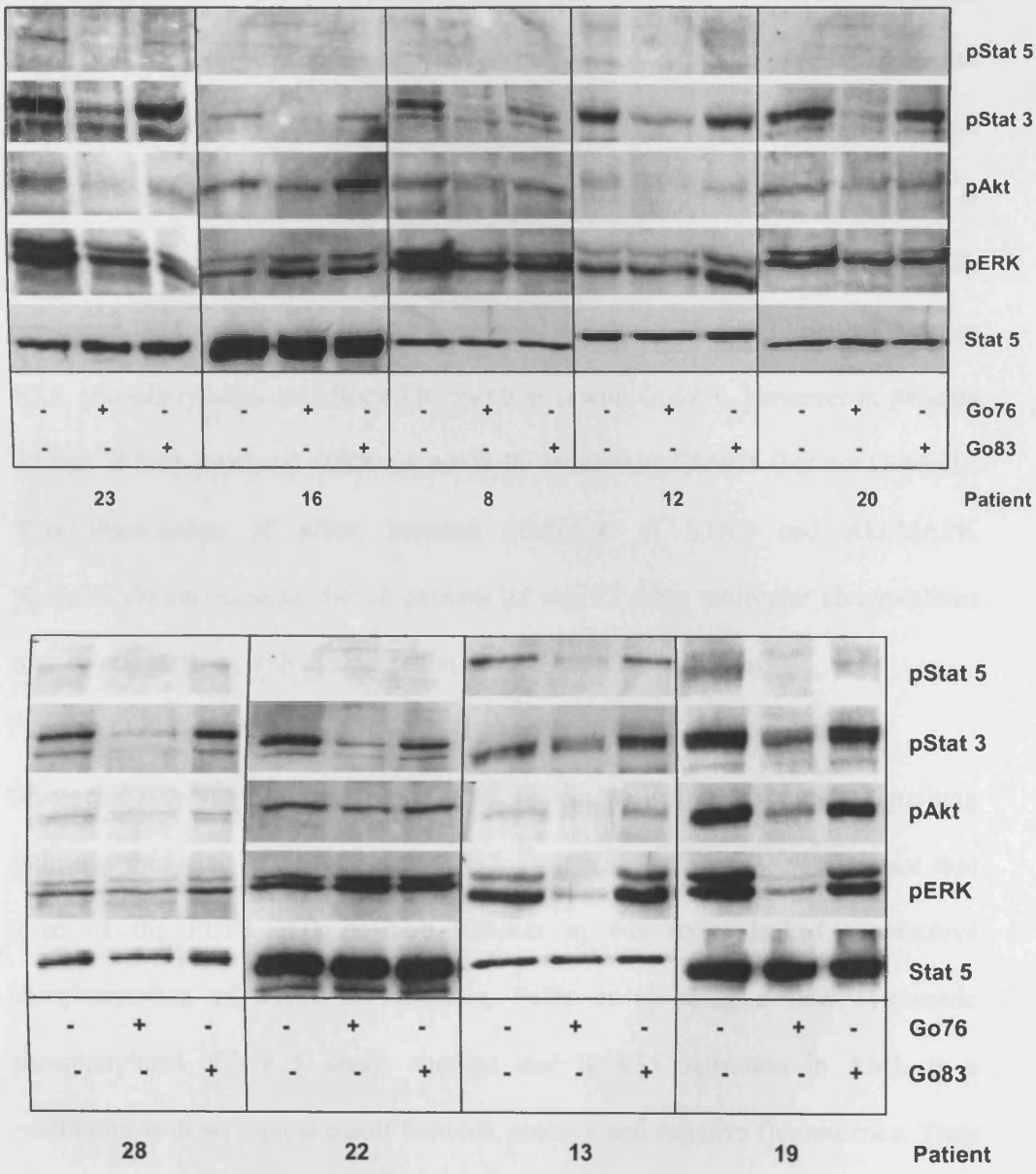


Figure 4.11 The effect of Gö6976 in primary AML cells.

Primary AML cells were cultured for a minimum of 6 hours with indicated inhibitor and total cell lysates were made. Immunoblot analysis was performed with the given antibodies. Total STAT 5 is shown as a protein loading control.

A number of different signalling patterns were seen. The second panel of Figure 4.11 which shows four patient examples illustrates this. It can be seen that examples 28, 13 and 19 show activation of STATs 3 and 5 whereas patient 22 shows activation of STAT 3 alone. All cases show a reduction in STAT phosphorylation after incubation with 1μM Gö6976 but not 1μM Gö6983. In all 4 examples there is evidence of constitutive activation of the PI3-Kinase and MAPK pathways with phosphorylation of Akt and ERK respectively. In patients 28 and 22 neither Akt nor ERK phosphorylation are affected by incubation with Gö6976. However in patients 13 and 19 both pAkt and pERK are markedly reduced by Gö6976 (but not Gö6983). This dissociation of effect between inhibition of STAT and Akt/MAPK phosphorylation suggests that in patients 28 and 22 other molecular abnormalities may be cooperating with dysregulation of the JAK/STAT pathway.

Patients 13, 23 and 28 have a FLT3 ITD and patient 16 has a Ras mutation.

Oncogenic signalling by FLT3 ITD is known to activate aberrant signalling pathways including activation of STAT 5 [79]. It may therefore seem unusual that some of the FLT3 ITD positive patients in our series lacked constitutive phosphorylation of STAT 5. However, Pallis et al, using a flow cytometric phosphorylated STAT 5 assay, showed that STAT5 activation in AML is a continuum with no logical cutoff between positive and negative fluorescence. They found just under 50% of their samples possessed a FLT3 ITD and found no association between the level of STAT 5 phosphorylation and the presence of a FLT3 ITD.

4.3.2 The effect of Gö6976 on survival and proliferation in primary AML cells

The biological effects of Gö6976 on primary AML cells were examined by measuring apoptosis at 24 hours and cell number at 48 hours. The results are shown in **Figure 4.12**. Incubation with Gö6976 led to a reduction in cell number to 63+/-3% (n=29) of control. The MTS results could be due to an increase in apoptosis, a reduction in proliferation or a combination of both. These results were at least partly due to an increase in apoptosis which was increased by 19+/-6% (n=15). Gö6983 had no effect on cell number or apoptosis. Although events at the signalling pathway level differed between patients it should be noted that 85% of samples showed a greater than 20% reduction in MTS activity in response to 1μM Gö6976. Further work carried out in our laboratory showed that when primary AML cells were cultured in the presence of IL-3, an increase in cell number was seen which was abrogated by incubation with 1μM Gö6976 (data not shown). In 2 patients the CD34+ CD38- subset was isolated by flow cytometric sorting; this subset is thought to include the leukaemic stem cell population. In one patient apoptosis was measured after incubation with 1μM Gö6976 or Gö6983 for 24 hours. Apoptosis in the bulk and CD34+38- populations increased by 8% and 26% respectively. In the second patient an MTS assay was carried out after 48hours incubation with Gö6976. This led to a 93% reduction in cell number in the CD34+38- group compared to 61% in the unselected cells. Gö6983 had no effect on apoptosis or cell number in either experiment.

Figure 4.8

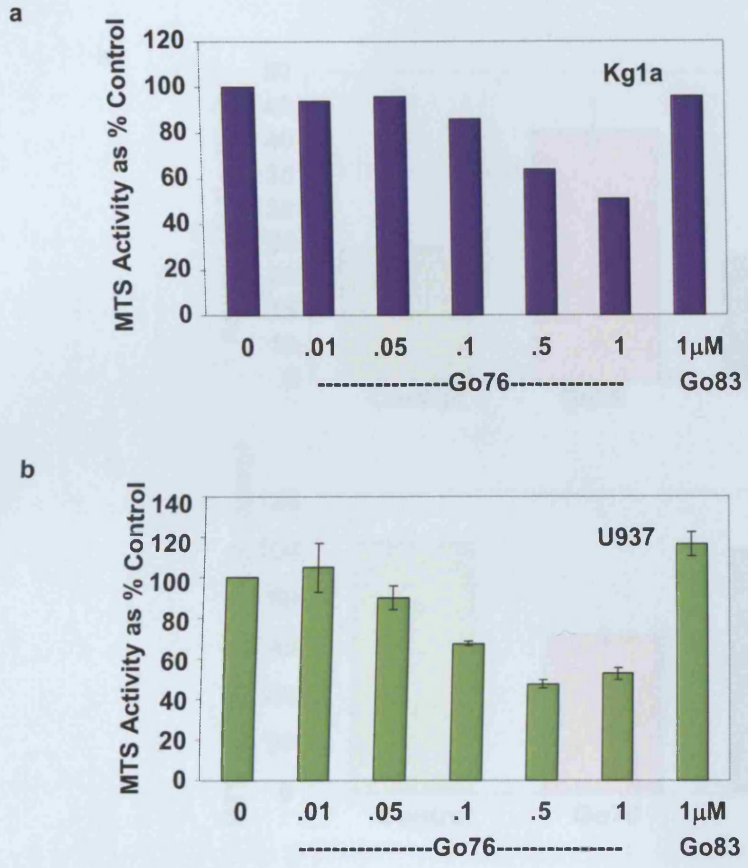


Figure 4.8 Gö6976 reduces proliferation in leukaemia cell lines.

a/b Leukaemic cell lines were cultured with indicated concentration of inhibitor and proliferation assessed by MTS assay at 48hours. Results are given as a percentage of control cells (no inhibitor).

Figure 4.12

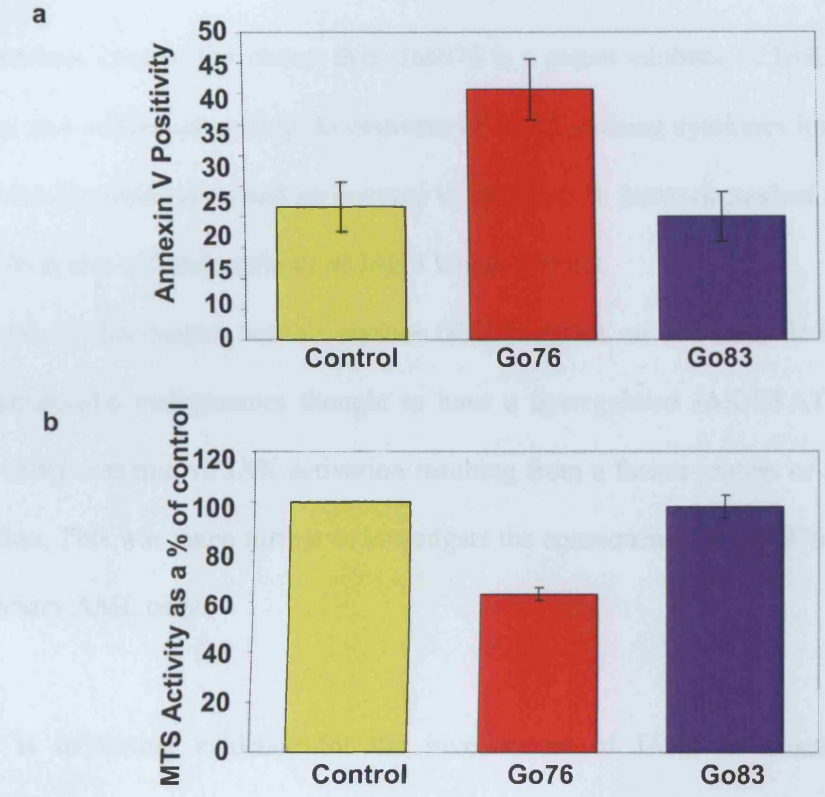


Figure 4.12 Gö6976 reduces cell number and increases apoptosis in AML cells.

Primary AML cells were incubated with 1μM Gö6976, 1μM Gö6983 or no inhibitor. Apoptosis was assessed at 24 hours by annexin V staining and flow cytometry (a) and cell number was assessed by MTS assay at 48hours (b).

4.6 Discussion

The previous chapter has shown that Gö6976 is a potent inhibitor of JAK 2 kinase activity and inhibits signalling downstream of JAK2 utilising cytokines leading to a reduction in proliferation and an increase in apoptosis in factor dependent cell lines. Gö6976 is also a direct inhibitor of JAK3 kinase activity.

The aim of this chapter was to explore Gö6976 effect on cell lines derived from haematopoietic malignancies thought to have a dysregulated JAK/STAT pathway with either constitutive JAK activation resulting from a fusion protein or activating mutation. This was taken further to investigate the consequence of Gö6976 exposure on primary AML cells.

There is increasing evidence for the involvement of JAK2 in haematological malignancies with the descriptions of Tel-JAK2 and PCM1-JAK2 fusion genes in leukaemia and myeloproliferative disease, [93,108] the amplification and overexpression of JAK2 in certain lymphomas [24,94,105,109] and most recently with the identification of the V617F mutation at very high frequencies in polycythaemia vera, essential thrombocythaemia and idiopathic myelofibrosis [68,95,104] and exon 12 mutations in polycythaemia and idiopathic erythrocytosis[110].

In order to further evaluate the effect of Gö6976 on JAK2 kinase, its effect on the constitutively active Tel-JAK2 kinase was investigated. The Translocated Ets leukaemia (TEL) protein gene is frequently rearranged in human malignancies. It

has been reported to be fused to the catalytic domain of JAK2 in pre B ALL (t(9;12)) and in an atypical CML (t(9;15;12). [92] This fusion protein is able to transform BAF3 cells and lead to growth factor independence. Mice transplanted with bone marrow infected with the fusion gene develop a rapidly fatal mixed myeloproliferative and lymphoproliferative disease which is thought to be dependent on STAT 5. [111] Tel-JAK2 fusion proteins have a diffuse cytoplasmic location and are not associated with the intracellular moiety of the receptor. Constitutive activation of STATs 3 and 5 in 32D cells expressing the Tel-JAK2 fusion was found which concurs with the findings of Ho[112] et al. Lacronique, however reports only constitutive activation of STAT 5.[108] Both Lacronique and Ho found that Tel-JAK2 does not phosphorylate wild type JAK2 or the IL-3βR suggesting that Tel-JAK 2 fusions are directly able to activate signalling pathways that lead to cell proliferation and inhibition of apoptosis independently of the cytokine receptor. Our results indicate that Gö6976 is able to inhibit the constitutively active Tel-JAK2 fusion protein and decrease phosphorylation of STATs 3, 5, Akt and ERK. Further work carried out in the laboratory has shown that Gö6976 also inhibits signalling downstream of the constitutively active JAK2 V617F mutation in HEL cells and reduces proliferation in this cell line. Although it would be predicted that tyrosines 1007 and 1008 would be phosphorylated in myeloproliferative disorders with activating JAK mutations this has not yet been formally demonstrated. JAK2 V617F retrovirally transfected into murine bone marrow cells produces a non fatal polycythaemia when transplanted into Balb/c mice. Continuous parenteral administration of the JAK2 inhibitor AG490 to polycythaemic mice over a 2 week period led to a reduction in

haematocrit and a fall in reticulocyte count[113]. Together these results show that Gö6976 can inhibit signalling from JAK2 in its wild type, fusion protein and V617F forms.

Figure 4.13

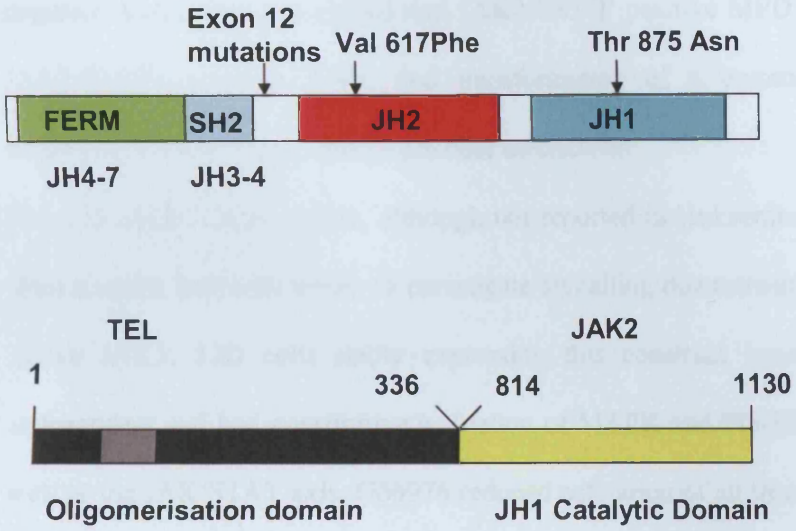


Figure 4. 13A schematic representation of JAK 2 illustrating the location of some of the mutations and fusion proteins occurring in haematological disorders. The T875N mutation has been reported in AMKL cell lines.[114]

Transformation to AML is a known complication of myeloproliferative disorders. The frequency of the JAK V617F somatic mutation in AML is low at around 1%[68,115-118]. Lee et al found 3 JAK 2 mutations in 113 samples screened giving a frequency of 2.7%. Only 2 of these were JAK2V617F with the other being K607F. The 2 JAKV617F mutations were found in patients with t(8;21) translocation[116]. Schnittger et al found 2 cases expressing JAK2V617F in 24 cases of AML expressing t(8;21) AML1/ETO, both of which were therapy related[119] and Desta et al found 2 cases in 140 cases of therapy related MDS and AML. The latter 2 cases

had myeloproliferative feaures[120]. Theocharides et al investigated the role of JAK V617F in leukaemic transformation and examined 27 patients with myeloproliferative disorders who had transformed to AML. The JAK2 V617F was found in 17/27 patients at diagnosis whereas 9/17 transformed to JAK2V617F negative AML. They concluded that JAK2-V617F positive MPD frequently yields JAK2-V617F negative AML and transformation of a common JAK2-V617F negative precursor represented a possible mechanism.

The Tel-JAK3 fusion protein, although not reported in leukaemia, has provided us with a useful tool with which to investigate signalling downstream of constitutively active JAK3. 32D cells stably expressing this construct were rendered factor independent and had constitutive activation of MAPK and PI3-Kinase pathways as well as the JAK/STAT axis. Gö6976 reduced activation of all three pathways which translated into a reduction in proliferation of these cells and an increase in apoptosis. It appears therefore that Gö6976 as well as being able to inhibit JAK3 kinase activity and wild type JAK3 is also able to inhibit this JAK3 fusion protein.

Constitutive STAT activation has been reported in many malignancies including multiple myeloma, acute myeloid leukaemia (AML), chronic myeloid leukaemia (CML), non Hodgkin's lymphoma and anaplastic large cell lymphoma (ALCL). [96,97] The effect of Gö6976 in 2 anaplastic lymphoma cell lines, Karpas 299 and SUDHL1 was investigated. The t(2;5), is thought to be present in greater than 50% of ALCL and leads to the formation of an 80kda fusion protein, fusing nucleophosmin (NPM) with the ALK kinase. Both STAT 5b and JAK2 contribute to cellular proliferation in

NPM-ALK expressing cell lines. [105] Activation of STATs 3 and 5 was seen in both cell lines which was reduced in a dose dependent manner after incubation with Gö6976. These effects were accompanied by a reduction in cell proliferation. Ruchatz et al [105] investigated the role of JAK2 activation in NPM-ALK transformed cells. They found that incubation of these cells with AG490, a tyrphostin JAK2 inhibitor led to a reduction in JAK phosphorylation and also a reduction in proliferation and increase in apoptosis, thus concurring with our results.

The effect of Gö6976 on K562 cells (chronic myeloid leukaemia in blast crisis) which possess the BCR-Abl fusion transcript was explored. These cells were found to be sensitive to the effect of JAK2 inhibition. Of interest, both STAT 3 and STAT 5 were found to be constitutively activated, however Gö6976 only reduced activation of STAT 3 and not of STAT 5. This was in contrast to the effect of Imatinib, a specific inhibitor of the BCR-Abl fusion, which reduced only STAT 5, thus suggesting that BCR-Abl is activating STAT 5 directly and not via JAK2. Xie et al found JAK2 phosphorylation in the absence of IL-3 in a BCR-Abl expressing cell line. They found that the C terminal of Abl was involved in a complex with JAK2 and that tumour formation by K562 cells in nude mice was JAK 2 dependent and inhibited by a kinase inactive JAK2 mutant. [121] Interestingly De Vos et al found that their K562 cells were insensitive to JAK2 inhibition by AG490[55].

IL-6, which signals via JAK2, has been widely implicated in the pathogenesis of Multiple Myeloma (MM) and is thought to be a major growth and survival factor in

this disease. Several MM lines, including U266 are thought to be dependent on autocrine IL-6 for survival. The effect of Gö6976 on 4 MM cell lines was investigated, including U266, and found none of them to be sensitive to this agent. This contrasts with the findings of De Vos et al who found that AG490 inhibited proliferation and induced apoptosis in U266 cells, together with reducing STAT 3 phosphorylation. [55] The findings of Alas et al agreed with this and they also found that JAK2 inhibition potentiated certain cytotoxic drugs. [122] The reason why our results differ may relate to the cells used and the possibility that further mutations have been acquired during passage that confer additional survival benefit. Alternatively, some of the effects of AG490 could be attributable to non-JAK2 inhibitory activity, such as its inhibition of CDK2 activation.

Having identified Gö6976 as a potent JAK inhibitor and investigated its effect in several haematopoietic cell lines the next step was to examine its effects on signalling, survival and proliferation in primary AML cells. Constitutive tyrosine phosphorylation of STATs 3 and/or 5 was found in 24 of 26 primary AML samples examined. This was reduced by incubation with Gö6976 in 23 of 24 cases. This result suggests that the STAT activating event in AML is occurring upstream of STATs rather than involving the STATs themselves and is due to the activation of JAKs rather than other tyrosine kinases implicated in STAT activation (such as src family kinases). [4] The FLT3-ITD abnormality has been reported to activate STAT 5 [106] but it is not clear if this is due to direct phosphorylation or via JAK activation. In our study, STAT5 activation in cases that were FLT3 positive was sensitive to Gö6976 suggesting that STAT activation is indirect, but further studies are required

to investigate this in more detail. STAT activation in AML could potentially result from novel fusion proteins such as Tel-JAK2 or PCM1-JAK2, [93,108] autocrine/paracrine growth factor secretion or activating mutations such as the V617F mutation within JAK2. [68] Activation of PI3-Kinase and MAPK pathways was also found in the majority of AML cases examined and is consistent with our previous findings. [123] In 12 out of 24 patients inhibition of JAKs by Gö6976 led to reduced activation of all three pathways suggesting that dysregulation of the JAK pathway was one of the main aberrant signaling events in these patients. Eight out of 24 patients had a reduction in STAT alone and the remainder of patients had more complex patterns of inhibition. There was no clear correlation between pattern of signaling inhibition and degree of reduction in MTS activity in response to Gö6976, but this may be a limitation of the sample number examined. It should be noted that despite the differences in pathway inhibition, a greater than 20% reduction in cell number was seen in 85% of patients tested.

Figure 4.14

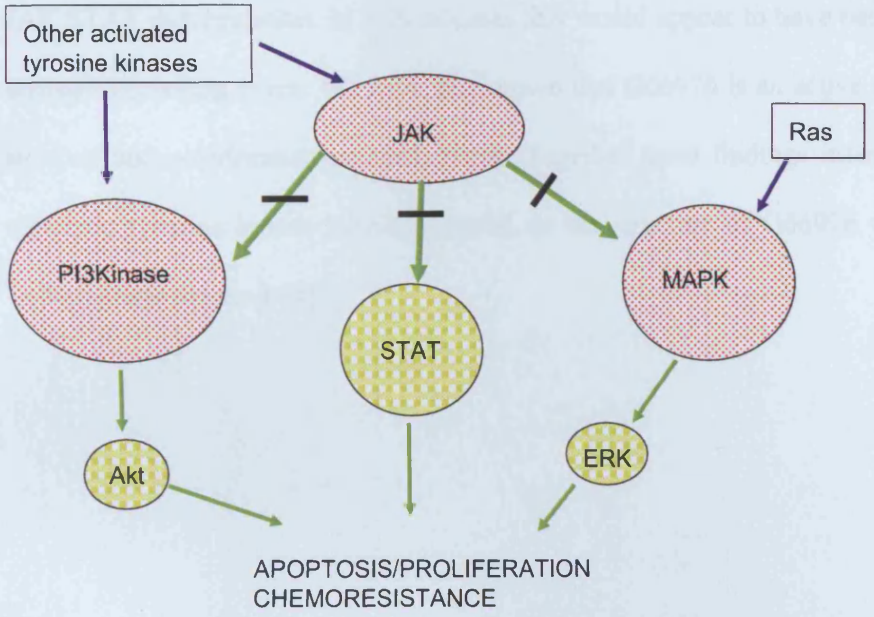


Figure 4. 14 Downstream signalling pathways in AML

4.7 Conclusions

In conclusion we have shown that Gö6976 is a potent inhibitor of JAK2 and that it is able to inhibit signalling downstream of wild type JAK2, JAK2 constitutively activated by fusion with Tel and mutant JAK2 V617F. We have also shown similar effects on JAK3 wild type and the Tel-JAK3 fusion protein. Investigation of several haematopoietic cell lines reported to have some dysregulation of the JAK/STAT pathway, revealed that Gö6976 was able to inhibit proliferation in 2/2 ALCL lines (Karpas 299, SUDHL1), 2/4 leukaemia lines (Kg1a, U937) and 1 CML line (K562) but had no effect on any of the 4 MM lines examined. When we investigated

primary AML cells we found STAT activation in 24/26 patient samples examined which was reduced by Gö6976 incubation in 23/24 cases, providing evidence of JAK STAT dysregulation. In 50% of cases this would appear to have been the main aberrant signalling event. We have also shown that Gö6976 is an active inhibitor of survival and proliferation in AML blasts. Together these findings infer that small molecule tyrosine kinase inhibitors based on the structure of Gö6976 would be a valid therapeutic modality.

CHAPTER 5-THE ROLE OF THE PI3-KINASE PATHWAY IN ACUTE MYELOID LEUKAEMIA

5.1 Introduction

The PI3-Kinases are important regulators of intracellular signal transduction pathways mediating cell proliferation, differentiation and survival. There is now increasing evidence for PI3-Kinase/Akt dysregulation in human malignancy. [28] The role of PI3-Kinase activation in AML is less clear. Recently constitutive phosphorylation of Akt has been described in a significant proportion of patients with AML. [124-126] The method of Akt activation is not clear but does appear to be important for leukaemia cell survival. [124,125] The presence of constitutive Akt phosphorylation has also been linked to a shorter overall survival in AML. [124] In this chapter the frequency of constitutive activation of PI3-Kinase in AML was investigated together with the effect of PI3-Kinase inhibition on survival in bulk primary AML blasts and on leukaemic progenitors. The potential for combining PI3-Kinase inhibition with conventional cytotoxics was examined and key downstream effector molecules that mediate the pro-survival effect of PI3-Kinase in AML, were identified.

5.2 Akt is constitutively active in primary AML Cells

In order to determine if Akt is activated in primary AML cells we utilised AML blasts from patients at presentation or relapse. For analysis both fresh and frozen samples were used. Primary cells were cultured overnight in RPMI 10% FCS in the absence of growth factors either with or without the PI3-Kinase inhibitor LY 294002. Total cell lysates were made and Akt phosphorylation was detected by western blot, using an antibody directed against phosphorylated Akt at serine 473. Twenty-four out of 25 assessable patients had activated Akt indicating that the PI3-Kinase pathway is activated in AML blasts. Overnight incubation with the selective PI3-Kinase inhibitor, LY294002 resulted in reduced or abolished Akt phosphorylation (**Figure 5.1**).

Figure 5.1

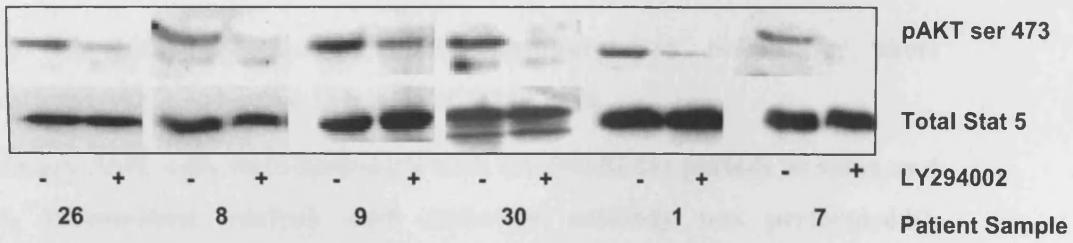


Figure 5.1 Constitutive Akt activation is reduced by LY294002 in AML cells.

Primary AML cells were incubated for 24 hours with or without LY294002 25μM and total cell lysates made. The blot was probed with phospho Akt ser473 and total STAT 5 to check for protein loading. The patient sample n° refers to Table 1.

This was also seen after incubations as short as 4 hours **(Figure 5.2a).** Using scanning densitometry the mean reduction was 53+/-4% (n=20). To illustrate that this was constitutive activation, Akt phosphorylation in the AML blasts was compared to that found in normal CD34+ cells grown in myeloid culture in the presence of SCF, IL-3 and GCSF (day 7) **(Figure 5.2b)**. Akt phosphorylation was not detected in the normal myeloid cells.

Figure 5.2

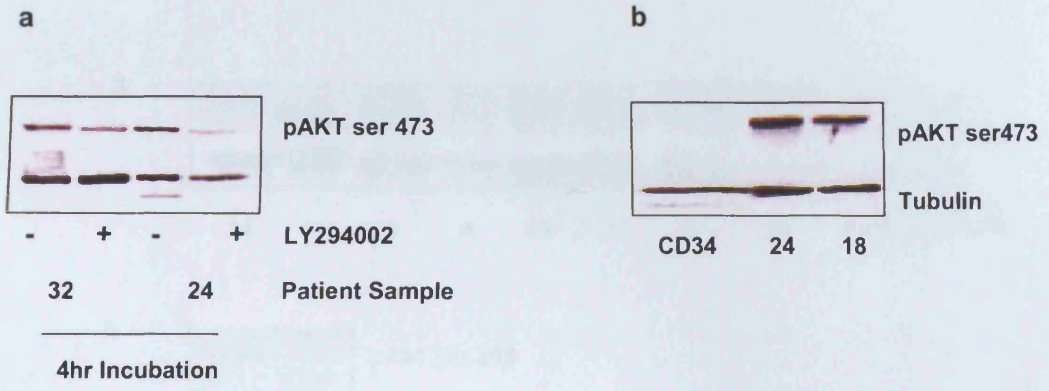


Figure 5.2 LY294002 reduces Akt phosphorylation even after short incubations; Akt is not active in normal CD34+ cells.

a) Primary AML cells were incubated with LY294002 for periods as short as 4 hours. Immunoblot analysis with indicated antibody was performed.b). Western blot to show activation of Akt in 2 patient samples but not in non-leukaemic myeloid cells (CD34+cells in myeloid culture, day 7). Tubulin is shown as a loading control.

Akt phosphorylation at serine (ser) 473 is thought to occur after initial phosphorylation at threonine (thr) 308 - phosphorylation at both residues is required for full activation of Akt. Primary AML blasts were incubated (8 cases) with LY294002 as described above and total cell lysates made. Phosphorylation at Thr 308 was examined by Western blot using a phosphospecific antibody. Constitutive activation of Akt at Thr 308 was seen in all samples examined and incubation with LY294002 led to a reduction in phosphorylation at this residue (**Figure 5.3**).

Figure 5.3

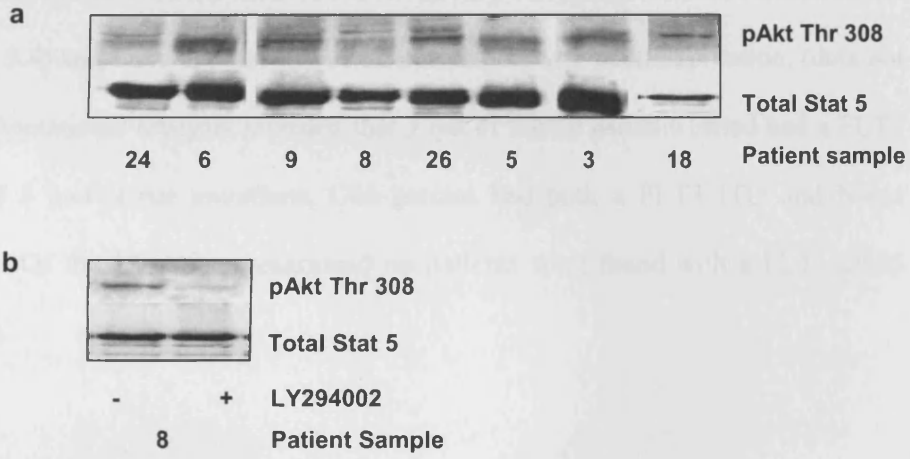


Figure 5.3 Akt is constitutively active at threonine 308 in primary AML cells.

Western blot showing constitutive activation of Akt at Thr 308 in primary AML blasts (8 samples shown here). b) Primary AML blasts were incubated with LY294002 for 24hours and total cell lysates made. Immunoblot analysis with phospho Akt Thr 308 was carried out.

Akt activation could be due to downregulation of one of the negative regulators of PI3-Kinase, such as PTEN. PTEN (phosphatase and Tensin homologue) is commonly deleted in a diverse array of cancers including haematological malignancies[32,33]. PTEN deletion in MX-1/Cre mice leads to a myeloproliferative disease within days and a transplantable leukaemia within weeks[127].Interestingly PTEN deletion also led to Haematopoietic stem cell (HSC) proliferation which led to a loss of self renewal capacity, a contrast to the leukaemia initiating stem cell. Treatment with rapamycin, a mTOR inhibitor, restored the self renewal capacity of HSC's and depleted leukaemia initiating cells [127]. PTEN expression in 12 patients with AML was at a similar level to a mononuclear cell fraction from a normal donor **(Figure 5.4)** and there appeared to be no correlation with pAkt expression. (data not shown) Mutational analysis revealed that 3 out of the 16 patients tested had a FLT3 ITD and 5 had N-*ras* mutations. One patient had both a FLT3 ITD and N-*ras* mutation. Of the 18 patients examined no patients were found with a FLT3 D835 mutation.

Figure 5.4

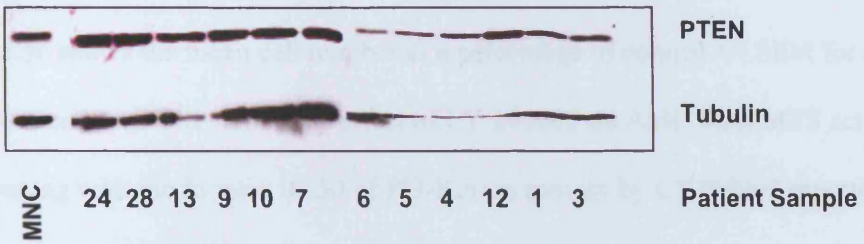


Figure 5.4 PTEN expression in primary AML cells.

Western blot showing PTEN protein in 12 patients with AML and a normal mononuclear cell preparation. The blot was re-probed with an anti-tubulin antibody to check protein loading.

5.3 Effect of PI3-Kinase blockade on leukaemic cell survival

In order to investigate the role of PI3-Kinase in AML survival primary AML blasts were incubated with 25μM LY294002 and an MTS assay carried out at 48 hours. Cell number was reduced by an average of 51+/-3.6% (range 6-85%, n=29 patient samples) **(Figure 5.5a).** This could represent a reduction in proliferation or an increase in apoptosis or both. The level of apoptosis was therefore examined after incubation with LY294002 using a flow-cytometric annexin V assay to detect the percentage of apoptotic cells. After 24hours, 19+/-4% of control cells and 43+/-5% of LY294002 treated cells were annexin positive (range 21-63%, n=9) **(Figure 5.5b)**, suggesting that the reduction in MTS activity was at least partly due to increased apoptosis. A dose response for LY294002 was carried out in blasts from 7

patients. AML blasts were incubated with increasing doses of LY294002 (0-50μM) and an MTS assay carried out at 48 hours. The mean IC50 was 7μM LY294002. Figure 5.5c shows the mean cell number as a percentage of control +/- SEM for each concentration used. The inhibitory effect of LY 294002 on AML blast MTS activity is in keeping with the in vitro IC 50 of PI3-Kinase activity by LY294002 reported as 10 μM by Davies et al. [128]

5.4 PI3-Kinase blockade reduces MAPK activation in some patients with AML

The ERK MAP-Kinase (MAPK) pathway is another key regulator of cellular processes including differentiation, proliferation and apoptosis. Primary AML samples were incubated with and without LY294002 for a minimum of 4 hours, total cell lysates made and examined by Western Blotting using a phospho-specific MAPK p42/44 antibody. We found that all of 18 AML samples investigated had constitutively active MAPK. When these cells were incubated with 25μM LY294002 MAPK phosphorylation was reduced in 10/18 patients **(Figure 5.6a)**. Quantification by scanning densitometry showed a decrease to 36+/-6% (mean+/-SEM) of control. This suggested that PI3-Kinase activity is upstream of MAPK, and raises the possibility that some of the effects of PI3-Kinase inhibition on leukaemic cell survival may be due to reduced MAPK activity. To assess this further the effect of U0126, a selective MEK inhibitor, on cell proliferation and apoptosis was

assessed. MTS assay at 48hours showed a reduction in cell number of 23+/-7% (range 0-69%, n=19) when compared to control cells (c.f. LY 51%) **(Figure 5.6b).** Annexin V assay, at 24 hours, showed apoptosis levels of 31+/-6% (range 14-61%, n=9) compared to control 19+/-4% **(Figure 5.6c)**. This indicated that although MAPK has a role in AML cell survival the effect of PI3-Kinase inhibition on AML survival could not solely be attributed to MAPK inhibition.

Figure 5.5

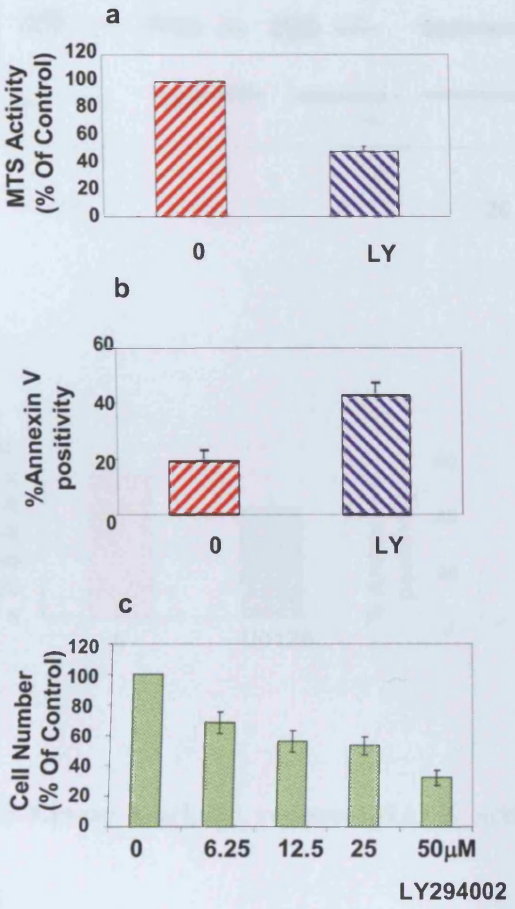
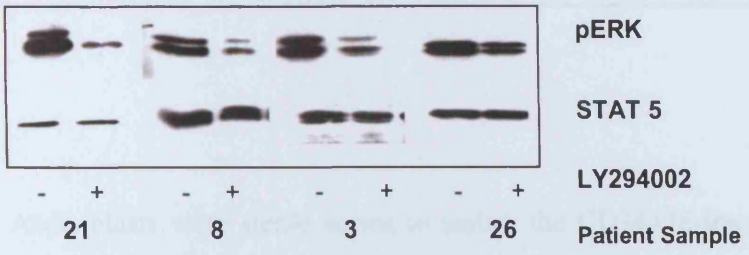


Figure 5.5 LY294002 reduces cell number and increases apoptosis in primary AML cells.

a/b) Primary AML cells were incubated with and without LY294002 25μM and apoptosis was assessed at 24hours by annexin V staining and an MTS assay was carried out at 48hours to assess cell number. c) Primary leukaemic blasts were incubated with increasing doses of LY294002 and an MTS assay carried out at 48 hours. The mean results for each concentration are shown +/- SEM (n=7).

Figure 5.6a



b

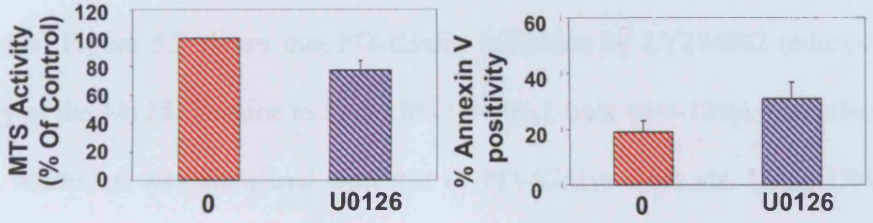


Figure 5.6 PI3-Kinase blockade reduces MAPK activation in some primary AML cells.

a/b Western blot showing ERK activation in four primary AML samples and reduction in phosphorylation after 24 hours incubation with LY294002 25μM. The blot was probed initially for Phospho ERK 1/2 and then re-probed with total Stat 5 to check for protein loading. b). Cells were incubated with U0126 10μM an MTS assay was carried out at 48 hours and apoptosis measured by annexin V staining afer 24 hours incubation. Results were expressed as a percentage of control. The mean reduction in MTS activity was 23+/-7% (range 0-69%, n=19). Apoptosis was 31+/-6% (range14-61%) compared to a control level of 19+/-4% (n=9).

5.5 Effect of PI3-Kinase inhibition on viability of CD34+38- AML cells

In four cases, AML blasts were sterile sorted to isolate the CD34+38-fraction in which the leukaemic stem cell is thought to reside. The cells were incubated with 25μM LY294002 and cell viability assessed after 24-48 hours by annexin V binding or MTS assay. The results were compared with results for the bulk (unsorted) blast population. Figure 5.7 shows that PI3-Kinase inhibition by LY294002 reduces the viability of the 34+38- fraction to 51+/-18% (n=4)(c.f. bulk 48+/-12%). The effect of MAPK inhibition was compared with that of PI3-Kinase blockade. Using U0126, cell viability was again assessed after 24-48 hours: MAPK blockade reduced bulk blast viability to 72+/-10% but did not affect the CD34+38- cells (n=3, 100+/-4%). These results suggested that inhibiting a given pathway in the bulk leukaemic blast population does not necessarily predict what would happen when the same pathway is inhibited in individual sub populations.

Figure 5.7

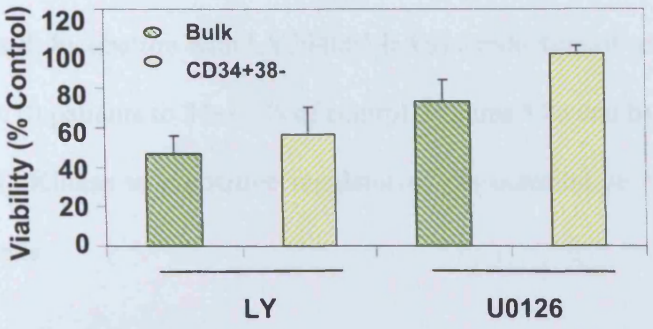


Figure 5.7 The effect of PI3-Kinase inhibition on viability of CD34+CD38- AML cells.

CD34+CD38- cell were isolated by sterile sorting and incubated with either LY294002 25μM or U0126 (MEK1 inhibitor) 10 μM for 24-48 hours. Either an MTS assay or annexin V staining was carried out to assess cell viability and expressed as a percentage of control.

5.6 PI3-Kinase regulates NF-kB and p53 activity in AML cells

NF-kB is a ubiquitously expressed transcription factor and increased activity is associated with anti-apoptotic effects. NF-kB has been reported to be constitutively active in a number of malignancies including AML, [129] and may be regulated by PI3-Kinase. Using a novel technique for high level transfection into primary AML cells ("Nucleofection") we measured the effects of PI3-Kinase blockade on NF-kB reporter gene activity. The cells were transfected with the NF-kB reporter gene (transfection efficiency by GFP was 39+/-6%). Transfected cells were incubated overnight (with or without LY294002) in RPMI/10% FCS. Cells were lysed and

induced NF-kB reporter activity was corrected for the constitutive renilla luciferase expression. Constitutive NF-kB activity (above background levels) was seen in all patients examined. Incubation with LY294002 led to a reduction of constitutive NF-kB activity in 9/10 patients to 34+/-7% of control (Figures 5.8a and b). These results suggest that PI3-Kinase is a positive regulator of the constitutive NF-kB activity seen in AML cells.

Figure 5.8 a **b**

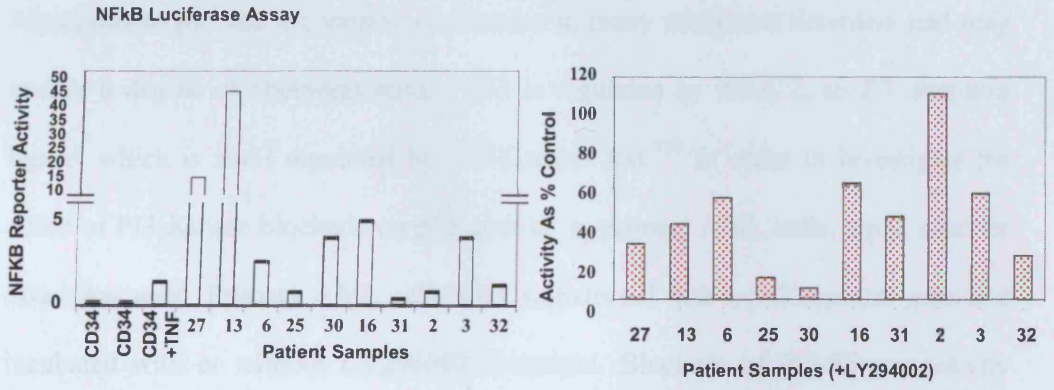


Figure 5.8 PI3-Kinase regulates NF-kB in primary AML cells.

a) Primary AML cells or normal CD34+ cells were transfected with an NF-kB reporter gene. The transfection efficiency for the CD34+cells was 54+/-9% and for AML blasts was 39+/-6%. All patients had constitutive activation of NF-kB, above levels seen in un-stimulated CD34+ cells. b) Incubation with LY294002 25μM led to a reduction in NF-kB activity in 9/10 patients. The activity was reduced to a mean of 34+/-7% of control (n=9). NF-kB reporter activity as a percentage of untreated control cells is shown for each individual.

Further work carried out in our laboratory investigated the contribution of the PI3-Kinase/p110δ isoform in regulating NF-kB in primary AML cells. Constitutive activation of the NF-kB construct was found in 6 of 6 AML samples investigated. When the cells were incubated with IC87114, a selective p110δ isoform inhibitor, the basal NF-kB activity was only slightly reduced (5/6 cases) in contrast to LY294002. This could be due to the more profound PI3-Kinase inhibition by LY294002 or to off-target effects of this compound.

P53 is a central mediator of the cells response to DNA damage and other stresses. Mutations in p53 are a common occurrence in many malignant disorders and may confer a degree of chemoresistance. p53 is regulated by mdm 2, an E3 ubiquitin ligase, which is itself regulated by PI3-Kinase /Akt.[130] In order to investigate the effect of PI3-Kinase blockade on p53 activity in primary AML cells, a p53 reporter assay was used. Primary AML cells were transfected with a p53 reporter gene and incubated with or without LY294002 overnight. Blockade of PI3-Kinase activity with LY294002 led to a more than 2 fold increase in p53 activity in 4 out of 9 patients tested **(Figure 5.9)**.

Figure 5.9

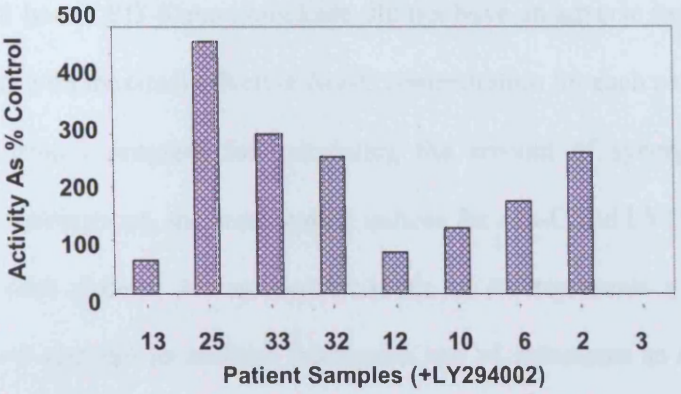


Figure 5.9 PI3-Kinase regulates p53 activity in AML cells.

Primary AML cells were transfected with a p53 reporter gene and incubated overnight with LY294002 25μM. PI3-Kinase inhibition led to a more than 2 fold increase in p53 activity in 4/9 patients examined. p53 reporter assay readings are shown after incubation with LY294002 25μM as a percentage of untreated control cells, for individual patients.

5.7 PI3-Kinase inhibition can enhance the cytotoxic effect of Ara-C

The enhancement of anti-apoptotic mechanisms by constitutively activated PI3-Kinase/Akt may reduce the effectiveness of cytotoxic agents. Therefore the effect of blocking the PI3-Kinase pathway on sensitivity to Ara-C, a central cytotoxic component of many AML treatment regimens was examined. As Ara-C exerts its cytotoxic activity at least partly in a cell cycle dependent manner, it was possible that PI3-Kinase blockade could reduce cell proliferation and have a deleterious

effect on the Ara-C response. AML cells were co-incubated with varying concentrations of LY294002 (0-50μM) and Ara-C (0-2μg/ml) and an MTS assay carried out at 48 hours. PI3-Kinase blockade did not have an adverse impact on the cell kill obtained with maximal effective Ara-C concentration for each patient tested. Using the Calcusyn® program for calculating the amount of synergy between different drug combinations, the combination indices for Ara-C and LY294002 were calculated for each patient. A combination index of <1 represents a synergistic interaction, of =1 signifies an additive interaction and >1 represents an antagonistic interaction. One patient out of the 8 patients examined was not sensitive to Ara-C and therefore synergy could not be calculated. Of the remaining patients, 5 out of 7 exhibited synergy (values at ED50 of 0.2, 0.7, 0.6, 0.5, 0.9), 1 showed an additive interaction (ED50 value 1) and 1 a moderately antagonistic interaction (ED50 value 1.4). Other groups have shown that PI3-Kinase blockade potentiates the response to several chemotherapeutic agents including paclitaxel, vincristine, doxorubicin, trastuzumab, and etoposide.[131-133] In our laboratory C Billotet et al incubated primary AML cells with IC87114 (1-5μM) together with low dose VP16 (0.5μM) and carried out an MTS assay at 72hours. They found an additive interaction between IC87114 and VP16 in 6 patients and a synergistic interaction in 8 patients. These results suggest that a more selective inhibition of just the p110δ isoform can also potentiate chemotherapeutic effect in primary AML cells.

5.8 The effect of activating Akt on chemotherapeutic effect

The results with primary AML cells indicate that PI3-Kinase is involved in basal cell survival and implicated in resistance to chemotherapy induced apoptosis. PI3-Kinase has a number of downstream targets of which Akt is one of the most important in survival signalling. To investigate if activation of Akt alone is sufficient to protect haematopoietic cells from cytotoxic effects of chemotherapy, an inducible activated Akt vector system was used. Myristolation of Akt targets it to the membrane, making it constitutively active. By fusing myristolated Akt to a mutant murine estrogen receptor hormone binding domain (mAkt-ER), the activation can be turned on and off by incubation with tamoxifen. A construct in which the membrane targeting ability of the myristolation sequence was abrogated by mutation of glycine to alanine at the second amino acid position, was used as a control (A2 mAkt-ER). [82] An IL-3 dependent murine line, 32D, was used to stably express these constructs. The cells were incubated overnight with or without tamoxifen (0.5μM), in the presence of murine IL-3. To demonstrate the activation of Akt on incubation with tamoxifen in the mAkt-ER but not the A2mAkt-ER expressing cells, total cell lysates were made and probed with phospho Akt (**Figure 5.10**).

Figure 5.10

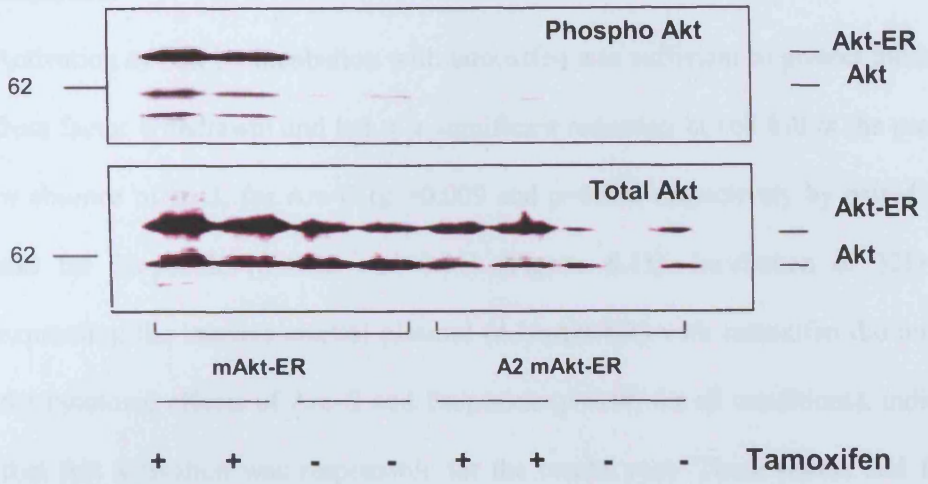


Figure 5.10 The effect of tamoxifen on Akt activation in mAkt-ER expressing cells.

32D cells stably expressing either the active mAkt-ER or control A2 mAkt-ER construct were incubated with or without tamoxifen (0.5μM) overnight. Western blot analysis illustrating that incubation with tamoxifen led to phosphorylation of mAkt in the mAkt-ER cells but not the A2 mAkt-ER expressing cells. The blot was then reprobed with total Akt to check for protein loading.

To assess the effect of Akt activation on chemotherapeutic effect the cells were incubated overnight with or without tamoxifen (0.5μM), in the presence of murine IL-3 (mIL-3) 10ng/ml The following day, the cells were washed and resuspended, at a concentration of $4x10^5$/ml, in RPMI/10% FCS with or without 10ng/ml mIL-3, in the presence or absence of tamoxifen. Ara-C (0-1μg/ml) or etoposide (0-50μM) was then added, mixed and incubated for 48 hours, when an MTS assay was carried out.

Results were normalised to the 10ng/ml mIL-3 cells (no cytotoxics) with or without tamoxifen.

Activation of Akt by incubation with tamoxifen was sufficient to protect these cells from factor withdrawal and led to a significant reduction in cell kill in the presence or absence of IL-3, for Ara-C ($p = 0.009$ and $p = 0.002$ respectively by paired t test) and for Etoposide ($p = 0.05$ and 0.01) (**Figure 5.11**). Incubation of 32D cells expressing the inactive control plasmid (A2mAkt-ER) with tamoxifen did not alter the cytotoxic effects of Ara-C and Etoposide ($p > 0.05$ for all conditions), indicating that Akt activation was responsible for the results seen. These results add further evidence to suggest that Akt and the pathways that it regulates are involved in chemoresistance in haematopoietic cells.

Figure 5.11

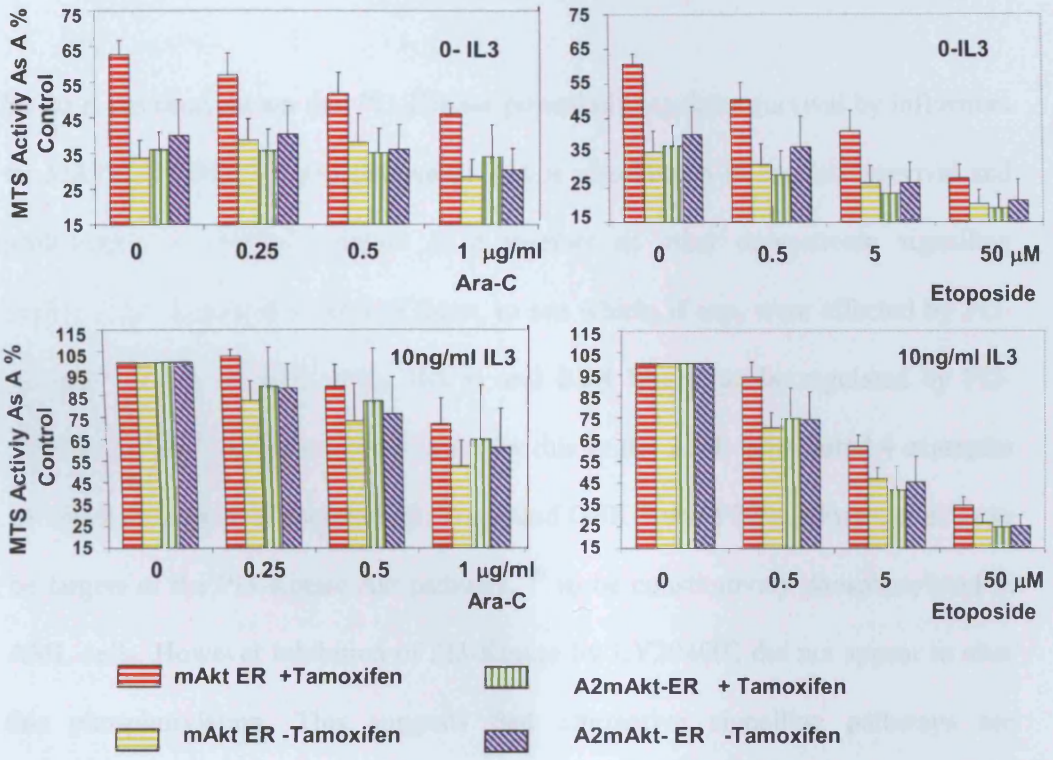


Figure 5.11 The effect of Akt activation on chemotherapeutic effect in a myeloid cell line.

Cells were incubated overnight with or without tamoxifen. The following day they were washed and resuspended in RPMI 10% FCS with or without murine IL-3 10ng/ml, in the presence or absence of tamoxifen. Ara-C (0-1µg/ml) or Etoposide (0-50µM) were added and the cells incubated at 37°C. An MTS assay was carried out at 48 hours. Results were normalised to cells stimulated with 10ng/ml IL-3, in the absence of cytotoxics.

5.9 The effect of PI3-Kinase blockade on other downstream signalling pathways

So far it has been shown that PI3-Kinase potentially regulates survival by influences on MAPK, NF-kB and p53 pathways. Akt is also known to regulate survival and proliferation by phosphorylation of a number of other downstream signalling proteins. We looked at several of these, to see which, if any, were affected by PI3-Kinase blockade in AML cells. Bcl xl and BIM levels can be regulated by PI3-Kinase, [28,134] but we found no evidence for this in the AML cells tested 4 examples of which are shown in **Figure 5.12**. We found GSK 3 and P70S6 Kinase, which can be targets of the PI3-Kinase/Akt pathway, [28] to be constitutively phosphorylated in AML cells. However inhibition of PI3-Kinase by LY294002 did not appear to alter this phosphorylation. This suggests that alternative signalling pathways are responsible for this phosphorylation and we have found this to be the case in several leukaemic lines tested (data not shown). In some AML cases constitutive phosphorylation of the Forkhead family member FKHRL1 was detected and found to be regulated in a PI3-Kinase dependent manner. Addition of LY294002 appeared to have no significant effect on the total levels of the cell cycle regulators p21 and p27.

Figure 5.12

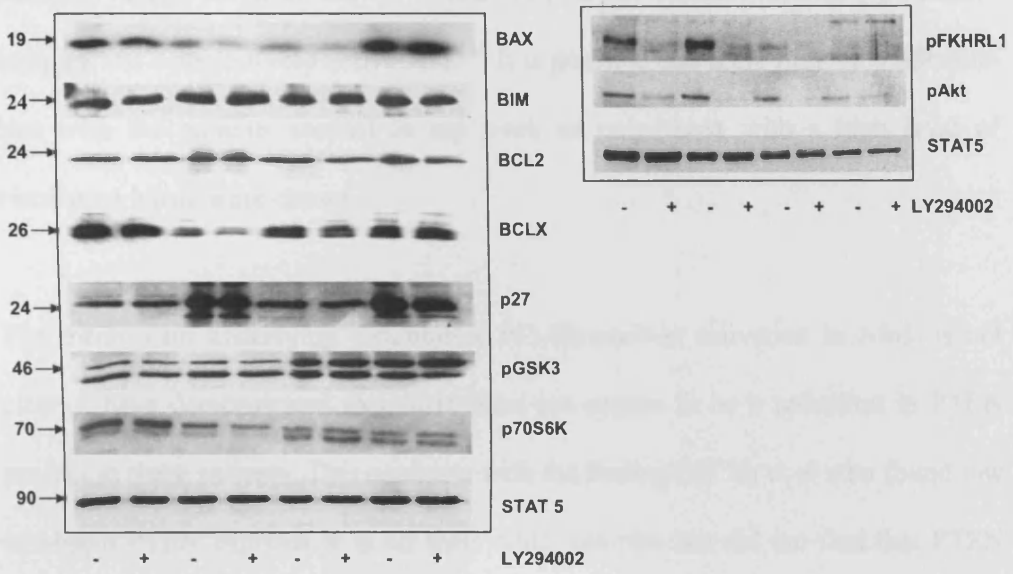


Figure 5.12 The effect of PI3-Kinase blockade on other downstream signalling pathways.

Primary AML cells were incubated with and with out LY294002 25μM overnight and total cell lysates made. $4x10^5$ cell equivalents were resolved by SDS-PAGE. The blot was probed with primary antibody annotated on the right and re-probed with total stat 5 to assess protein loading (4 examples shown).

5.10 Discussion

I have found that the PI3-Kinase pathway is activated in all the AML samples I tested. This is demonstrated by the presence of phosphorylated Akt ser473 on western blotting which was reduced or abolished when the cells were incubated with the selective PI3-Kinase inhibitor, LY294002. Previous studies in AML have shown

PI3-Kinase/Akt activation. Min et al reported that 72% of their samples showed phosphorylation of Akt at ser473 [124] and Xu et al showed that most of their patient samples had constitutively active Akt.[125] It is possible that there may be a selection bias with the patients studied in my work as only those with a high level of circulating blasts were chosen.

The mechanism underlying constitutive PI3-Kinase/Akt activation in AML is not clear. I have demonstrated that there does not appear to be a reduction in PTEN protein in these patients. This contrasts with the findings of Xu et al who found low or absent PTEN expression in all their AML samples but did not find that PTEN expression correlated with Akt phosphorylation. [125] Of the patients I examined, 3/18 had FLT3 ITDs and 5 had *N-ras* mutations (exon 1 and 2). One patient had both FLT3 ITD and *N-ras* mutations and no patient had a D835 FLT3 mutation. In this series there was no clear difference in the level of Akt activation between patients with or without mutations. These experiments were carried out without exogenous cytokines, but autocrine cytokine production is another possible mechanism of PI3-Kinase activation. [135,136]

It appears that PI3-Kinase activation is important for leukaemic cell survival, as incubation with LY294002 resulted in a reduction in cell number related to an increase in apoptosis. The MAPK pathway can also regulate cell survival and was found to be activated in these samples. When this pathway was inhibited, there was an increase in apoptosis, but to a lesser extent than that seen with PI3-Kinase

blockade. Milella et al found constitutive activation of MAPK in 74% of their patients with AML and showed that MEK inhibition led to a net increase in apoptosis of 13% (10-23%), [137] this is similar to the data presented here (12% increase). I demonstrated that PI3-Kinase blockade brings about a reduction in MAPK activation in 10/18 patients, suggesting that in AML, PI3-Kinase can act upstream of MAPK. Although MAPK can be regulated by PI3-Kinase, this is unlikely to be the major anti-apoptotic pathway in AML because the reduction in cell number after MAPK inhibition was considerably less than after PI3-Kinase blockade (23% v 51% respectively) and PI3-Kinase blockade had effects in cells where it was not involved in MAPK activation. Several possible mechanisms have been described for PI3-Kinase regulation of MAPK. Yart et al have shown that in EGF signalling, there is a PI3-Kinase dependent pathway for MAPK activation involving SHP2, GAB1, Ras and thus Raf/MEK/ERK. [138] MAPK activation by IL-3 in BAF/3 cells appears to be under PI3-Kinase regulation due to influences on SHP2 and GAB2 phosphorylation. [138] Another possible mechanism involves PKC activation by PDK1, a PI3-Kinase target, and PKC activation of MAPK. [139,140] In addition, PI3-Kinase activation of Rac, a member of the Rho family of GTPases, and its effector Pak (p21 activating kinase) has been described which can activate Raf [141] and facilitate MEK/ERK association and ERK activation.[142] It is not evident at present which, if any, of these mechanisms is relevant in AML.

AML stem cells are thought to reside in the CD34+/CD38- compartment of leukaemic blasts.[143] Inhibition of PI3-Kinase in leukaemic stem cells led to a similar

fall in cell viability as blockade in the bulk population, suggesting that this pathway is equally necessary for survival in both populations. This concurs with the findings of Xu et al who treated leukaemic cells with LY294002 for 16 hours prior to injecting them into NOD/SCID mice. They found reduced engraftment after LY294002 treatment compared to control cells. In contrast to the result with PI3-Kinase blockade, inhibition of the MAPK pathway appears to have a more prominent effect on the bulk population compared to the stem cell subset, implying that this pathway may not be as important for survival in the leukaemic stem cell population. These results should be interpreted with caution however, as only a small number of patient samples have been examined.

Having found that PI3-Kinase/Akt activation was important for AML cell survival I went on to investigate downstream effector molecules that could mediate this process. Akt has been reported to regulate several proteins involved in the control of apoptosis, for example by phosphorylating serine 136 of the Bad protein leading to reduced binding to Bcl 2/Bcl X_L and increased cell survival.[144] Phosphorylated Bad was not detected in my AML samples (Data not shown). This contrasts with the findings of Zhao et al, who found constitutive BAD phosphorylation in primary AML cells. [126] Akt is also able to promote cell survival by inhibition of FKHRL1, a member of the Forkhead family of transcription factors. In some AML cases studied constitutive phosphorylation of the Forkhead family member FKHRL1 was detected and found to be regulated in a PI3-Kinase dependent manner. PI3-Kinase/Akt may also regulate apoptosis by upregulation of Bcl X mRNA in response to IL-3 and

IGF-1, leading to increased expression of the anti-apoptotic factor.[134] No alteration in Bcl X levels was found on incubation with LY294002 in the cases studied here.

These findings suggest that alternative targets of PI3-Kinase/Akt may be responsible for regulating survival in AML. Therefore further investigation of other mediators of PI3-Kinase/Akt survival signals was necessary. Constitutive activation of NF-kB was found in AML cells using a novel transfection technique (Nucleofection) and reporter assay.[129] Inhibition of the PI3-Kinase pathway led to a reduction in constitutive NF-kB activity in the majority of the AML samples tested, suggesting that PI3-Kinase/Akt can stimulate signalling pathways that upregulate the activity of NF-kB. This transcription factor is a heterodimeric protein composed of p50 and p65 subunits which is regulated by the I kappa B (IkB) family of proteins that restrain NF-kB in the cytoplasm. Following cellular stimulation, IkB becomes phosphorylated by IkB kinases, leading to its ubiquitination and degradation allowing NF-kB to translocate to the nucleus and regulate transcription. Several groups have reported that PI3-Kinase and Akt are involved in the activation of NF-kB, which may be mediated by phosphorylation and activation of IKKs or by up regulation of the transcriptional activity through the transactivation domain of the p65 subunit. [145-147] Inhibition of endogenous Akt activity can result in the loss of H-*ras* induced NF-kB transcriptional activity and sensitisation of fibroblast cells to apoptosis. [145,148] There have been many reports of constitutively active NF-kB in malignancy.[149-152] My findings are consistent with those of Guzman et al who found NF-kB activation in all the AML specimens they examined (n=11). NF-kB was

constitutively active in leukaemic (CD34+, CD38-, CD123+) but not normal stem cells. Leukaemic stem cells underwent apoptosis with the proteasomal inhibitor MG132 (which reduces NF-kB activity via increasing IkB) whereas normal stem cells did not.[129]

I have also shown that inhibition of PI3-Kinase/Akt leads to an increase in constitutive p53 activity by reporter assay in primary cells. Genes trans-activated by p53 can either induce cell cycle arrest or promote apoptosis. Low p53 activity due to mutation can lead to aggressive tumour behaviour and reduced therapeutic sensitivity but mutations are uncommon in AML (5-15%).[153-155] p53 normally has a very short half life because of rapid binding to mdm2, ubiquitination, and degradation. [130] Elevated mdm2 levels can cause genomic instability by inactivating DNA damage responses and have been found in multiple human malignancies. [156] Phosphorylation of mdm2 by Akt allows mdm2 to translocate to the nucleus, bind p53, and target it for degradation. Downregulation would be expected to enhance the p53 response by shifting the balance from degradation to stabilisation. This could be a pathway for overactive PI3-Kinase to promote resistance to cytotoxic agents - blockade of PI3-Kinase/Akt could lead to increased chemosensitivity by increasing p53 levels. Regulation of both NF-kB and p53 by PI3-Kinase/Akt in AML cells could reduce the apoptotic response to cytotoxic chemotherapy. My data showing that PI3-Kinase blockade appears to be synergistic with Ara-C supports this possibility. Madrid et al have shown that Akt induced activation of NF-kB was able to protect Akt expressing cells from etoposide induced apoptosis.[145] Other groups

have shown that PI3-Kinase blockade potentiates the response to several chemotherapeutic agents including paclitaxel, vincristine, doxorubicin, trastuzumab, and etoposide. [131-133]

PI3-Kinase has several downstream effectors which could be responsible for the reduction in chemotherapy induced apoptosis in AML cells. Using an inducible myristolated Akt construct stably expressed in 32D cells I have shown that activation of Akt alone is sufficient to protect these factor dependent cells from cytokine withdrawal induced apoptosis. This is consistent with the finding that PI3-Kinase blockade in primary AML cells leads to a reduction in basal survival. Activation of Akt also protects 32D cells from the cytotoxic effect of both Ara-C and etoposide. This was seen even in the presence of maximal concentrations of IL-3 (10ng/ml) which activate PI3-Kinase/Akt. However, in contrast to the transient Akt activation seen following IL-3 stimulation, with a return to baseline after 2-3 hours, (Data not shown), the 32D cells expressing the inducible activated Akt-ER construct have sustained Akt activation. This indicates that persistent Akt activity, as is seen in the primary AML cells, has an enhanced anti-apoptotic effect and may make a significant contribution to chemoresistance.

Figure 5.13

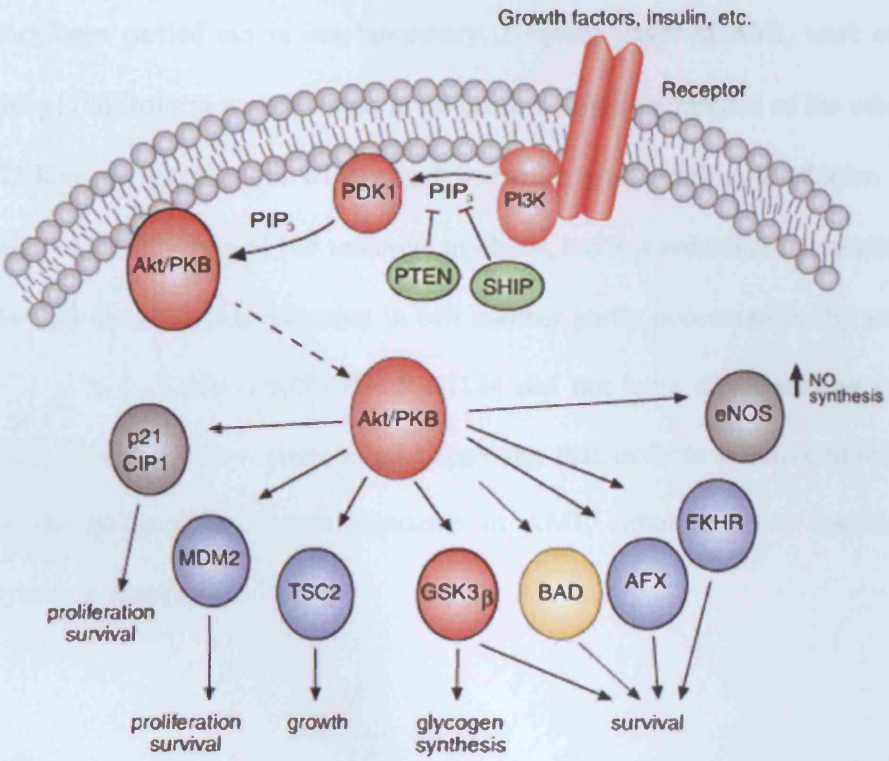


Figure 5. 13 PI3-Kinase activity is involved in many cell functions

5.11 Conclusions

In conclusion, we have found that the PI3-Kinase pathway is activated in all the AML samples we tested. We have shown that inhibition of this pathway leads to an increase in apoptosis in the leukaemic stem cell pool, unlike MAPK blockade, and that it potentiates the response to cytotoxic chemotherapy. We have demonstrated that PI3-Kinase/Akt positively regulates the anti apoptotic NF-kB pathway and negatively regulates the P53 pathway in AML blasts. These results indicate the

potential for PI3-Kinase inhibition in the treatment of AML. Further work investigating the role of the different class I isoforms of PI3-Kinase in AML has since been carried out in our laboratory. Fourteen cases of AML were examined. The p110δ isoform was detected in all cases whereas expression of the other class I PI3-Kinases varied more widely and was often undetectable. Incubation of AML cells with IC87114, a p110δ selective inhibitor, led to a reduction in constitutive Akt phosphorylation and a reduction in cell number partly accounted for by an increase in apoptosis. Unlike LY294002, IC87114 did not have direct adverse effects on normal haematopoietic progenitors suggesting that isoform selective inhibitors may be the preferred therapeutic approach in AML either alone or combined with cytotoxic therapy.

CHAPTER 6 CONCLUSIONS

6.1 JAK/STAT Pathway

I have shown that Gö6976 is a potent inhibitor of JAK 2 in in vitro kinase assays and also in whole cell systems, where it inhibits signaling downstream of multiple JAK2 coupled cytokines including IL-3, GM-CSF and EPO. Gö6976 is also a potent inhibitor of JAK 3 but its effects on JAK1 and Tyk 2 are less clear. Further work would include delineation of the effect of Gö6976 on these kinases using in vitro kinase and cell based systems.

Gö6976 was also found to inhibit signalling downstream of disease-associated forms of JAK2 such as JAK2 constitutively activated by fusion with TEL and mutant JAK2 V617F. Investigation of several haematopoietic cell lines reported to have some dysregulation of the JAK/STAT pathway, revealed that Gö6976 was able to inhibit proliferation in 2/2 ALCL lines (Karpas 299, SUDHL1), 2/4 acute myeloid leukaemia lines (Kg1a, U937) and 1 CML line (K562) but had no effect on any of the 4 MM lines examined.

I found that the majority of primary AML cells investigated had constitutive STAT activation. This was reduced by incubation with Gö6976 in 23 of 24 cases. In 12 out of 24 patients inhibition of JAKs by Gö6976 led to reduced activation of all three

pathways (MAPK, PI3K, STAT) suggesting that dysregulation of the JAK pathway was one of the main aberrant signalling events in these patients. I have also shown that Gö6976 is an active inhibitor of survival and proliferation in AML blasts.

This work leads to the proposal that JAK2 inhibition would be a possible therapeutic strategy in AML. However, given that JAK2 is utilised by the major haematopoietic cytokines any JAK2 inhibitor could potentially have severe adverse effects on normal haematopoiesis. Some initial work carried out in our laboratory supports this in that Gö6976 appears to reduce colony numbers in a colony assay. However, it is possible that the phenomenon of oncogene addiction may lead to a therapeutic window narrow enough for AML cells to be susceptible to inhibition without toxicity to normal haematopoiesis. In fact Meydan et al reported that AG490, a tyrphostin JAK2 inhibitor, selectively blocked leukaemic cell growth in vitro and in vivo by inducing programmed cell death, with no deleterious effect on normal haematopoiesis. [54] CP-690-550 is a potent JAK3 inhibitor. It has an IC50 of 1nM for JAK3 but also inhibits JAK2, although it is 20 times less potent, with an IC50 of 20nM. It has been used in various murine models to prevent rejection of organ allografts. Dose limiting anaemia, presumed to be due to JAK2 inhibition was only seen in the higher dosing groups and the animals in the four fold lower dosing groups only had minor decreases in haemoglobin which recovered to baseline values. [157] This suggests that it may be possible to develop therapeutically useful JAK2 inhibitors.

6.2 PI3-Kinase Pathway

I have found that the PI3-Kinase pathway is activated in AML as demonstrated by the presence of phosphorylated Akt ser473 on western blotting. This was reduced or abolished when the cells were incubated with the PI3-Kinase inhibitor, LY294002. The mechanism underlying constitutive PI3-Kinase/Akt activation in AML is not clear but did not appear to be directly related to PTEN expression or the presence of FLT3 or Ras mutations. It appears that PI3-Kinase activation is important for leukaemic cell survival, as incubation with LY294002 resulted in a reduction in cell number related to an increase in apoptosis. This is also true for the leukaemic stem cell population. MAPK inhibition appeared to have a more prominent effect on the bulk population compared to the stem cell subset thus implying that this pathway may not be as important for survival in the leukaemic stem cell population.

The search for downstream effector molecules of the PI3-Kinase pathway in AML led me to investigate the role of NF-kB in AML cell survival. Inhibition of the PI3-Kinase pathway led to a reduction in constitutive NF-kB activity in the majority of the AML samples tested, suggesting that PI3-Kinase/Akt can stimulate signalling pathways that upregulate the activity of NF-kB. I have also shown that inhibition of PI3-Kinase/Akt leads to an increase in constitutive p53 activity in primary AML cells.

From the work with 32D cells stably expressing an activated AKT-ER construct it appears that activation of Akt alone is sufficient to protect factor dependent cells from cytokine withdrawal induced apoptosis and also from the cytotoxic effects of Ara-C and Etoposide. This Akt activation is persistent and mirrors the activity seen in AML cells, suggesting that activation of the PI3-Kinase pathway may contribute to chemoresistance.

The specific mechanism for PI3-Kinase activation in AML will differ between patients. It may result from mutations of receptor tyrosine kinases, such as FLT3, or of the Ras proto-oncogene or be the result of chromosomal translocations leading to the creation of aberrant kinase activity. Small molecule signalling pathway inhibitors are increasingly being investigated for use in acute leukaemia. One approach to therapy has been to try and find selective inhibitors to particular aberrant pathways such as farnesyl transferase inhibitors directed at the Ras/MEK/ERK pathway or FLT3 inhibitors. Theoretically this could be advantageous by reducing the likelihood of excessive non-specific toxicity. However it seems unlikely, especially in the case of acute leukaemia, that the malignancy would be dependent on just one pathway for its growth and survival. It also seems likely that there would be a significant level of cross talk between molecules within different signaling pathways. Another approach would be to use less selective kinase inhibitors and target several pathways simultaneously. This has been successfully used in the case of Imatinib for the treatment of gastrointestinal stromal tumours where response is thought to be due to the inhibition of KIT and PDGFR. The draw back for some agents relates to the

differential in IC50's for each target. This can sometimes vary by 10-100 fold and so it may not be possible to inhibit both targets safely in vivo. Inhibition of so called 'nodal' pathways, such as the PI3-Kinase pathway is a further option. These pathways are common to many activating events and may therefore be applicable to more tumours whilst avoiding the problems associated with combining several agents or using multitargeted drugs. Billotet at al have shown that the leucocyte restricted isoform, p110δ, of PI3-Kinase appears to be expressed at higher levels than the other class 1 PI3-Kinases in acute myeloid leukaemia. It is also susceptible to inhibition with an isoform selective compound, IC87114, leading to an increase in apoptosis and reduction in proliferation of primary AML cells. Thus the development of isoform specific inhibitors may have the advantage of inhibiting a nodal pathway without the toxicity related to pan PI3-Kinase inhibition.

The development of specific small molecule inhibitors of tyrosine kinases such as Imatinib is a landmark in anticancer therapy. However in contrast to CML, deregulated receptor tyrosine kinases are only one of several targets involved in the pathophysiology of AML. As discussed in chapter 1.8 class I mutations including fusion tyrosine kinases and activating mutations of receptor tyrosine kinases provide a proliferative advantage whereas class II mutations impair cellular differentiation. In addition other chromosomal abnormalities may cooperate in leukaemogenesis. The complex nature of these changes may explain the poor efficacy of some tyrosine kinase inhibitors in AML so far. As increasing numbers of deregulated enzymes are identified in AML, so this therapeutic challenge will intensify. It seems likely that

future therapeutic regimens in AML will consist of rationally designed combinations of specific agents with or without classical chemotherapy which will hopefully lead to an improvement in overall outcome.

6.3 Future Directions

I have shown that the majority of cases of primary AML exhibit constitutive STAT phosphorylation indicating activation of the JAK/STAT pathway. The mechanisms resulting in this activation are not clear. The somatic JAK2 V617F mutation has been reported in a small number of de novo AML cases although it has been found to be present in a larger number of cases secondary to myeloproliferative disorders[115]. Another novel JAK2 mutation has been described in acute megakaryoblastic cell lines resulting from a threonine to aspargine switch at position 875. this also results in aa contitutively activated JAK2[114]. Several gain of function mutations in JAK3 have also been described in acute megakaryoblastic cells lines [158]. In the light of these discoveries further mutational analysis of JAKs 1,2,3 and Tyk2 to locate other novel activating mutations would be a valid approach to further clarifying this situation.Autocrine/paracrine production of growth factors such as IL-6 and GMCSF is well described in AML and may result in JAK/STAT activation. Therefore the use of neutralizing monoclonal antibodies to block these signals would further elucidate what contribution autocrine/paracrine cytokine production played in these cases.

It is important to fully characterize the spectrum of Gö6976 activity. Although the anti-JAK2 and 3 activity has been clearly demonstrated, the effect of Gö6976 on JAK1 and Tyk2 has not. This could be investigated by utilising both in vitro kinase assays and whole cell systems.

Further characterization of the effects of Gö6976 on normal progenitor cell growth and engraftment is needed to help to clarify if there is preferential activity against AML cells. This could be achieved by using CD34 initiated colony assays, long-term cultures (LT-CIC) and looking at effects on engraftment in NOD SCID mice.

In clinical practice it is likely that small molecule inhibitors of JAK2 will be used in combination with classical chemotherapeutic agents. Therefore the effect of blocking the JAK/STAT pathway on sensitivity to central cytotoxic components of AML treatment regimens should be examined. This work could then be further developed by investigating the effect of Gö6976 on leukaemic stem cell engraftment in NOD SCID mice alone and in combination with chemotherapeutic agents.

Constitutive PI3-Kinase activation is frequently found in AML although the reasons for this are less clear. PIK3CA mutations have been described in other malignancies but have been rarely described in AML. I have screened 92 AML samples and found no PIK3CA mutations - however other PI3-Kinase isoforms, in particular PI3KCD, have not been extensively examined and therefore mutational analysis of would be crucial in furthering our current knowledge. The results of this analysis would guide

us in the trial of isoform specific inhibitors on proliferation and apoptosis in AML cases. The logical extension of this work would be to investigate the effect of PI3-Kinase isoform inhibitors on the ability of leukaemic progenitors to engraft NOD SCID mice.

APPENDIX 1

Amino Acid abbreviations

A	**ala**	**alanine**	**M**	**met**	**methionine**
C	**cys**	**cysteine**	**N**	**asn**	**asparagine**
D	**asp**	**aspartate**	**P**	**pro**	**proline**
E	**glu**	**glutamate**	**Q**	**gln**	**glutamine**
F	**phe**	**phenylalanine**	**R**	**arg**	**arginine**
G	**gly**	**glycine**	**S**	**ser**	**serine**
H	**his**	**histidine**	**T**	**thr**	**threonine**
I	**ile**	**isoleucine**	**V**	**val**	**valine**
K	**lys**	**lysine**	**W**	**trp**	**tryptophan**
L	**leu**	**leucine**	**Y**	**tyr**	**tyrosine**

REFERENCE LIST

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

(2) **Enver T, Heyworth CM, Dexter TM. Do stem cells play dice?**
Blood. 1998;92:348-351.

(3) **Lu X, Gross AW, Lodish HF. Active conformation of the erythropoietin receptor: random and cysteine-scanning mutagenesis of the extracellular juxtamembrane and transmembrane domains**
J Biol Chem. 2006;281:7002-7011.

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

(5) **Ihle JN. The Janus protein tyrosine kinases in hematopoietic cytokine signaling**
Semin Immunol. 1995;7:247-254.

(6) **Jiang N, He TC, Miyajima A, Wojchowski DM. The box1 domain of the erythropoietin receptor specifies Janus kinase 2 activation and functions mitogenically within an interleukin 2 beta-receptor chimera**
J Biol Chem. 1996;271:16472-16476.

(7) **Aringer M, Cheng A, Nelson JW et al. Janus kinases and their role in growth and disease**
Life Sci. 1999;64:2173-2186.

(8) **Feng J, Witthuhn BA, Matsuda T et al. Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop**
Mol Cell Biol. 1997;17:2497-2501.

(9) **Saharinen P, Takaluoma K, Silvennoinen O. Regulation of the Jak2 tyrosine kinase by its pseudokinase domain**
Mol Cell Biol. 2000;20:3387-3395.

(10) **Zhou YJ, Hanson EP, Chen YQ et al. Distinct tyrosine phosphorylation sites in JAK3 kinase domain positively and negatively regulate its enzymatic activity**
Proc Natl Acad Sci U S A. 1997;94:13850-13855.

(11) **Argetsinger LS, Kouadio JL, Steen H et al. Autophosphorylation of JAK2 on tyrosines 221 and 570 regulates its activity**
Mol Cell Biol. 2004;24:4955-4967.

(12) **Kisseleva T, Bhattacharya S, Braunstein J, Schindler CW. Signaling through the JAK/STAT pathway, recent advances and future challenges.**
Gene. 2002;285:1-24.

(13) **Zhou YJ, Chen M, Cusack NA et al. Unexpected effects of FERM domain mutations on catalytic activity of Jak3: structural implication for Janus kinases**
Mol Cell. 2001;8:959-969.

(14) **O'Shea JJ, Gadina M, Schreiber RD. Cytokine signaling in 2002: new surprises in the Jak/Stat pathway.** Cell. 2002;109 Suppl:S121-S131.

(15) **Vinkemeier U, Moarefi I, Darnell JE, Jr., Kuriyan J. Structure of the amino-terminal protein interaction domain of STAT-4**
Science. 1998;279:1048-1052.

(16) **Decker T, Kovarik P. Serine phosphorylation of STATs**
Oncogene. 2000;19:2628-2637.

(17) **Chen X, Vinkemeier U, Zhao Y et al. Crystal structure of a tyrosine phosphorylated STAT-1 dimer bound to DNA**
Cell. 1998;93:827-839.

(18) **Ward AC, Touw I, Yoshimura A. The Jak-Stat pathway in normal and perturbed hematopoiesis**
Blood. 2000;95:19-29.

(19) **de la CA, Traskelin AL, Juvonen E. Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis**
Proc Natl Acad Sci U S A. 1993;90:4495-4499.

(20) **Valentino L, Pierre J. JAK/STAT signal transduction: regulators and implication in hematological malignancies**
Biochem Pharmacol. 2006;71:713-721.

(21) **Alexander DR. Kinase regulation: competing phosphatases in JAK dephosphorylation**
Curr Biol. 2002;12:R288-R290.

(22) **Ilangumaran S, Ramanathan S, Rottapel R. Regulation of the immune system by SOCS family adaptor proteins**
Semin Immunol. 2004;16:351-365.

(23) **Starr R, Willson TA, Viney EM et al. A family of cytokine-inducible inhibitors of signalling**
Nature. 1997;387:917-921.

(24) **Guiter C, Dusanter-Fourt I, Copie-Bergman C et al. Constitutive STAT6 activation in primary mediastinal large B-cell lymphoma**
Blood. 2004;104:543-549.

(25) **Melzner I, Bucur AJ, Bruderlein S et al. Biallelic mutation of SOCS-1 impairs JAK2 degradation and sustains phospho-JAK2 action in the MedB-1 mediastinal lymphoma line**
Blood. 2005;105:2535-2542.

(26) **Okkenhaug K, Vanhaesebroeck B. PI3K in lymphocyte development, differentiation and activation**
Nat Rev Immunol. 2003;3:317-330.

(27) **Anderson KE, Jackson SP. Class I phosphoinositide 3-kinases**
Int J Biochem Cell Biol. 2003;35:1028-1033.

(28) **Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer**
Nat Rev Cancer. 2002;2:489-501.

(29) **Dong LQ, Liu F. PDK2: the missing piece in the receptor tyrosine kinase signaling pathway puzzle**
Am J Physiol Endocrinol Metab. 2005;289:E187-E196.

(30) **Bader AG, Kang S, Vogt PK. Cancer-specific mutations in PIK3CA are oncogenic in vivo**
Proc Natl Acad Sci U S A. 2006;103:1475-1479.

(31) **Lee JW, Soung YH, Kim SY et al. PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas**
Oncogene. 2005;24:1477-1480.

(32) **Sakai A, Thieblemont C, Wellmann A, Jaffe ES, Raffeld M. PTEN gene alterations in lymphoid neoplasms**
Blood. 1998;92:3410-3415.

(33) **Gronbaek K, Zeuthen J, Guldberg P, Ralfkiaer E, Hou-Jensen K. Alterations of the MMAC1/PTEN gene in lymphoid malignancies**
Blood. 1998;91:4388-4390.

(34) **Dahia PL, Aguiar RC, Alberta J et al. PTEN is inversely correlated with the cell survival factor Akt/PKB and is inactivated via multiple mechanisms in haematological malignancies** Hum Mol Genet. 1999;8:185-193

(35) **Blume-Jensen P, Hunter T. Oncogenic kinase signalling**
Nature. 2001;411:355-365.

(36) **Staal SP. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma**
Proc Natl Acad Sci U S A. 1987;84:5034-5037.

(37) **Cheng JQ, Godwin AK, Bellacosa A et al. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas.** Proc Natl Acad Sci U S A. 1992;89:9267-9271.

(38) **Cheng JQ, Ruggeri B, Klein WM et al. Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA.** Proc Natl Acad Sci U S A. 1996;93:3636-3641.

(39) **Chang F, Steelman LS, Lee JT et al. Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: potential targeting for therapeutic intervention**
Leukemia. 2003;17:1263-1293.

(40) **Stirewalt DL, Meshinchi S, Radich JP. Molecular targets in acute myelogenous leukemia.** Blood Rev. 2003;17:15-23.

(41) **Morgan MA, Reuter CW. Molecularly targeted therapies in myelodysplastic syndromes and acute myeloid leukemias**
Ann Hematol. 2006;85:139-163.

(42) **Haluska FG, Tsao H, Wu H et al. Genetic alterations in signaling pathways in melanoma**
Clin Cancer Res. 2006;12:2301s-2307s.

(43) **Schindler T, Bornmann W, Pellicena P et al. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase**
Science. 2000;289:1938-1942.

(44) **Schnittger S, Kohl TM, Haferlach T et al. KIT-D816 mutations in AML1-ETO-positive AML are associated with impaired event-free and overall survival**
Blood. 2006;107:1791-1799.

(45) **Shah NP, Tran C, Lee FY et al. Overriding imatinib resistance with a novel ABL kinase inhibitor**
Science. 2004;305:399-401.

(46) **Jorgensen HG, Allan EK, Jordanides NE, Mountford JC, Holyoake TL. Nilotinib exerts equipotent antiproliferative effects to imatinib and does not induce apoptosis in CD34+ CML cells**
Blood. 2007;109:4016-4019.

(47) **Blagosklonny MV. Gefitinib (iressa) in oncogene-addictive cancers and therapy for common cancers**
Cancer Biol Ther. 2004;3:436-440.

(48) **Litzow MR. Imatinib resistance: obstacles and opportunities**
Arch Pathol Lab Med. 2006;130:669-679.

(49) **White DL, Saunders VA, Dang P et al. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib**
Blood. 2006;108:697-704.

(50) **Graham SM, Jorgensen HG, Allan E et al. Primitive, quiescent, Philadelphia-positive stem cells from patients with chronic myeloid leukemia are insensitive to STI571 in vitro**
Blood. 2002;99:319-325.

(51) **Bhatia R, Holtz M, Niu N et al. Persistence of malignant hematopoietic progenitors in chronic myelogenous leukemia patients in complete cytogenetic remission following imatinib mesylate treatment**
Blood. 2003;101:4701-4707.

(52) **Copland M, Hamilton A, Elrick LJ et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction**
Blood. 2006;107:4532-4539.

(53) **Jorgensen HG, Allan EK, Graham SM et al. Lonafarnib reduces the resistance of primitive quiescent CML cells to imatinib mesylate in vitro**
Leukemia. 2005;19:1184-1191.

(54) **Meydan N, Grunberger T, Dadi H et al. Inhibition of acute lymphoblastic leukaemia by a Jak-2 inhibitor**
Nature. 1996;379:645-648.

(55) **De Vos J, Jourdan M, Tarte K, Jasmin C, Klein B. JAK2 tyrosine kinase inhibitor tyrphostin AG490 downregulates the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription (STAT) pathways and induces apoptosis in myeloma cells**
Br J Haematol. 2000;109:823-828.

(56) **Burdelya L, Catlett-Falcone R, Levitzki A et al. Combination therapy with AG-490 and interleukin 12 achieves greater antitumor effects than either agent alone.** Mol Cancer Ther. 2002;1:893-899.

(57) **Wang LH, Kirken RA, Erwin RA, Yu CR, Farrar WL. JAK3, STAT, and MAPK signaling pathways as novel molecular targets for the tyrphostin AG-490 regulation of IL-2-mediated T cell response**
J Immunol. 1999;162:3897-3904.

(58) **Levitzki A. Tyrosine kinases as targets for cancer therapy**
Eur J Cancer. 2002;38 Suppl 5:S11-S18.

(59) **Kleinberger-Doron N, Shelah N, Capone R, Gazit A, Levitzki A. Inhibition of Cdk2 activation by selected tyrphostins causes cell cycle arrest at late G1 and S phase**
Exp Cell Res. 1998;241:340-351.

(60) **Thompson JE, Cubbon RM, Cummings RT et al. Photochemical preparation of a pyridone containing tetracycle: a Jak protein kinase inhibitor**
Bioorg Med Chem Lett. 2002;12:1219-1223.

(61) **Lucet IS, Fantino E, Styles M et al. The structural basis of Janus kinase 2 inhibition by a potent and specific pan-Janus kinase inhibitor**
Blood. 2006;107:176-183.

(62) **O'Shea JJ, Pesu M, Borie DC, Changelian PS. A new modality for immunosuppression: targeting the JAK/STAT pathway**
Nat Rev Drug Discov. 2004;3:555-564.

(63) **Uckun FM, Sudbeck EA, Mao C et al. Structure-based design of novel anticancer agents**
Curr Cancer Drug Targets. 2001;1:59-71.

(64) **Stepkowski SM, Erwin-Cohen RA, Behbod F et al. Selective inhibitor of Janus tyrosine kinase 3, PNU156804, prolongs allograft survival and acts synergistically with cyclosporine but additively with rapamycin**
Blood. 2002;99:680-689.

(65) **Grimwade D, Walker H, Harrison G et al. The predictive value of hierarchical cytogenetic classification in older adults with acute myeloid leukemia (AML): analysis of 1065 patients entered into the United Kingdom Medical Research Council AML11 trial**
Blood. 2001;98:1312-1320.

(66) **Kottaridis PD, Gale RE, Frew ME et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials**
Blood. 2001;98:1752-1759.

(67) **Growney JD, Clark JJ, Adelsperger J et al. Activation mutations of human c-KIT resistant to imatinib mesylate are sensitive to the tyrosine kinase inhibitor PKC412**
Blood. 2005;106:721-724.

(68) **Steensma DP, Dewald GW, Lasho TL et al. The JAK2 V617F activating tyrosine kinase mutation is an infrequent event in both "atypical" myeloproliferative disorders and the myelodysplastic syndrome**
Blood. 2005.

(69) **Bowen DT, Frew ME, Hills R et al. RAS mutation in acute myeloid leukemia is associated with distinct cytogenetic subgroups but does not influence outcome in patients younger than 60 years**
Blood. 2005;106:2113-2119.

(70) **Grimwade D. The pathogenesis of acute promyelocytic leukaemia: evaluation of the role of molecular diagnosis and monitoring in the management of the disease**
Br J Haematol. 1999;106:591-613.

(71) **Licht JD, Sternberg DW. The molecular pathology of acute myeloid leukemia**
Hematology (Am Soc Hematol Educ Program). 2005;137-142.

(72) **Falini B, Mecucci C, Tiacci E et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype**
2 N Engl J Med. 2005;352:254-266.

(73) **Bernardi R, Scaglioni PP, Bergmann S et al. PML regulates p53 stability by sequestering Mdm2 to the nucleolus**
Nat Cell Biol. 2004;6:665-672.

(74) **Chen W, Rassidakis GZ, Medeiros LJ. Nucleophosmin gene mutations in acute myeloid leukemia**
Arch Pathol Lab Med. 2006;130:1687-1692.

(75) **Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biologic and clinical features**
Blood. 2007;109:874-885.

(76) **Rosnet O, Birnbaum D. Hematopoietic receptors of class III receptor-type tyrosine kinases.** Crit Rev Oncog. 1993;4:595-613.

(77) **Rosnet O, Buhring HJ, Marchetto S et al. Human FLT3/FLK2 receptor tyrosine kinase is expressed at the surface of normal and malignant hematopoietic cells**
Leukemia. 1996;10:238-248.

(78) **Reindl C, Spiekermann K. From kinases to cancer: leakiness, loss of autoinhibition and leukemia**
Cell Cycle. 2006;5:599-602.

(79) **Choudhary C, Schwable J, Brandts C et al. AML-associated Flt3 kinase domain mutations show signal transduction differences compared with Flt3 ITD mutations**
Blood. 2005;106:265-273.

(80) **Vogt PK. PI 3-kinase, mTOR, protein synthesis and cancer**
Trends Mol Med. 2001;7:482-484.

(81) **Recher C, Beyne-Rauzy O, Demur C et al. Antileukemic activity of rapamycin in acute myeloid leukemia**
Blood. 2005;105:2527-2534.

(82) **Kohn AD, Barthel A, Kovacina KS et al. Construction and characterization of a conditionally active version of the serine/threonine kinase Akt**
J Biol Chem. 1998;273:11937-11943.

(83) **Hirai T, Chida K. Protein kinase Czeta (PKCzeta): activation mechanisms and cellular functions**
J Biochem (Tokyo). 2003;133:1-7.

(84) **Newton AC. Protein kinase C: structure, function, and regulation**
. J Biol Chem. 1995;270:28495-28498.

(85) **Martiny-Baron G, Kazanietz MG, Mischak H et al. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976.** J Biol Chem. 1993;268:9194-9197.

(86) **Kurzer JH, Argetsinger LS, Zhou YJ et al. Tyrosine 813 is a site of JAK2 autophosphorylation critical for activation of JAK2 by SH2-B beta**
Mol Cell Biol. 2004;24:4557-4570.

(87) **Luo C, Laaja P. Inhibitors of JAKs/STATs and the kinases: a possible new cluster of drugs**
Drug Discov Today. 2004;9:268-275.

(88) **Ihle JN, Kerr IM. Jaks and Stats in signaling by the cytokine receptor superfamily**
Trends Genet. 1995;11:69-74.

(89) **Fabbro D, Parkinson D, Matter A. Protein tyrosine kinase inhibitors: new treatment modalities?** Curr Opin Pharmacol. 2002;2:374-381.

(90) **Boggon TJ, Li Y, Manley PW, Eck MJ. Crystal structure of the Jak3 kinase domain in complex with a staurosporine analogue**
Blood. 2005;106: 996-1002.

(91) **Nagar B, Bornmann WG, Pellicena P et al. Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571)**
Cancer Res. 2002;62:4236-4243.

(92) **Peeters P, Raynaud SD, Cools J et al. Fusion of TEL, the ETS-variant gene 6 (ETV6), to the receptor-associated kinase JAK2 as a result of t(9;12) in a lymphoid and t(9;15;12) in a myeloid leukemia.** Blood. 1997;90:2535-2540.

(93) **Reiter A, Walz C, Watmore A et al. The t(8;9)(p22;p24) is a recurrent abnormality in chronic and acute leukemia that fuses PCM1 to JAK2**
Cancer Res. 2005;65:2662-2667.

(94) **Joos S, Kupper M, Ohl S et al. Genomic imbalances including amplification of the tyrosine kinase gene JAK2 in CD30+ Hodgkin cells**
Cancer Res. 2000;60:549-552.

(95) **James C, Ugo V, Le Couedic JP et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera**
Nature. 2005;434:1144-1148.

(96) **Bowman T, Garcia R, Turkson J, Jove R. STATs in oncogenesis**
Oncogene. 2000;19:2474-2488.

(97) **Sternberg DW, Gilliland DG. The role of signal transducer and activator of transcription factors in leukemogenesis.** J Clin Oncol. 2004;22:361-371.

(98) **Biethahn S, Alves F, Wilde S, Hiddemann W, Spiekermann K. Expression of granulocyte colony-stimulating factor- and granulocyte-macrophage colony-stimulating factor-associated signal transduction proteins of the JAK/STAT pathway in normal granulopoiesis and in blast cells of acute myelogenous leukemia**
Exp Hematol. 1999;27:885-894.

(99) **Hayakawa F, Towatari M, Iida H et al. Differential constitutive activation between STAT-related proteins and MAP kinase in primary acute myelogenous leukaemia**
Br J Haematol. 1998;101:521-528.

(100) **Benekli M, Xia Z, Donohue KA et al. Constitutive activity of signal transducer and activator of transcription 3 protein in acute myeloid leukemia blasts is associated with short disease-free survival**
Blood. 2002;99:252-257.

(101) **Xia Z, Baer MR, Block AW, Baumann H, Wetzler M. Expression of signal transducers and activators of transcription proteins in acute myeloid leukemia blasts.** Cancer Res. 1998;58:3173-3180.

(102) **Tefferi A, Spivak JL. Polycythemia vera: scientific advances and current practice**
Semin Hematol. 2005;42:206-220.

(103) **Tefferi A, Gilliland DG. JAK2 in myeloproliferative disorders is not just another kinase**
Cell Cycle. 2005;4:1053-1056.

(104) **Levine RL, Wadleigh M, Cools J et al. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis**
Cancer Cell. 2005;7:387-397.

(105) **Ruchatz H, Coluccia AM, Stano P, Marchesi E, Gambacorti-Passerini C. Constitutive activation of Jak2 contributes to proliferation and resistance to apoptosis in NPM/ALK-transformed cells**
Exp Hematol. 2003;31:309-315.

(106) **Yu H, Jove R. The STATs of cancer--new molecular targets come of age**
Nat Rev Cancer. 2004;4:97-105.

(107) **Pallis M, Seedhouse C, Grundy M, Russell N. Flow cytometric measurement of phosphorylated STAT5 in AML: lack of specific association with FLT3 internal tandem duplications**
Leuk Res. 2003;27:803-805.

(108) **Lacronique V, Boureux A, Monni R et al. Transforming properties of chimeric TEL-JAK proteins in Ba/F3 cells**
Blood. 2000;95:2076-2083.

(109) **Pileri SA, Zinzani PL, Gaidano G et al. Pathobiology of primary mediastinal B-cell lymphoma**
Leuk Lymphoma. 2003;44 Suppl 3:S21-S26.

(110) **Scott LM, Tong W, Levine RL et al. JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis**
N Engl J Med. 2007;356:459-468.

(111) **Schwaller J, Frantsve J, Aster J et al. Transformation of hematopoietic cell lines to growth-factor independence and induction of a fatal myelo- and lymphoproliferative disease in mice by retrovirally transduced TEL/JAK2 fusion genes**
EMBO J. 1998;17:5321-5333.

(112) **Ho JM, Beattie BK, Squire JA, Frank DA, Barber DL. Fusion of the ets transcription factor TEL to Jak2 results in constitutive Jak-Stat signaling**
Blood. 1999;93:4354-4364.

(113) **Zaleskas VM, Krause DS, Lazarides K et al. Molecular Pathogenesis and Therapy of Polycythemia Induced in Mice by JAK2 V617F**
PLoS ONE. 2006;1:e18.

(114) **Mercher T, Wernig G, Moore SA et al. JAK2T875N is a novel activating mutation that results in myeloproliferative disease with features of megakaryoblastic leukemia in a murine bone marrow transplantation model**
Blood. 2006;108:2770-2779.

(115) **Frohling S, Lipka DB, Kayser S et al. Rare occurrence of the JAK2 V617F mutation in AML subtypes M5, M6, and M7**
Blood. 2006;107:1242-1243.

(116) **Lee JW, Kim YG, Soung YH et al. The JAK2 V617F mutation in de novo acute myelogenous leukemias**
Oncogene. 2006;25:1434-1436.

(117) **Levine RL, Loriaux M, Huntly BJ et al. The JAK2V617F activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia**
Blood. 2005;106:3377-3379.

(118) **Theocharides A, Boissinot M, Girodon F et al. Leukemic blasts in transformed JAK2-V617F positive myeloproliferative disorders are frequently negative for the JAK2-V617F mutation**
Blood. 2007.

(119) **Schnittger S, Bacher U, Kern W, Haferlach C, Haferlach T. JAK2 seems to be a typical cooperating mutation in therapy-related t(8;21)/ AML1-ETO-positive AML**
Leukemia. 2007;21:183-184.

(120) **Desta F, Christiansen DH, Andersen MK, Pedersen-Bjergaard J. Activating mutations of JAK2V617F are uncommon in t-MDS and t-AML and are only observed in atypic cases**
Leukemia. 2006;20:547-548.

(121) **Xie S, Wang Y, Liu J et al. Involvement of Jak2 tyrosine phosphorylation in Bcr-Abl transformation**
Oncogene. 2001;20:6188-6195.

(122) **Alas S, Bonavida B. Inhibition of constitutive STAT3 activity sensitizes resistant non-Hodgkin's lymphoma and multiple myeloma to chemotherapeutic drug-mediated apoptosis**
Clin Cancer Res. 2003;9:316-326.

(123) **Grandage VL, Gale RE, Linch DC, Khwaja A. PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kappaB, Mapkinase and p53 pathways**
Leukemia. 2005;19:586-594.

(124) **Min YH, Eom JI, Cheong JW et al. Constitutive phosphorylation of Akt/PKB protein in acute myeloid leukemia: its significance as a prognostic variable**
Leukemia. 2003;17:995-997.

(125) **Xu Q, Simpson SE, Scialla TJ, Bagg A, Carroll M. Survival of acute myeloid leukemia cells requires PI3 kinase activation**
Blood. 2003;102:972-980.

(126) **Zhao S, Konopleva M, Cabreira-Hansen M et al. Inhibition of phosphatidylinositol 3-kinase dephosphorylates BAD and promotes apoptosis in myeloid leukemias**
Leukemia. 2004;18:267-275.

(127) **Yilmaz OH, Valdez R, Theisen BK et al. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells**
Nature. 2006;441:475-482.

(128) **Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors**
Biochem J. 2000;351:95-105.

(129) **Guzman ML, Neering SJ, Upchurch D et al. Nuclear factor-kappaB is constitutively activated in primitive human acute myelogenous leukemia cells**
Blood. 2001;98:2301-2307.

(130) **El Deiry WS. The role of p53 in chemosensitivity and radiosensitivity**
Oncogene. 2003;22:7486-7495.

(131) **Clark AS, West K, Streicher S, Dennis PA. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells.** Mol Cancer Ther. 2002;1:707-717.

(132) **Shingu T, Yamada K, Hara N et al. Synergistic augmentation of antimicrotubule agent-induced cytotoxicity by a phosphoinositide 3-kinase inhibitor in human malignant glioma cells**
Cancer Res. 2003;63:4044-4047.

(133) **O'Gorman DM, McKenna SL, McGahon AJ, Knox KA, Cotter TG. Sensitisation of HL60 human leukaemic cells to cytotoxic drug-induced apoptosis by inhibition of PI3-kinase survival signals**
Leukemia. 2000;14:602-611.

(134) **Leverrier Y, Thomas J, Mathieu AL et al. Role of PI3-kinase in Bcl-X induction and apoptosis inhibition mediated by IL-3 or IGF-1 in Baf-3 cells**
Cell Death Differ. 1999;6:290-296.

(135) **Bradbury D, Rogers S, Reilly IA, Kozlowski R, Russell NH. Role of autocrine and paracrine production of granulocyte-macrophage colony-stimulating factor and interleukin-1 beta in the autonomous growth of acute myeloblastic leukaemia cells--studies using purified CD34-positive cells.** Leukemia. 1992;6:562-566.

(136) **Schuringa JJ, Wierenga AT, Kruijer W, Vellenga E. Constitutive Stat3, Tyr705, and Ser727 phosphorylation in acute myeloid leukemia cells caused by the autocrine secretion of interleukin-6**
Blood. 2000;95:3765-3770.

(137) **Milella M, Kornblau SM, Estrov Z et al. Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia**
J Clin Invest. 2001;108:851-859.

(138) **Yart A, Laffargue M, Mayeux P et al. A critical role for phosphoinositide 3-kinase upstream of Gab1 and SHP2 in the activation of ras and mitogen-activated protein kinases by epidermal growth factor**
J Biol Chem. 2001;276:8856-8864.

(139) **Le Good JA, Ziegler WH, Parekh DB et al. Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1**
Science. 1998;281:2042-2045.

(140) **Schonwasser DC, Marais RM, Marshall CJ, Parker PJ. Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes**
Mol Cell Biol. 1998;18:790-798.

(141) **Chaudhary A, King WG, Mattaliano MD et al. Phosphatidylinositol 3-kinase regulates Raf1 through Pak phosphorylation of serine 338.** Curr Biol. 2000;10:551-554.

(142) **Eblen ST, Slack JK, Weber MJ, Catling AD. Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes**
Mol Cell Biol. 2002;22:6023-6033.

(143) **Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell.** Nat Med. 1997;3:730-737.

(144) **Downward J. How BAD phosphorylation is good for survival**
Nat Cell Biol. 1999;1:E33-E35.

(145) **Madrid LV, Wang CY, Guttridge DC et al. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB**
Mol Cell Biol. 2000;20:1626-1638.

(146) **Ozes ON, Mayo LD, Gustin JA et al. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase**
Nature. 1999;401:82-85.

(147) **Romashkova JA, Makarov SS. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling**

4 Nature. 1999;401:86-90.

(148) **Kane LP, Shapiro VS, Stokoe D, Weiss A. Induction of NF-kappaB by the Akt/PKB kinase**

Curr Biol. 1999;9:601-604.

(149) **Bargou RC, Emmerich F, Krappmann D et al. Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells**

J Clin Invest. 1997;100:2961-2969.

(150) **Kordes U, Krappmann D, Heissmeyer V, Ludwig WD, Scheidereit C. Transcription factor NF-kappaB is constitutively activated in acute lymphoblastic leukemia cells**

Leukemia. 2000;14:399-402.

(151) **Sovak MA, Arsura M, Zanieski G, Kavanagh KT, Sonenshein GE. The inhibitory effects of transforming growth factor beta1 on breast cancer cell proliferation are mediated through regulation of aberrant nuclear factor-kappaB/Rel expression**

. Cell Growth Differ. 1999;10:537-544.

(152) **Wang W, Abbruzzese JL, Evans DB et al. The nuclear factor-kappa B RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells**

Clin Cancer Res. 1999;5:119-127.

(153) **Nakano Y, Naoe T, Kiyoi H et al. Prognostic value of p53 gene mutations and the product expression in de novo acute myeloid leukemia**

Eur J Haematol. 2000;65:23-31.

(154) **Stirewalt DL, Kopecky KJ, Meshinchi S et al. FLT3, RAS, and TP53 mutations in elderly patients with acute myeloid leukemia**
Blood. 2001;97:3589-3595.

(155) **Wattel E, Preudhomme C, Hecquet B et al. p53 mutations are associated with resistance to chemotherapy and short survival in hematologic malignancies**
Blood. 1994;84:3148-3157.

(156) **Chene P. Inhibiting the p53-MDM2 interaction: an important target for cancer therapy**
Nat Rev Cancer. 2003;3:102-109.

(157) **Changelian PS, Flanagan ME, Ball DJ et al. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor**
Science. 2003;302:875-878.

(158) **Walters DK, Mercher T, Gu TL et al. Activating alleles of JAK3 in acute megakaryoblastic leukemia**. Cancer Cell. 2006;10:65-75.