Characterisation of the in vitro properties of pacritinib (SB1518) in Acute Myeloid Leukaemia

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ABSTRACT

Acute Myeloid Leukaemia remains an incurable malignancy in the majority of cases, with long term survival rates of 30-40% in those under 60 years old and less than 15% in older patients. FMS like Tyrosine kinase 3 (FLT3) is a trans-membrane receptor tyrosine kinase that is mutated in around one third of cases of AML, making FLT3 an attractive therapeutic target and paving the way for the development of a range of FLT3 Tyrosine Kinase Inhibitors (TKIs) over the last 10 years. Progress has been hampered by the limited efficacy of the majority of FLT3 TKIs in targeting the bone marrow blast population. Pacritinib is a second generation TKI with equimolar efficacy for both the FLT3 and Janus Kinase 2 (JAK2) receptors. FLT3 occupies a strategic position at the head of a complex array of downstream pathways which control transduction of signals from the extracellular environment to the nucleus. These pro-survival pathways may be enhanced on stroma leading to the creation of leukaemia niches where AML blasts can evade the effects of treatment. Cross talk between pathways such as dual activation of Signal Transducer and Activator of Transcription 5 (STAT5) by FLT3 and JAK2 has been implicated in this phenomenon and can be targeted by pacritinib.

Pacritinib showed good efficacy in cell lines and in primary AML mononuclear cells with increased potency seen in cases which harboured a FLT3 ITD mutation compared to wild type FLT3. There was no correlation between surface FLT3 level, as measured by CD135 expression, and in vitro sensitivity to pacritinib. Western blotting experiments demonstrated inhibition of phosphorylated FLT3 protein in MV4-11 cell lines and rapid inhibition of phosphorylated-STAT5 in MV4-11 and primary AML cells in a dose dependent manner following pacritinib treatment. There was minimal effect on the Extracellular Regulated Kinase (ERK) pathway which is known to generate pro-survival signals in AML cells and may be upregulated on stroma.

Short term resistance to pacritinib treatment was seen when primary AML cells were cultured on mouse (MS-5) stroma with supplemented medium as compared to culture in standard medium alone, potentially related to delayed apoptotic induction on stroma as seen by Annexin V Binding

Assay. Further western blotting experiments in the co-culture setting showed basal up regulation of both p-STAT5 and p-ERK in primary AML when cultured on MS-5 stroma which was not entirely inhibited by pacritinib treatment, supporting the argument that this is a possible mechanism of drug resistance.

This observation led to the rationale to combine pacritinib with two different agents, which could potentially be used to eradicate residual disease within the stroma niche. A good synergistic effect was seen using the combination of pacritinib with cytarabine and with PD0325901 (a small molecule inhibitor of the ERK pathway) and this latter combination may be able to overcome stroma-induced protection of AML blasts. This justifies the on-going laboratory and clinical development of pacritinib in targeting environment mediated drug resistance in acute myeloid leukaemia.

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List of Abbreviations

AML	acute myeloid leukaemia
AraC	cytosine arabinoside (cytarabine)
AKT	protein kinase B (PBK)
APC	allophycocyanin
ATP	adenosine triphosphate
AVB	annexin V binding assay
BAD	BCL-2 antagonist of cell death
BCL-2	B cell leukaemia/lymphoma 2
BCR	breakpoint cluster region
BM	bone marrow
BSA	bovine serum albumin
CBF	core binding factor
CC3	cleaved caspase 3
CD	cluster differentiation
CEBPA	CCAAT/enhancer binding protein (C/EBP) alpha
CI	combination index
CML	chronic myeloid leukaemia
CR	complete remission
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNMT	DNA (cytosine-5-)-methyltransferase 3 alpha
ECOG	Eastern Cooperative Oncology Group
EC_{50}	50% inhibitory concentration
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular regulated kinase
FAB	French-American-British
FCS	fetal calf serum
FITC	fluorescein isothiocyanate

FL	FLT3 ligand
FLK-2	fetal liver kinase-2
FLT3	FMS-like tyrosine kinase
HDAC	histone deacetylase
GCSF	granulocyte colony stimulating factor
IC_{50}	50% inhibitory concentration (100% starting point)
IDH-1/2	isocitrate dehydrogenase-1/2
IL-3	interleukin-3
IMDM	Isocove's modified Dulbecco's medium
ITD	internal tandem duplication
JAK	janus kinase
JM	juxtamembrane
LSC	leukaemic stem cell
LTC	long term culture
LDS	lithium dodecyl sulphate
MAPK	mitogen-associated protein kinase
MEM	minimal essential medium
MFI	mean fluorescence intensity
MRC	Medical Research Council
MRD	minimal residual disease
MS-5	mouse stroma-5
mTOR	mammalian target of rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NCRI	National Cancer Research Institute
NPM-1	nucleophosphim-1
OS	overall survival
PB	peripheral blood
PBS	phosphate buffered serum

PDGFR	platelet derived growth factor
PDK-1	3-phosphoinositide-dependent protein kinase 1
PE	phycoerythrin
PI	propidium iodide
PI3k	phosphatidyl-inositol 3’-kinase
PKC	protein kinase C
PM	point mutation
PR	partial remission
PS	phosphatidyl serine
PVDF	polyvinylidene difluoride
RAS	reticular activating system
RLU	relative light unit
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
RR	relapse risk
RTK	receptor tyrosine kinase
SEM	standard error of the mean
STAT	signal transducer and activator of transcription
TBS	tris-buffered saline
TKD	tyrosine kinase domain
TKI	tyrosine kinase inhibitors
TM	transmembrane
TNF	tumour necrosis factor
TPO	thrombopoeitin
VEGF	vascular endothelial growth factor
WCC	white cell count
WHO	World Health Organisation
WT	wild type

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Chapter 1

Introduction

Chapter 1: Introduction

1.1 Overview of Acute Myeloid Leukaemia

Acute Myeloid Leukaemia is the commonest myeloid malignancy in adults and results from an accumulation of immature cells in the marrow (Estey, 2012, AJH 87(1)) and peripheral blood. The presence of this abnormal clone disrupts normal haematopoiesis leading to bone marrow failure. Patients with AML typically present with features of bone marrow failure such as anaemia, bleeding due to thrombocytopenia and the secondary effects of either a high white cell count (leucostasis) or a low count (opportunistic infections). Less frequently, malignant cells may also infiltrate other tissues, spreading the leukemic effect throughout the lungs, CNS and soft tissue.

1.2 Classification of AML

AML is a clonally heterogeneous disorder and different patients show variable degrees of maturation and differentiation within the leukaemic clone. The original French-American-British (FAB) classification system was largely based on these characteristics and morphological findings; recently it has been superseded by the World Health Organisation (WHO) criteria, which also incorporates clinical and cytogenetic features. Both classification systems are summarised in the table below, although now outdated the FAB system is included given that is the system used for stratification of patients entered into the NCRI trials.

Table 1

FAB type	Description
M0	Undifferentiated acute myeloblastic leukaemia
M1	Acute myeloblastic leukaemia with minimal maturation
M2	Acute myeloblastic leukaemia with maturation
M3	Acute promyelocytic leukaemia (APL)
M4Eo	Acute myelomonocytic leukaemia with eosinophilia
M4	Acute myelomonocytic leukaemia
M5	Acute monocytic leukaemia
M6	Acute erythroid leukaemia
M7	Acute megakaryoblastic leukaemia

Table 2

Group	Description
AML with certain genetic abnormalities	t (8,21), inv(16), t(15,17)
AML with multilineage dysplasia	More than one myeloid cell involved
AML related to previous chemotherapy or radiation	
AML not otherwise specified	
Undifferentiated or bi-phenotypic leukaemia	

Tables 1 + 2: FAB and WHO Classification of AML taken from www.cancer.org.uk

It has become increasingly clear from attempts to classify the disease using clinical, morphological, cytogenetic or molecular techniques that AML is much more than one disease entity. Partly in an attempt to develop better treatments, a great deal of work has been invested into improving

knowledge of how best to stratify patients at diagnosis and deliver risk adapted or tailored therapy thereby improving patient outcomes. This work has identified a variety of therapeutic targets over recent years of which the FMS like tyrosine kinase-3 (FLT3) receptor is one of many and represents the focus for this work.

1.3 Pathogenesis of AML

Most cases of AML are sporadic in that there is no underlying genetic factor but there are certain factors which are known to increase an individual's risk of developing the condition such as exposure to high levels of radiation or benzene, smoking, increased body mass index and several congenital or acquired bone marrow failure syndromes. For example AML commonly occurs following a leukaemic transformation of a chronic myeloproliferative or myelodysplastic neoplasm such as polycythaemia vera or a myelodysplastic syndrome. Less frequently AML can result from transformation of one of the rare congenital clonal myeloid disorders such as Fanconi Anaemia (2014). There are around 2,600 new cases of AML in the UK every year which corresponds to an annual incidence of around 3 per 100,000.

All of these initiating factors are associated with the presence of a mutation in a somatic stem cell (Dash A & Gilland DG, 2001) which results in the development of a leukaemic clone. These genetic changes lead to a variety of outcomes which affect transcription or translation of genes regulating myeloid cell development, proliferation of cells of the myeloid lineage or regulate apoptotic signalling to curb the development of abnormal clones.

Structural chromosomal abnormalities are known to be associated with the prognosis of AML in recognised risk-scores (Burnett AK *et al*, 2006). For example there are several 'good risk' lesions such as t (8,21) or inv(16) where in general patients with these abnormalities have good outcomes with chemotherapy. In contrast, poor risk features include monosomy 7 or complex karytotypes and these patients have been shown to require more intensive treatment to improve survival including

allogeneic transplant. Nevertheless, more than 50% of patients have a normal karyotype even with the most sensitive techniques which is considered 'standard risk' and yet carries a very heterogeneous outlook. This observation has led to a more targeted genetic sequencing approach and at a molecular level it has been shown that the abnormalities underlying AML are heterogeneous and often polyclonal entities where new mutations may arise at relapse or in response to treatment. Consequentially AML has the worst prognosis of all leukemias and is a disease which lacks effective targeted therapies.

1.4 Summary of Molecular Lesions in AML

The Genome Atlas Study (Ley TJ et al, 2013) identified a total of 13 mutations within the average AML genome, 6 of which encode genes which are recurrently mutated, which are *FLT3*, *DNMT3a, NPM1, CEPBA, IDH1/2 and RUNX1*. The commonest mechanistic effects of these genetic abnormalities involved disruption to cell signalling (59%), DNA-methylation (44%) and chromatin modification (30%). The authors conclude that nearly all AML samples carry at least one potential 'driver' mutation; however it is known that this alone is not sufficient to give rise to leukaemia. A summary of common molecular lesions in AML is shown in Table 3.

Class of Mutation	**Class I**	**Class II**	**Epigenetic**	**Mixed**
Main Mutations described	KIT	CBFβ-MYH11	DNMT3a (15-25%)	JAK2 (Class 1 and Epigenetic)
	FLT3 (25-30%)	PML-RARα	TET2 (7-23%)	WT1 (Class I & II)
	N-RAS (15-30%)	RUNX1-RUNX1T1	EZH2 (Rare)	MLL translocations
	K-RAS (15-30%)	RUNX1	IDH1/2 (15-30%)	MLL-PTD
	PTPN11	NPM1	AXSL1 (10-15%)	(both epigenetic and class II)
		CEBPα		
		EVI1		

Table 3: Overview of molecular lesions in AML adapted from Ley TJ et al. Percentages are that of cases with the mutation.

One of the best described theories to explain the pathogenesis of AML is the "2 hit hypothesis" which relates to the fact that more than one different type of genetic mutation is required for malignant transformation of a myeloid precursor cell. In simple terms two mutations are required to drive proliferation and inhibit differentiation so that the primitive clone survives (Ofran Y, 2014). This theory has been borne out in mouse models of AML where co-operative events such as *C-KIT* mutations (Wang Y *et al*, 2010) in mice who carry full length *AML1-ETO* fusion gene or *FLT3* internal tandem duplication (ITD) in an MLL-partial tandem duplication model (Zorko N *et al*, 2012) have been shown to be required for leukaemogenesis. FLT3 ITD mutations have also been shown to co-operate with the inversion of chromosome 16 acute myeloid leukaemias where expression of the fusion product core binding factor (CBF) beta subunit-smooth muscle myosin heavy chain (SMMHC) only promotes development of AML in a mouse model when there is co-expression of a FLT3 ITD mutation (Kim H-G *et al*, 2008). Although the paper acknowledged that FLT3 ITD mutations are relatively rare in inv(16) AML (approximately 3-8% cases) the model does demonstrate proof of principle for a 'two-hit' mechanism of disease pathogenesis. The association has also been borne out in an *in vivo* setting involving 176 patients where clinical heterogeneity within inversion 16 acute myeloid leukaemia was reflected by genetic findings and *FLT3* mutations were found to be one of a handful of prognostic marker lesions (Paschka P *et al*, 2013).

There is increasing awareness that interactions between mutations are of utmost importance from a prognostic standpoint (Rowe J, 2014) for example in determining the overall significance of combination of a 'good risk' cytogenetic marker (e.g. inv(16)) with a 'poor risk' molecular lesion (isolated FLT3 ITD mutation). There is a large body of scientific and clinical evidence to suggest that in these cases FLT3 ITD is probably a weak driver mutation (Smith C *et al*, 2012) so that it's presence is required for leukaemogenesis but it is not a determinant of its maintenance . This may explain the lack of potency of early FLT3 inhibitors (Smith C *et al*, 2012) which will be discussed later. There is some disagreement in the literature regarding this point however, and other authors have proposed more of a driver role for FLT3 mutations given that in up to 84% of patients the ITD mutation is

present at diagnosis and at relapse suggesting that these FLT3-mutated cells may include 'leukaemic stem cells' (Shih L-Y *et al*, 2002). This is also supported by the increase in allelic ratio seen at relapse (Levis M, 2011) suggesting that as other clones are killed by chemotherapy, the ITD mutated clone carries a survival advantage, possibly due to interaction with the bone marrow stroma (Shih L-Y *et al*, 2002).

As previously stated AML is a polyclonal disease and it is now thought to arise via an initial assembly of founding clones which are likely to contain mutations in epigenetics-modifying genes such as *DNMT3a*, *IDH1/2* and *TET2* many of which can be found at diagnosis and at relapse (Wakita S *et al*, 2013). At the time of relapse however, new sub-clones can emerge and the same authors showed that FLT3-ITD mutations were commonly expressed by these cells. This suggests that the initial mutations in epigenetic-modifying genes such as *DNMT3a* or *IDH1/2* results in epigenetic changes and the promotion of secondary lesions such as *FLT3-ITD* (Wakita S *et al*, 2013). This has relevance for minimal residual disease (MRD) monitoring and also there has recently been preliminary data using an inhibitor to mutant mitochondrial *IDH2* which shows potential in overcoming the ability of this lesion to block cellular differentiation (Stein E, 2014). In a phase 1 study of 48 patients, the drug was well tolerated by oral dosing and led to triggering of differentiation of leukaemic blasts and ultimately to objective responses in patients including some complete remissions.

1.5 Overview of treatment of AML

With knowledge of the complex aetiology and molecular genetic makeup of AML it becomes clear that all of these factors combine to confound efforts to stratify patients and tailor treatment accordingly. It is clear that a 'one size fits all' approach to treatment does not produce satisfactory outcomes in the majority of patients because AML is simply not one disease. Traditional (cytarabine-daunorubicin based) induction regimens can produce wildly different results in patients who may seem to be similar according to standard methods of disease classification. This can range from primary refractory disease to patients who achieve a durable remission with just 2 or 3 courses of

standard therapy. This molecular heterogeneity and variation in response to therapy is one of the principal clinical features of AML and partly explains why overall responses remain poor with less than 50% long term survivors across all risk groups (Burnett AK, 2012).

The success of treatment of AML rests on two main factors: firstly patient factors which define tolerance to chemotherapy such as age and performance status (Dohner H *et al*, 2014). In clinical practice most patients diagnosed with AML are over 60 years old and increasing age is an adverse prognostic factor even after accounting for other risk factors such as cytogenetics, molecular genetics and type of AML (i.e. *de novo* or secondary). The second main factor is specific characteristics of the leukemic clone which may lead to greater or lesser intrinsic resistance to therapy as described above. Approximately one third of cases of AML have normal cytogenetics in that no aberrant karyotypic lesions are found within the clone. Prognostic significance within cytogenetically normal AML has been consistently been shown for mutations in the *NPM1*, *CEBPA* and *FLT-3* genes alone or in combination in younger adult patients. With this evidence in mind, one of the key aims of this project is to explore the mechanism and potency of FLT3 signalling as a determinant of *in vitro* response to drugs targeting this pathway in primary AML samples taken from patients at diagnosis.**1.6 The role of FLT-3 mutations in disease behaviour**

FLT3 is a receptor tyrosine kinase of the same family as Steel factor receptor (KIT), Macrophage-colony stimulating factor (M-CSF) and the receptors for PGDFRα and β (Grafone T *et al*, 2012) all of which are found to be aberrantly expressed in many malignancies including leukaemia (Stirewalt DL & Radich JP, 2003). FLT3 plays a key role in cellular signalling for proliferative growth and survival pathways and it's downstream effectors include Signal Transducer and Activator of Transcription 5 (STAT5), Janus Kinase (JAK) family, phosphatidylinositosol (PI)-3-kinase (PI3K/AKT and Reticular Activating System (RAS)/mitogen-activated protein kinase (MAPK) and Extracellular Regulated Kinase (ERK) pathways (Grafone T *et al*, 2012).

The human gene for FLT3 encodes a 993-amino acid protein, which is comprised of an immunoglobulin-like extracellular ligand binding domain, a transmembrane domain, a juxtamembrane dimerization domain and a cytoplasmic domain with a split tyrosine kinase motif. The inactivated form of the receptor is a monomer which resides within the plasma membrane, the structure of the FLT3 receptor is shown in Figure 1.1.

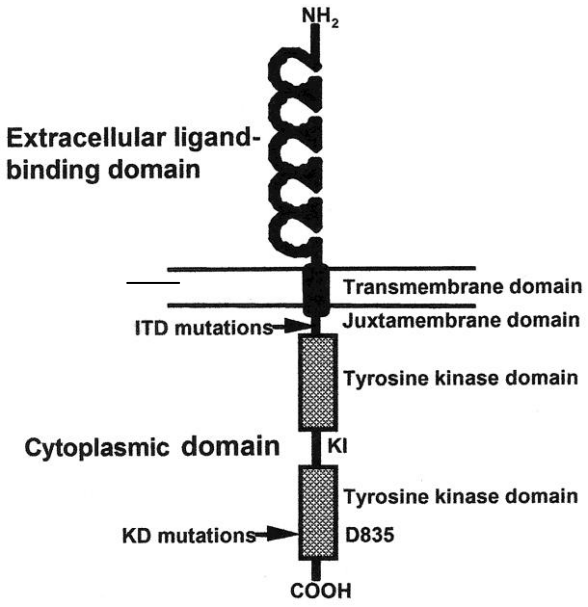


Figure 1.1: Schematic representation of FLT3 structure which shows its 5 immunoglobulin-like folds that make up the ligand binding extracellular domain, single transmembrane domain and cytoplasmic domain which comprises a kinase domain interrupted by a kinase insert. The arrows highlight the juxtamembrane domain where internal tandem duplications (ITDs) occur and residue aspartic acid 835 where most kinase domain mutations occur. Many FLT3 TKIs interrupt the ATP binding pocket of the kinase domain. (ASH Education Book **January 1, 2006** vol. 2006 no. 1 **178-184)**

Exposure of FLT3 to its ligand is a crucial step in regulating activity of the receptor (Grafone T *et al*, 2012). FLT3 ligand is produced by many cells of the haemopoeitic system and binds to the extracellular domain of FLT3, promoting dimerization, phosphorylation and activation of the FLT3 receptor which results in phospho-activation of secondary mediators. High levels of *FLT3* expression and additional paracrine/autocrine stimulation by FLT3 ligand may in some cases render FLT3 wild

type (WT) blasts dependent on FLT3 signalling and therefore more sensitive to FLT3-TKI therapy despite the lack of a FLT3 mutation (Kindler T *et al*, 2010).

FLT3 is mutated in approximately 1/3 of AML patients making this receptor a major molecular abnormality in AML. It is thought that activating FLT-3 receptor mutations lead to a proliferation and survival benefit for the leukemic clone. Such mutations confer growth factor independence to myeloid cells in mouse models which can lead to leukaemogenesis (Parmar A *et al*, 2011) as described above. Mutations can occur in 2 principal regions of the FLT3 receptor, namely Internal Tandem Duplication (ITD) and tyrosine kinase domain (TKD) mutations.

FLT3 activating ITD mutations occurring in exon 14 & 15 of the juxtamembrane domain are activating mutations found in 25-30% cytogenetically normal AML and are widely accepted to confer a poor prognosis. The length of the duplicated region measured in base pairs (bp) is variable between 3 and more than 400 bp, however its length always increases in multiples of three which preserves the reading frame and therefore a functional protein is always produced. FLT-3 ITD mutations result in a mis-localised and constitutively activated receptor (Taylor SJ *et al*, 2015) which, through ligand-independent auto-phosphorylation, leads to an impact on downstream pro-survival pathways (Zirm E *et al*, 2011). ITD mutations also impair intracellular trafficking which promotes localisation of the receptor within the endoplasmic reticulum (ER) rather than at the cell surface (Choudhary C *et al*, 2009). Constitutively activated FLT3-ITD kinase stimulates aberrant proliferative signalling pathways, including PI3K/AKT, RAS/ERK, NF-κB (nuclear factor kappa-B) and STAT5 (Gerloff D *et al*, 2015) which leads to a variety of effects including resistance to apoptosis, abnormal cell growth and differentiation block (Stanicka J *et al*, 2015) through alteration in expression of target genes (Taylor SJ *et al*, 2015). Other authors have suggested that activation of downstream pathways by the oncogenic mutant of FLT3 (FLT3-ITD) is compartment-dependent with different effects seen depending on if the receptor is localised at the endoplasmic reticulum

(reduced impact on PI3K and MAPK) or at the cell membrane (lesser effect on STAT5 signalling) (Choudhary C *et al*, 2009) as described in Figure 1.2.

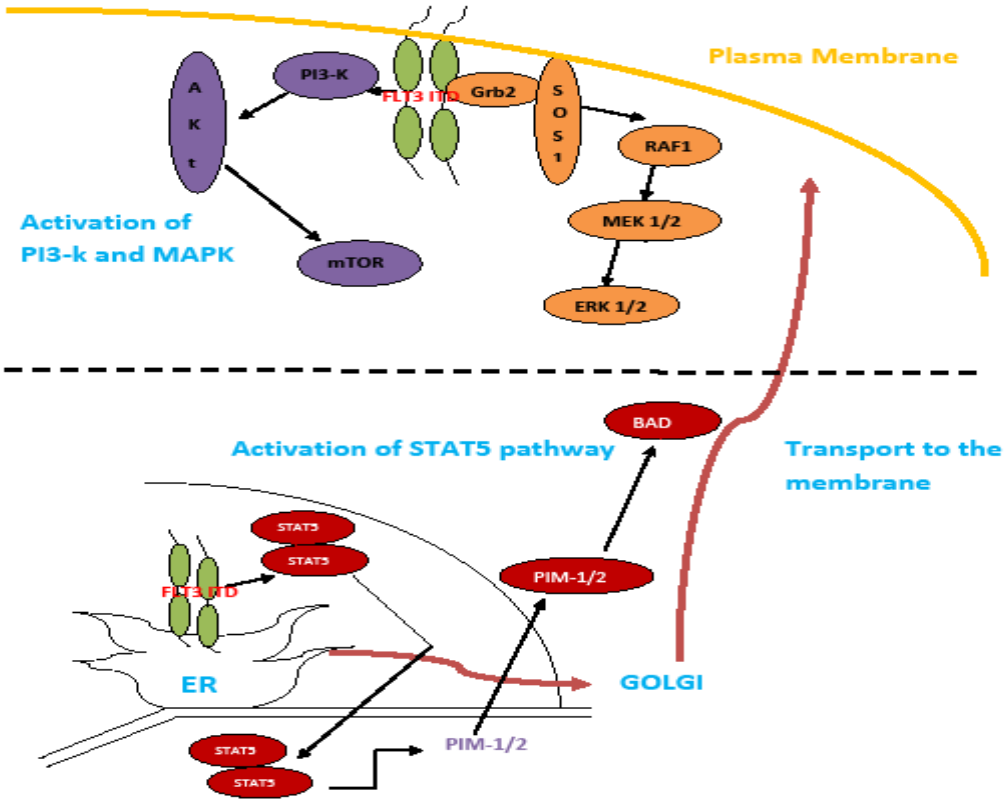


Figure 1.2: Schematic presentation of FLT3-ITD signalling compartmentalisation adapted from (Choudhary C *et al*, 2009) to show only main downstream effectors at cell surface and ER.

FLT3 ITD mutations are associated with high presenting white cell count and bone marrow blast percentage reflecting a more proliferative disease phenotype. FLT3 ITD mutations are also associated with a greater propensity to relapse, and relapse quickly in first remission with median time to relapse being 6-7 months (Levis M & Small D, 2003) . These factors have combined to

develop a body of opinion that allogeneic transplant in first response or additional novel therapies should form the recommended standard or care for patients with cytogenetically normal AML who harbour a FLT3 ITD mutation at diagnosis.

The extent to which FLT3 mutation status can predict outcome depends on several factors such as the FLT3 mutated to wild type allelic ratio, other characteristics of the study population such as age and interaction with other lesions, principally the nucleophosmin-1 (*NPM1)* mutation. An interaction between the two lesions has been described such that isolated presence of *FLT3-ITD* mutation in the absence of *NPM1* mutation is a poor risk feature for survival in both younger (Gale R *et al*, 2008) and older patients (Lazenby M *et al*, 2014). In contrast several authors suggest that isolated NPM1 mutation has a positive effect on survival and when the two mutations are balanced there is no overall effect (Kindler T *et al*, 2010).

In younger patients (less than 60 years old at diagnosis) the role of FLT3 mutations in conferring an increased relapse risk has been well described (Gale R *et al*, 2008;Swords R *et al*, 2012). As previously described however, FLT3-ITD positive patients remain a molecularly heterogeneous group and FLT3 status may well be a surrogate for additional poor risk markers. There have also been attempts to correlate abnormalities of FLT3 expression with clinical features, for example, high WBC, prior myelodysplasia and performance score >2.

The other main category of FLT3 mutations are, mainly single base point mutations within the tyrosine kinase domain (TKD) of FLT3 which frequently involve a change of amino acid from aspartic acid to tyrosine (D835Y or Asp835Tyr). TKD mutations can be found at diagnosis but have also been shown to be acquired following treatment with FLT3 TKIs in FLT3-ITD positive cell lines (Moore AS *et al*, 2012;Zimmerman E *et al*, 2013) and also in clinical studies (Baker S *et al*, 2014)). These 'treatment emergent' mutations include a new D835Y TKD mutation of the FLT3 ITD+ allele and have been found at several other sites including F691L (Baker S *et al*, 2014). FLT3 TKD mutations occur in 5-10%

of AML cases at diagnosis, they can also coexist with ITD mutations and are sometimes seen as a feature of disease relapse (Baker S *et al*, 2014) . Like ITD mutations, FLT3 TKD mutations promote ligand independent auto-phosphorylation thus stabilising the activation loop in its open binding configuration (Knapper S, 2011) and resulting in constitutive receptor activation. The association of TKD mutations with disease characteristics (e.g. presenting white cell count) is not as clear as in the case of ITD mutations. Also TKD point mutations may be present at diagnosis in a small percentage of malignant cells, which are then not necessarily detectable at relapse suggesting that this clone is non-dominant.

1.7 Development and Clinical Experience of FLT3 inhibitors in AML

In view of its frequency of expression in AML, importance as a proliferative signalling molecule and association with poorer risk features such as relapse and non-favourable cytogenetic profile, FLT 3 ITD is an attractive therapeutic target (Gozgit JM *et al*, 2011) and to this end several FLT3 inhibitors have been trialled in AML. Relatively modest clinical activity has been reported to date (Gozgit JM *et al*, 2011) partly explained by the fact that preclinical studies show that FLT3 inhibition needs to be sustained to facilitate destruction of AML cells carrying a FLT3 ITD mutation to overcome the proliferative advantage of the clone. This sustained inhibition of the receptor may be demonstrated in the laboratory by means of suppression of FLT3 auto-phosphorylation and several authors have shown that a level of <15% of baseline phosphorylation is the minimum cut off to achieve simultaneous cytotoxic effect (Smith BD, 2004) .To achieve maximum therapeutic benefit, continuous, near complete inhibition of FLT3 kinase with minimal off target effects on other tyrosine kinases within the same class III family (e.g. cKIT, FMS, Platelet Derived Growth Factor receptors PDGFR α and β) which are more likely to lead to undesirable physical side effects such as flushing and diarrhoea would be ideal although such specificity is very difficult to achieve due to overlap between domains.

A variety of small molecule tyrosine kinase inhibitors with variable potency against FLT3 have been in development for more than ten years both in *in vitro* and in clinical trials. (Knapper S, 2011) (Levis M & Small D, 2003). In summary their development has followed a structured progression from single agent studies into combination chemotherapy regimens and advanced stage international studies in treatment both at diagnosis and relapse. Progress has been such that these agents may now be sub divided into first and second generation inhibitors, where the first generation (e.g. Lestaurtinib or Midostaurin) were largely 'off the shelf' compounds taken from compound libraries with broad spectrum tyrosine kinase inhibitory properties not designed specifically with FLT3 in mind. In contrast the second generation of agents (e.g. AC220 or Crenolanib) have a more targeted mode of action; they are more potent in terms of FLT3 inhibition and have been developed to specifically target FLT3 (Table 4).

Compound	**Chemical Class**	**Generation**	**FLT3 IC_{50}**	**Other targets**	**Clinical trial development**
Semaxinib (SU5416)	3-Substituted indolinone	1st	100nM	c-KIT, VEGF	Phase II
Sunitinib (SU11248)	3-Substituted indolinone	1st	50nM	c-KIT, PDGFR, VEGFR	Phase II
Lestaurtinib (CEP701)	Indolocarbazole	1st	3nM	TrkA, VEGFR, JAK2	Phase III
Midostaurin (PKC412)	Indolocarbazole	1st	10nM	c-KIT, PDGFR, c-FMS	Phase III
Tandutinib (MLN518)	Piperazinyl quinazoline	1st	30nM	c-KIT, PDGFR	Phase II
Sorafenib (BAY 43-9006)	Bi-aryl urea derivative	1st	3nM	c-RAF, PDGFR, VEGFR, c-KIT	Phase III
KW-2449	n/a	2nd	13nM	Aurora kinase, ABL	Phase II
AC220	Bis-aryl urea derivative	2nd	1nM	c-KIT, PDGFR, RET, CSF1R	Phase III
Ponatanib	n/a	2nd	3.8nM	Pan BCR-ABL c-KIT	Phase II planned (NCRi AML 19 pilot)
Crenolanib	n/a	2nd	<10nM	PDGFR	Phase II

Table 4: Chemical properties of FLT3 inhibitors used in clinical trials adapted from (Knapper S, 2011)

Although there have been a multitude of phase I-III clinical trials involving the first generation compounds, their development has been hindered by the facts that they are generally poorly tolerated, they have minimal activity against FLT3 TKD point mutations (Tandutinib being entirely inert) and none have shown genuine efficacy in terms of inducing sustainable marrow remissions.

Sorafenib (BAY-43-9006) has a slightly improved pedigree compared to most other FLT3 TKIs, having been trialled extensively in both AML and solid tumours. In exception to all other FLT3 inhibitors it is a licensed drug in renal and hepatocellular carcinoma and can therefore be prescribed outside the context of a clinical trial. It has a wide range of therapeutic targets with inhibition of a variety of kinases including FLT3, PDGFR, VEGF and c-KIT (Knapper S, 2011). It has been used both as a single agent in a phase-I dose finding study in refractory AML (Zhang W *et al*, 2008), in several compassionate use programmes, often in relapsed-refractory patients as a 'bridge' to allogeneic transplant or in the setting of relapse post transplant. It has also been combined with a range of therapies including standard induction regimens in the upfront setting (Rollig C *et al*, 2014) where sorafenib treatment was associated with longer relapse free and overall survival in patients carrying FLT3 ITD mutation and there was generalised improvement in event free survival regardless of FLT3 status.

AC220 (Quizartinib) is a novel bis-aryl derived second generation drug that was produced in response to screening a cohort of kinases against a scaffold-focused compound library (Knapper S, 2011) . It has the advantage of both potency and specificity for key interaction sites within the FLT3 molecule and has been shown to be the most potent and targeted in comparison to lestaurtinib, midostaurin, sunitinib, KW-2449 and sorafenib (Pratz KW *et al*, 2010) .

Figure 1.3

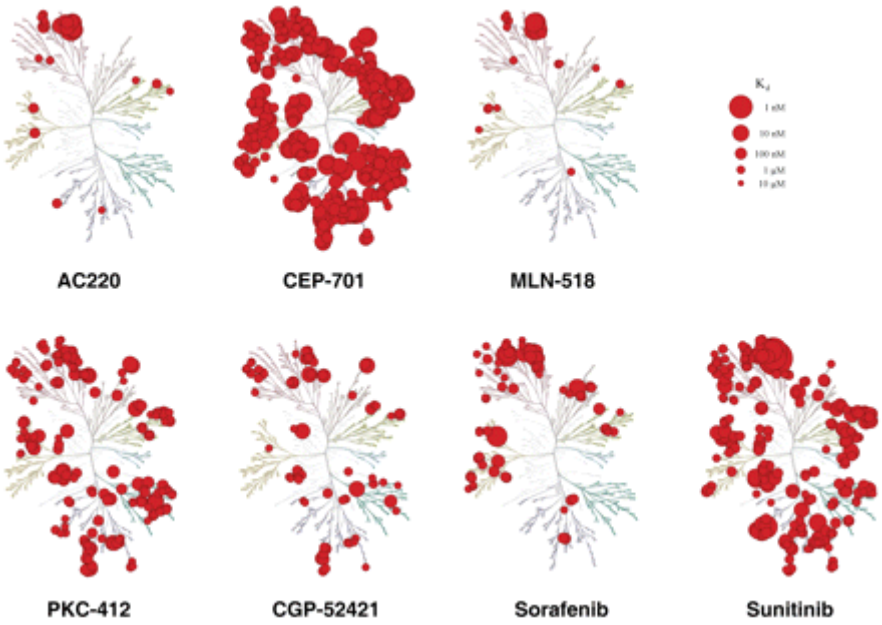


Figure 1.3: Results of KinomeScan binding assay for 7 FLT3 TKIs, the red circles represent geographical areas of the human kinome which are inhibited by the relevant compound and the relative size of the circle is inversely proportional to the IC_{50} binding affinity. Compounds with a smaller number of binding sites are said to be more 'specific' with fewer 'off target effects' image taken from Knapper 2011 1-19 (original source Cell Signaling Technology, Inc).

Safety and efficacy of AC220 was assessed during a phase II open label monotherapy study in patients with relapsed/refractory FLT3 ITD positive AML. Of those who were refractory to their last therapy, 31% achieved a CR with AC220 monotherapy which represents the highest level of single agent activity to date for FLT3-targeted therapy in FLT3 ITD mutated patients but the results were also encouraging for those with FLT3 WT disease suggesting a potential wider application for the drug. It was also highly significant that 1/3 patients in the study were successfully 'bridged' to allogeneic stem cell transplant with AC220, a treatment which represents their only real chance of durable remission and survival.

This study has been followed up with a phase I dose escalation study of AC220 in combination with standard induction and consolidation chemotherapy in patients aged 18-60 years with newly diagnosed AML (Altman J *et al*, 2013). The study showed that safety and tolerability was good and has paved the way for a number of on-going phase III studies looking at the use of AC220 in a similar setting including the NCRI AML 18 and LI-1 studies which are looking, respectively, at the

combination of AC220 with intensive and non-intensive therapeutic strategies in older patients with newly-diagnosed AML.

Almost before it has had a chance to prove its worth, however, AC220 has become somewhat notorious for the development of 'treatment emergent' resistance-conferring mutations in patients treated with the drug. Similarly to the story of TKI development in Chronic Myeloid Leukaemia it seems likely that disease evolution and perhaps 'clonal tiding' as a driver for treatment resistance is relevant in FLT3 mutated AML as in other haematological malignancies and it is this resistance which represents one of the major barriers to on-going drug development in this area. It could be argued that compounds such as AC220 which have been specifically developed to target treatment emergent resistance-conferring mutations such as D835V in the FLT3 molecule are perhaps failing to tackle the primary cause of resistance to treatment in AML. If relapse is determined by the inability to eradicate the disease at an MRD level during first line therapy this lends weight to the argument supporting the use of drugs which can combat mechanisms that allow sub-populations of the leukaemic clone to evade treatment. One such protective factor is the bone marrow microenvironment and several of the newer generation of drugs used to treat AML in recent MRC studies (such as pacritinib) have been developed to target this mechanism of 'environment mediated resistance' in AML.

1.8 Summary of resistance mechanisms to FLT3 inhibitors

One of the main lessons from early preclinical and clinical studies of FLT3 inhibitors has been that some patients with FLT3 mutations are resistant to treatment, in spite of laboratory evidence of suppression of FLT3 phosphorylation or silencing of further downstream targets as evidenced by Western blot readouts. This lack of direct correlation between the bench and bedside observations can be explained by four major 'resistance mechanisms'.

Firstly, the degree of 'addiction' to FLT3 signalling varies within the same patient between samples taken at diagnosis and at relapse. Data compiled from phase I and II monotherapy studies suggests that around 30% of patients with FLT3-ITD mutated AML have primary resistance to FLT3 TKIs (Stone RM *et al*, 2005). At diagnosis the dependence on FLT3 signalling is generally less and therefore patients are less likely to benefit from targeted FLT3 inhibition. Inhibition of FLT3 alone is not enough to induce apoptosis in a significant proportion of FLT3 ITD positive samples taken from newly diagnosed patients (Levis M, 2011) such that some patients demonstrate inherent resistance despite evidence of almost complete inhibition of FLT3 tyrosine phosphorylation. Also an individual's sensitivity to treatment may vary depending on the physical properties of their given mutation, particularly relevant to point mutations, for example different FLT3 TKIs show distinct inhibitory activity against different FLT3 TKD mutations (Baker S *et al*, 2014) . Finally in this area, up-regulation of anti-apoptotic proteins such as B cell lymphoma 2 (BCl-2) has been shown in FLT3-expressing cell lines and primary AML blasts that have developed resistance to FLT3 TKIs which can be overcome in vitro using the BH3-mimetic ABT-737 (Kohl TM *et al*, 2007).

Secondly, responses to FLT3 inhibitors are often transient with a relatively rapid loss of response. Possible explanations include insufficient plasma drug levels or short plasma half life due to either poor oral bioavailability or hepatic metabolism which may lead to incomplete/transient inhibition of FLT3 autophosphorylation and therefore incomplete elimination of the leukaemic clone (Kindler T *et al*, 2010).

Alternatively, resistance may be explained by the emergence of new acquired mutations in response to treatment, again particularly TKD point mutations. This phenomenon has hampered treatment strategies with TKIs in CML and in AML an in vitro screen to detect mutations in the adenosine-tri-phosphate (ATP) binding pocket of FLT3 identified 4 mutations which conferred resistance to SU5614, midostaurin and K-252a. In an attempt to overcome this, sorafenib has been used in combination with sunitinib in FLT3 ITD mutated AML to explore whether dual-TKI therapy could

target ‘treatment emergent’ FLT3-TKD mutations (Baker S *et al*, 2014). The contrasting, non-overlapping profiles of resistance of cells expressing FLT3 TKD mutations (based on cell line models and binding assays) to these two drugs was given as an explanation for how sunitinib may be effective if given following therapy with sorafenib that results in resistant disease. Importantly there was some evidence in this trial that sunitinib could retain activity against F691L and D835H mutations both *in vitro* and *in vivo* suggesting potential clinical benefit for combinations of FLT3 inhibitors.

Thirdly, as previously mentioned, an increase in levels of FLT3 ligand (FL) in response to chemotherapy treatment has been suggested as a mechanism of resistance to FLT3 TKIs. FLT3 ligand hyperactivates FLT3 signalling and its downstream cascade, including the MEK/ERK and mTOR/S6K pathways, potentially promoting cell survival and enhancing disease activity (Smith C *et al*, 2012).

Finally and perhaps conceptually the most simple to grasp, if FLT3 is over activated by means of a mutation, then other pathways may become more dominant to negate this effect and make FLT3 signalling effectively redundant. Potential ‘survival mechanisms’ include persistent activation of STAT5 via JAK2 or ongoing MAPK/ERK pathway activation (Kindler T *et al*, 2010). Treatment may also induce activation of compensatory survival pathways such as MEK/ERK where up-regulation may lead to a reduction in dependence of the clone on FLT3 signalling, thereby turning the mutation into a ‘bystander’ at relapse. These factors are felt to be increasingly important when AML cells are in contact with the bone marrow stroma, reflecting the alteration in signalling pathway activity within the bone marrow microenvironment compared to the peripheral circulation.

1.9: The role of the bone marrow stroma

A recurring feature of treatment with FLT3 TKIs over the years has been the achievement of significant reduction in peripheral blood blast percentage without any meaningful, sustained effect on bone marrow disease. This leaves a reservoir of disease within the stroma niche that may remain

quiescent for a given period of time until conditions are favourable to induce a proliferative response and subsequently disease relapse. The quiescent nature of this population may be part of the mechanism by which it evades both cytotoxic clearance and eradication by usual mechanisms of programmed cell death.

Previous studies have identified that FLT3 ITD mutated clones show a tendency to emerge and disappear at diagnosis and at the time of relapse (Kottardis PD *et al*, 2002). It is felt that the high incidence of relapse in those who carry a FLT3 ITD mutation may be linked to persistence of a low level resistant; 'non-cycling' clone within the bone marrow microenvironment which can evade treatment in contrast to the active cycling population in the peripheral blood. The persistence of this leukemic stem cell (LSC) population in the bone marrow after chemotherapy is thought to be responsible for the high rate of relapse .This has been confirmed in an animal model where the non-cycling fraction was shown to retain CD34 expression and this marker was used to demonstrate that this population can engraft the non-obese severe combined immunodeficiency (NOD-SCID) mouse. It has been suggested that these non-cycling cells are particularly resistant to treatment with FLT3 inhibitors possibly due to the observation that they may be less reliant on FLT3 signalling for survival than their actively cycling counterparts, despite the fact that the FLT3 ITD mutation can still be detected amongst the stroma adherent population in *in vitro* experiments again potentially due to a lack of reliance on FLT3 signalling for survival (Alvares C *et al*, 201). This suggests a potential therapeutic niche for TKIs with dual target inhibitory properties. So far none of the established FLT3 inhibitors have demonstrated robust evidence of their ability to overcome this stroma-derived protection in laboratory experiments which therefore represents an area of developmental need.

Growth and survival of normal progenitors is tightly regulated and their ability to migrate to other areas of the haematopoietic system is controlled so that cells in circulation are mature and functional. One of the hallmarks of acute myeloid leukaemia is a loss of this regulatory control so that immature forms are able to proliferate and enter the peripheral circulation and it is thought

that this loss of control is greatly influenced by signals derived from the bone marrow microenvironment which may be up-regulated, blocked or modified compared to the normal state.

Organisation of normal BM stroma can be summarised as two distinct compartments: the osteoblastic (endosteal) niche and the vascular niche. Together these areas modulate HSC quiescence, proliferation, differentiation and migration. The stem cells interact with their niche by a process of adhesion and release (migration) which is governed by the exchange of stimuli through a variety of receptors such as CXCR4 and molecular signalling pathways such as those involving JAK, STAT and CD44 expression. Under normal conditions, the vascular and cellular compartments within the BM work in harmony and loss of this regulatory control is critical to disease survival in AML and other malignancies such as multiple myeloma (Percy L *et al*, 2014). The microenvironment provides cytokine support and cell-contact mediated signals to LSCs (Raaijmakers MH *et al*, 2010) through the same mechanisms used by normal cells. For example SDF-1-mediated CXCR4 signalling for homing and mobilisation within the bone marrow (Messinger Y *et al*, 1996) can also act as a chemo-attractant for malignant cells. Another key effector of this abnormal stroma response is CD44, which has been shown to alter gene expression at a microRNA level through its interaction with its ligand/RTK signalling and regulation of promoter methylation sites. This may lead to effective re-programming of leukaemia cells to exhibit a more stem cell like behaviour and remain within the marrow, therefore limiting the potential for successful treatment with conventional agents that are not able to penetrate the stroma niche (Williams K *et al*, 2013). This adhesive signal may also determine disease phenotype through its influence on the level of the presenting peripheral white cell count.

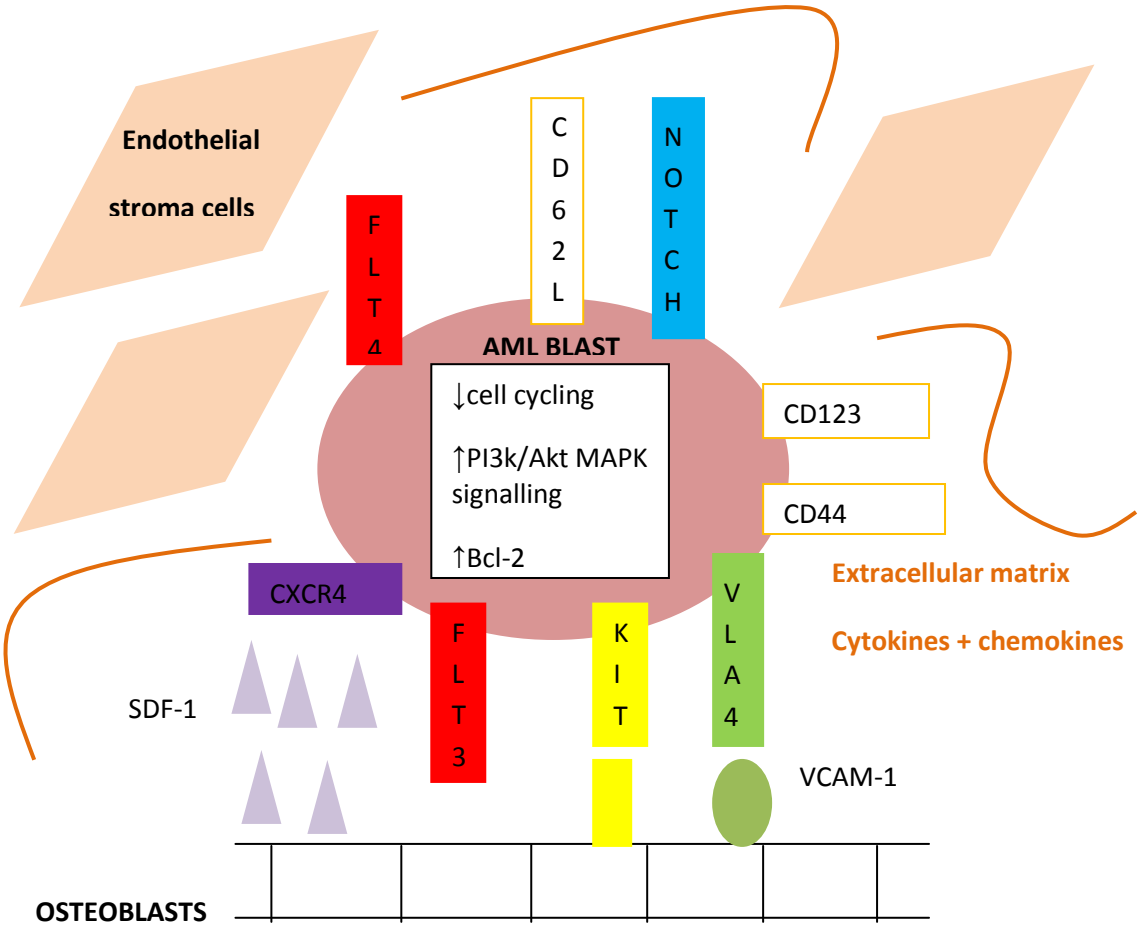


Figure 1.4: Diagrammatic representation of the protective effect of the marrow microenvironment which highlights the interaction between extracellular matrix components, osteoblasts, stromal/mesenchymal cells and receptors expressed on AML blasts, including FLT3 adapted from (Abboud C, 2009).

Certainly, the bone marrow microenvironment provides a supportive background to growth and maturation of normal hematopoietic stem cells, this being its major evolutionary function. Recently it has been proposed that leukemic stem cells may not only benefit from this support network but may also be able to manipulate it to their own advantage over normal progenitors (Tabe Y & Konopleva M, 2014). This interaction may influence the efficacy of FLT3 TKIs in the eradication of these cells.

1.10 Pacritinib overview and mechanism of action

Pacritinib (SB1518) is one of the new generation of tyrosine kinase inhibitors which has been specifically chosen for *in vitro* and clinical study in AML due to its potential to overcome the protective effect of the stroma. This drug is the main focus of laboratory work in the project and its biology, pre-clinical and clinical development will now be discussed in greater detail.

Pacritinib is an innovative pyrimidine based macrocycle with a unique kinase profile showing almost equipotent activity for both Janus Kinase-2 (JAK-2; EC_{50}=23 and 19nM for $JAK2^{wt}$ and $JAK2^{V617F}$ respectively) and FLT-3 (EC_{50}=22nM) (Hart S *et al*, 2011b). It also shows activity against other members of the JAK family (EC_{50}=1280, 520 and 50nM for JAK1, JAK3 and TYK2 respectively) although the potency against JAK1 is particularly weak. A very recent piece of work (Singer J et al) , identified that pacritinib has potent inhibitory activity against all members of the JAK family at low nM concentrations other than JAK 1 and this may explain the relative lack of myelosuppression seen in patients treated with this drug compared to other JAK inhibitors (Hatzmichael E *et al*, 2014).

Pacritinib is a type I inhibitor, meaning that it only binds to the active state ('DFG-in' conformation) of tyrosine kinases. Microarray expression profiling of AML cells treated with pacritinib revealed alterations in CCAAT/enhancer-binding protein α (C/EBPα) and PU.1 expression (known to be impaired by FLT3 signalling and leading to differentiation arrest and a survival advantage for the leukaemic clone) (Mizuki M *et al*, 2003). Following this initial cell line work pacritinib has also shown efficacy in a mouse model context (Zheng R *et al*, 2004). Two models were selected on the basis of their relevance to the molecular targets: Ba/F3-$JAK2^{V617F}$ and MV4-11 allograft and xenograft studies representing cell lines dependent on JAK2 and FLT3 signalling respectively (William AD *et al*, 2015). The MV4-11 xenograft was selected to evaluate the effect of pacritinib on FLT3 signalling and here the drug showed survival benefits at very well tolerated doses.

Other pathways that may also be important in the mechanism by which AML blasts may be able to overcome the inhibitory effect of FLT3 TKIs include Extracellular Regulated Kinase (ERK) 1 and 2 downstream of Janus Kinase (JAK) and STAT5. Activation of both FLT3 and JAK2 receptors leads to phosphorylation of STAT proteins which facilitates dimerisation of this molecule and ultimately governs cell proliferation. Pacritinib can inhibit STAT5 protein activity both via FLT3 and JAK pathways (Hart S *et al*, 2011a) and this dual pronged effect is of potential therapeutic benefit in a variety of haematological malignancies.

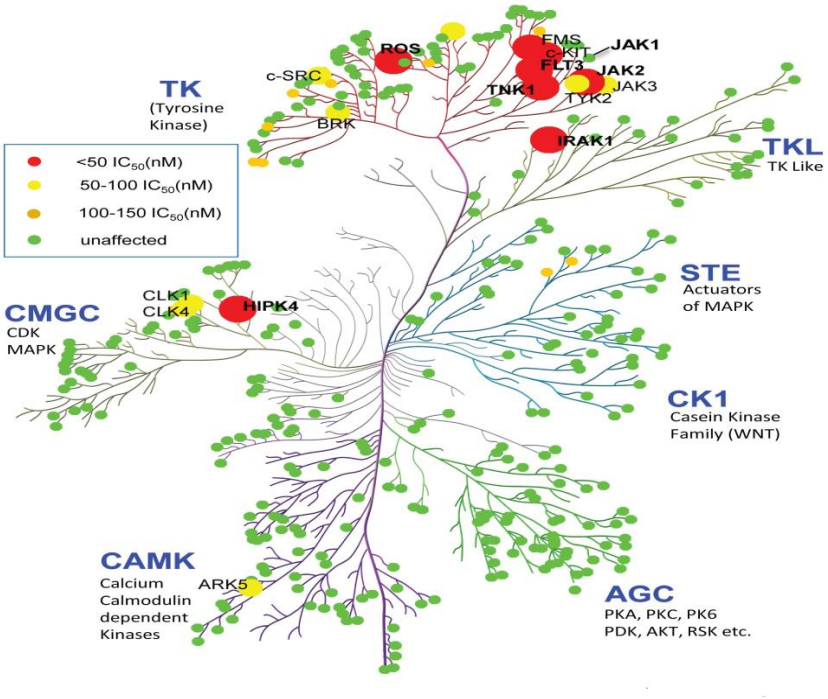


Figure 1.5: Kinome inhibitory activity of pacritinib

1.11 The clinical development of pacritinib in haematological malignancy

Pacritinib has been evaluated in a number of early phase clinical trials where it has displayed considerable activity and acceptable toxicity. Its side effect profile is largely manageable and largely consists of GI side effects such as diarrhoea (Hatzmichael E *et al*, 2014). The patient groups included in these studies included those with lymphoid malignancies where aberrant activation of JAK-STAT signalling is known to play a role in disease pathogenesis (Hatzmichael E *et al*, 2014) . The bulk of its

clinical interest however appears to lie in the treatment of myeloproliferative neoplasms due to the frequency of expression of the $JAK2^{V617F}$ mutation in these disorders and this has been the major driving force behind the therapeutic development of JAK inhibitors in recent years with minimal data on the use of these drugs in AML.

1.12 Clinical trial development of pacritinib

The following table (Table 5) summarises the development of pacritinib in preclinical studies and one recent phase III clinical trial. To date there have been 9 clinical trials in total where pacritinib has been evaluated, 6 of which have completed, 1 terminated and 2 are still in progress .

Study title;identifier	**Phase;number enrolled;status**	**Primary endpoint**	**Diseases**	**Dosing schedule**
Phase I/II study of SB1518 for the Treatment of Advanced Myeloid Malignancies NCT00719836	Phase I/II, 76, completed	Phase I: establish MTD Phase II: assess CBR	AML, CML, CMML, MDS, PMF	Dose escalation:100-600mg Oral 25 days #1 then 28 day cycle
Phase I study of SB1518 for the Treatment of Advanced Lymphoid Malignancies NCT00741871	Phase I, 35, complete	Establish MTD	HL, MCL, FL, SLL, MZL	Dose escalation 100-600mg Oral, 28 day cycle
Phase I/II Study of SB1518 in Subjects with Chronic Idiopathic Myelofibrosis (CMF) NCT00745550	Phase I/II,54,complete	Phase I: establish MTD Phase II; assess CBR	CMF including PET-MF, PPV-MF	Dose escalation 100-600mg orally up to 1 year if tolerated.
A Phase 2 Safety and Efficacy Study of SB1518 for the Treatment of Advanced Lymphoid Malignancies	Phase II,28,completed	Assess overall tumour response by CT/FDG-PET scan and bone marrow biopsy as a measure of efficacy	HL, MCL, FL, MZL, SLL	Oral, 400mg per day, 28 day cycle up to and beyond 12 cycles as tolerated.

NCT01263899				
PERSIST-1 Oral Pacritinib versus Best Available Therapy to treat Myelofibrosis NCT01773187	Phase III,completed recruitment (n=270)	Compare efficacy versus 'best available therapy' in patients with PPV-MF or PET-MF	PMF, PPV-MF, PET-MF	Oral, 400mg per day, 28-day cycle given continuously while patient clinically benefitting.
PERSIST-2 Oral Pacritinib versus Best Available Therapy to treat Myelofibrosis with Thrombocytopenia NCT02055781	Phase II, estimated 300, recruiting	Compare efficacy of two dosing schedules (pooled once daily and twice daily) with that of BAT in patients with PMF, PPV-MF or PET-MF	PMF, PPV-MF, PET-MF	
AML 17 EudraCT 2007-003798-16	Phase III, closed	Patients with FLT3-ITD mutated AML treated within the AML 15 or 17 studies who have relapsed pre or post allogeneic stem cell transplant	Relapsed AML	Oral 200mg twice daily for 28 days. Patient assessed at day 14 and dose may be increased to 300mg BD. Treatment for up to 12 months + 6 month extension if benefit shown

Table 5: Summary of clinical trial experience with pacritinib, adapted from (Hatzmichael E *et al*, 2014) . Key: MTD (maximum tolerated dose), CBR (Clinical benefit rate), PET-MF (post-essential thrombocythaemia-myelofibrosis), PPV-MF (post-polycythaemia vera-myelofibrosis), PMF (primary myelofibrosis), HL (Hodgkin lymphoma), MCL (mantle cell lymphoma), MZL (marginal zone lymphoma), FL (follicular lymphoma), SLL (small lymphocytic lymphoma), CML (chronic myeloid leukaemia), CMML (chronic myelomonocytic leukaemia).

The NCRI AML 17 trial closed to recruitment in December 2014 but continues to gather data regarding the use of pacritinib in relapsed AML patients who harbour a FLT3-ITD mutation. This will offer the strongest indication yet as to the potential efficacy of pacritinib in the treatment of AML in the refractory phase of the disease. As previously mentioned however, the drug may have a therapeutic target in preventing the development of primary resistance to FLT3 TKI therapy in the

upfront setting due to its potential to overcome the protective effect of the bone marrow stroma and this will now be discussed in the final section of the introduction to this project.

1.13 The potential advantage of pacritinib in overcoming the protection of the bone marrow microenvironment

FLT3 ITD activation of signal transduction pathways entails the interaction of numerous protein kinases and underscores the possible therapeutic advantage of targeting tyrosine kinases acting downstream of the receptor. Increasing dependence on JAK2 signalling has been proposed as a mechanism of resistance of FLT3 ITD mutated AML, particularly in the population of cells adherent to the bone marrow microenvironment. It is well described that resistance of MPNs to JAK2 inhibitor treatment is mediated by cytokines secreted by stroma cells, namely IL-6, FGF4 and CXCL-10/IP-10 (Manshouri T *et al*, 201) for example because IL-6 is known to activate JAK/STAT signalling (Weisberg E *et al*, 2012). High circulating levels of IL-6, IP-10, KC and MCP-1 have been measured in AML mouse models in the absence of drug treatment where addition of single agent pacritinib lead to normalisation of IL-6, IP-10 and KC levels (Novotny-Diermayr V *et al*, 2012) suggesting that the drug may be able to overcome the protective effect of the bone marrow microenvironment. Similarly, Weisberg et al tested a panel of JAK inhibitors for the ability to potentiate FLT3 inhibition in an attempt to overcome cytoprotection mediated by stroma derived cytokines. They found that JAK inhibitors synergise with FLT3 inhibitors against mutant FLT3 expressing cells in a stroma co-culture environment.

There are some data to suggest that treating FLT3 ITD positive AML cells with FLT3 TKIs that have no anti-JAK2 activity (e.g. sunitinib) may lead to additional up regulation of this pathway so that the cells become resistant to treatment (Hart S *et al*, 2011a), presumably because constitutive FLT3 activity is no longer the major factor driving survival. With all this evidence in mind, it is logical to conclude that due to its dual target effectiveness pacritinib may be a viable therapeutic option for

both patients with FLT3 ITD mutated disease and those patients who do not carry a FLT3 ITD mutation, which represents the majority (approximately 70%) of patients at diagnosis and could eventually support it's usage as part of induction therapy for all. The concurrent inhibition of both FLT3 and JAK2 pathways induced by pacritinib may carry potential to override the environment mediated resistance that results from interactions between stromal cells and leukaemic cells which is frequently observed in AML (Hatzmichael E *et al*, 2014). This direction of therapeutic design is novel compared with previous mechanisms to tackle resistance induced by for example 'treatment emergent' mutations. If targeting the stroma niche population holds the key to MRD erradication then theoretically treatment emergent mutations could become a feature of the past in this aspect of AML therapy.

1.14 Study Aims

In summary, it is clear that there remains a paucity of effective therapies which can target the FLT3 signalling pathway in frontline therapy for AML. The laboratory and clinical development of existing agents has been limited by a lack of durable potency and a particular inability to clear blasts from the bone marrow niche. There are a variety of underlying explanations for this at a mechanistic level and therefore the aim of this project is to analyse the potency of pacritinib, a next generation dual-targeted tyrosine kinase inhibitor, in inducing a cytotoxic response in primary AML samples and also investigate its mechanism of action and potential to overcome environment mediated resistance in AML.

The aims are as follows:

1. To determine the mean EC_{50} for primary AML cells treated with pacritinib and assess whether potency varies according to FLT3 mutation status, FLT3 cell surface expression, % allelic expression and any other patient characteristics including clinical outcome.

2. To determine the mechanism of cell death in response to pacritinib and to analyse the effect of pacritinib on signalling pathways downstream of FLT3
3. To assess whether pacritinib can target primary AML cells cultured on a stroma monolayer as an *in-vitro* model of environment mediated resistance to therapy
4. To investigate the effect of pacritinib treatment on FLT3 downstream signalling in a co-culture setting.
5. To investigate combination treatment of pacritinib with conventional and novel targeted agents to address signalling pathways arising from a co-culture setting.

The next chapter will describe the materials and methods used to carry out these investigations.

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

All reagents and plasticware were obtained from Sigma Aldrich UK Ltd (Dorset, UK) or Fisher Scientific UK Ltd (Loughborough, UK) unless otherwise stated.

2.1: Composition of Stock Solutions

Primary Cell Thawing reagent 900µl 0.45µM filtered Fetal Calf Serum (FCS Labtech, FCS-SA/500-40507) 20µl DNase 10µg/ml

2.1.1 Western blotting reagents:

Western blotting cell Lysis buffer stock: 1.5ml 5M NaCl, 1ml 1M Tris buffer (pH 7.4), 5ml Glycerol, 0.5ml Igepal NP40, 1ml 0.5M EDTA, 5ml 1M NaF, 30ml ddH_2O

Homogenisation buffer working solution: 1 MiniComplete EDTA free tablet (Roche) dissolved in 1ml ddH_2O, 8.7ml lysis buffer stock, 300µl 0.1M Sodium orthovanadate

Western blotting Running buffer: 950ml sterile water, 50 ml running buffer

Western blotting Transfer buffer: 100ml methanol, 1ml NuPage antioxidant, 50ml transfer buffer, 849ml distilled water

Blocking buffer for immunoprecipitation experiments: 500ml TBS-T, 10% Bovine serum albumin

Blocking buffer for other western blotting experiments: 500ml TBS-T, 25g milk powder

TBS-T: 10ml 1M Tris (pH 7.4), 20ml 5M NaCl, 10ml 10% Tween-20, 960ml ddH_20

Western blotting Stripping buffer: 15g Glycine, 1ml 10% Sodium azide, 6ml HCl, 5ml 10% Tween-20, 988ml ddH_20

2.1.2 Tissue culture Media:

IMDM: Dulbecco's medium, HEPES buffer, sodium bicarbonate, 10% L-glutamine, 10% FCS, Gentamicin

LTC Medium: α-MEM, 12.5% Horse serum (HS *biosera*, Boussens France), 12.5% FCS 1uM Hydrocortisone, 9mM Beta-mercaptopurine, , TPO (0.2%) , IL-3 and GCSF (0.1%) (PeproTech EC Ltd.)

MEM dilution medium: MEM, Penicillin/Streptomycin, 20u/ml Heparin sodium (CP Pharmaceuticals)

MEM complete medium: MEM (Biowest, Ringmere,UK), 10% FCS

RPMI culture medium: RPMI 1640 medium containing 25mM HEPES and L-glutamine,

10% Foetal calf serum, 1% Penicillin/Streptomycin

Flow cytometry Staining buffer: 0.5% BSA, 1x PBS, 0.02% Na Azide

TBS: 10ml 1M Tris (pH 7.4), 20ml 5M NaCl, 970ml ddH_20

7AAD staining buffer 100ug/ml diluted in PBS

2.2: Drug preparations:

Pacritinib (molecular weight 472.58) was supplied by CTI Biopharma Corporation, Seattle, WA, US in 0.5% methylcellulose (w/v) and 0.1% Tween-80 in H_2O. PD035901 (molecular weight 482.19) was purchased from Sigma Aldrich as a white powder. AraC (cytosine arabinoside) (molecular weight 243.2) was supplied by the University Hospital of Wales pharmacy in a glass vial containing 100mg dissolved in 5ml water for injections. All three drugs were dissolved in DMSO at 10mM, aliquoted and stored at -20°C. When required, drugs were diluted in media to an intermediate concentration prior to well dilution.

2.3: Antibodies

2.3.1: Antibodies used in flow cytometry

Anti-CD135 (FLT3) allophycocyanin (APC) mouse monoclonal antibody (Biolegend 313308), Anti-CD45 flourescin isothiocyante (FITC) (Biolegend 304038) and anti-CD34 phycoerythrin (PE) (BD Bioscience 345802) mouse monoclonal antibodies and their respective IgG labelled control antibodies (IgG APC Biolegend 400120, IgG FITC Biolegend 400108 and IgG PE BD 340270) were used.

2.3.2 Antibodies used in Western Blotting

Anti-phospho-STAT5 (pSTAT5) (Tyr 694), anti-STAT5 (9363S), anti-Map-kinase (p44/42) (4695), anti-caspase3 (9664S) and anti-phospho-Map-kinase (p44/42) (9102s) rabbit polyclonal antibodies were produced by Cell Signalling Technology (Massachusetts, US). ECL anti-rabbit IgG and anti-mouse IgG, horseradish peroxidase-linked, species specific secondary antibodies were obtained from Amersham Biosciences (Little Chalfont, UK).

2.4: Tissue Culture

The following experiments were carried out in class II laminar flow biological cabinets on work surfaces sterilised with 70% ethanol in water prior to commencing work. All materials used in tissue culture were either purchased sterile or filter-sterilised prior to use and contaminated waste was disposed of using an autoclave.

2.4.1: Primary cells: Freezing and Thawing

Bone marrow and peripheral blood diagnostic samples were obtained at both the University Hospital of Wales and other institutions throughout the UK from patients newly diagnosed with AML and enrolled in the UK MRC AML 15, AML 16, AML 17 and AML LI-1 studies. All patients gave informed consent for storage of excess material for research purposes at the time of trial entry and samples were collected in preservative-free heparin or EDTA.

A nucleated cell count was performed on a 40µl aliquot of sample diluted in 20ml Isoton II diluents using a Coulter Z2 Particle Count and Size Analyzer (Beckman Coulter). 6-7ml aliquots of patient sample were then layered over equal volumes of Ficoll-Histopaque 1077 (Sigma) using a syringe with 0.8mm aluminium hub needle. Samples were diluted according to the baseline count with warmed MEM dilution medium and FCS to give a maximum of 100 x 10^6 nucleated cells per Ficoll gradient. This suspension was then centrifuged twice and the monolayer pellet was re-suspended in 500µl MEM dilution medium and pooled in a universal container. A further nucleated cell count was then performed.

Mononuclear cells, suspended in MEM dilution medium, were divided into 500ul aliquots in 1.8ml cryovials, to ensure a maximum of 100 x 10^6 cells per vial. 400µl FCS and 100ul dimethylsulphoxide (DMSO) were added to each vial. Cryovials were then transferred to a freezing container half filled with propan-1-ol and placed at -80°C overnight to ensure controlled-rate freezing. Cryovials were then moved to cryovats containing liquid nitrogen.

Cells were recovered from the liquid nitrogen cryostore and 900ul 0.45µM filtered FCS and 20µl DNase 10µg/ml) were added followed by transfer to a water bath heated to 37°C. After 3 minutes the vials were removed and swabbed with alcohol then transferred to a universal container. Next a doubling volume of IMDM complete medium was added slowly, dropwise over 3 minutes in triplicate to prevent osmotic damage up to a volume of 14ml and then the

cells were centrifuged at 1000rpm for 10 minutes prior to resuspension in MEM complete media at $5x10^6$/ml for further analysis.

2.4.2: Cell viability assessment

Prior to spinning, an aliquot of cells was removed for counting, mixed 1:1 volume with 0.4% Trypan Blue viability stain and cells counted by light microscopy using Fast-Read Disposable Counting Chambers (Immune Systems Ltd), where live cells exclude the Trypan blue stain and dead cells stain blue and a minimum of 200 cells was counted. Cell viability was expressed as a percentage of live cells out of the total number of cells. A minimum pre-treatment viability of 80% was required for drug culture experiments.

From the average count the total number of cells in the sample may be calculated using the formula:

Total cells = Average cell count $x10^{6/ml}$ x 14ml volume x 2 trypan dilution factor

2.4.3: Cell Glo Cytotoxicity/Proliferation Assay

Primary AML blasts were cultured with a range of concentrations of pacritinib, PD035901 and AraC used alone and in combination. After 48 hours, Cell Titer Glo (Promega) assay was performed to determine the number of viable cells that remained; this is a luminescent viability-based assay where the number of viable cells in a culture can be determined based on their ATP content, which signals the presence of metabolically active cells.

Opaque walled 96 well plates were marked and then 50ul of IMDM was placed in each required well, excluding the outside wells where evaporation is more likely to occur. Top dose wells were made up to 100ul with further medium and drug added from a working

solution of appropriate concentration (~100-150µM solutions) to give a top dose drug concentration of 2x the desired final concentration. Serial dilution using 50µl from the top wells was applied across the 96well plate resulting in 10 doses in triplicate. Control wells were set up using DMSO (Pacritinib and PD035901) at equivalent concentrations to that found in the top dose wells. Finally 50ul of cells diluted in IMDM from 5 x 10^6 /ml suspension were added to all the drug and DMSO wells (8x10^4/well) and then plates were incubated at 37°C, 5% CO_2 for 48 hours.

At the end of 48 hours, Cell Glo reagent was defrosted at room temperature and shielded from light. Plates were removed from the incubator and allowed to equilibrate to room temperature for 1 hour (to prevent temperature gradients which may affect the rate of the luciferase reaction), then 100ul Cel Glo was added to each well and the plates were then shaken protected from light on an orbital shaker for 2 minutes to allow complete lysis of cells. Plates were read using a Chameleon automated plate reader (Hidex, Finland) on single direct luminescence setting with the aperture adjusted for RLU in the range of20,000 – 5,000,000. The amount of ATP and therefore the luminescence reading is directly proportional to the number of LIVE cells present in culture within this range.

Dose response curves were established for cell viability as a percentage of the untreated/DMSO control according to the formula:

$$\% \text{ Cell Viability} = \frac{\text{Mean luminescence (treated cells)} - \text{background control}}{\text{Mean luminescence (DMSO/untreated control)} - \text{background control}}$$

Calcusyn version 2.1 (Biosoft, Cambridge. UK) was used to calculate a 50% inhibitory concentration (EC_{50}) for cytotoxic responses to pacritinib based on luminescence data from cell glo experiments. and, where relevant, the combination index (CI) for each drug combination.

2.4.4: Stroma Co-Culture Cytotoxicity/Proliferation Assay

MS-5 mouse stroma cells were used for the co-culture experiments purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany www.dsmz.de).

The stroma co-culture plates were set up in exactly the same way as the basic cell glo assay described above to assess the effects of stroma on drug sensitivity. Additionally plates were set up for AML in LTC medium alone to assess the effects of cytokine/serum interaction on AML drug sensitivity.

MS5 control plates with monolayer plus drug at identical concentrations were run to determine the effect of the drug on the monolayer itself. The change in luminescent signal from the drug + MS5 only was later subtracted from for the combined AML + MS5 co-culture plates, to give the change in output purely based on the effect on the AML cells in the co-culture setting.

2.5 Flow Cytometry

Flow Cytometry was performed using a Becton-Dickinson Accuri C6 cytometer coupled to a Dell Optiplex 765 Personal Computer running C-Flow Plus software for data acquisition and analysis.

2.5.1: Immunophenotyping and quantification of cell surface FLT3 expression on primary AML blasts

$1x10^5$ cells were placed in a universal container and centrifuged at 1200 rpm for 5 minutes. The supernatant was removed and re-suspended in 40ul of ice cold staining buffer and then 20ul of this suspension was placed in duplicate in a 96 well V-bottom plate. 10ul each of the following antibodies was added to the first well: anti-CD135 APC, anti-CD34 PE and anti-CD45 FITC to give a total volume of 50ul per well. In the second well 10ul of the relevant anti-IgG1 isotope control for APC, PE and FITC was added. The plate was covered and vortexed gently and incubated at 4°C for 30 minutes. After this time, 150ul of cold staining buffer was added to stop the reaction and the plate was centrifuged at 1200 rpm for 3 minutes and the supernatant aspirated. Cell pellets were re-suspended in 50ul 1ug/ml 7AAD/FACS buffer and then this suspension was transferred to flow tubes for analysis where a minimum of 10,000 live events were recorded. Using C-Flow software blasts were selected using a CD45/side scatter gating strategy and FLT3 expression levels quantified by mean fluorescence intensity.

2.5.2: Annexin V apoptosis assay

MV4-11 cell lines were plated out at a density of $2x10^5$/ml into 24 well plates in triplicate for three, 24, 48 and 72 hours in IMDM medium. Varying concentrations of drug at twice the required final concentration were added to make up to a total volume of 1ml per well. At each time point cells were harvested into a universal container with 3ml ice cold PBS added to wash the cells. Following centrifugation at 1200 rpm for 5 minutes, the supernatant was aspirated and the pellet re-suspended in a cocktail of 97.5ul Binding buffer Annexin V binding buffer: (pH 7.4) 10mM HEPES, 10mM NaOH, 140mM NaCl, 2.5mM $CaCl_2$ (Alexis Biochemicals + 2.5ul Annexin V FITC per well in a 96 well plate. This was incubated at room

temperature for 10 minutes then 100ul of binding buffer was added and the plate spun at 1200rpm for 5 minutes.

After centrifugation the pellet was resuspended in a second cocktail of 190ul binding buffer + 10ul propidium iodide (PI from stock) and transferred to FACS tubes for analysis by flow cytometry with at least 10,000 events per sample recorded. Results were analysed using CFlow Plus software looking at changes in percentage annexin V and propidium iodide positivity with time. Populations were stratified into viable cells (annexin V-/PI-), early apoptotic (annexin V+/PI-) and late apoptotic (annexin V +/PI+) and in particular the percentage increase in the 'double positive' population with time was selected to represent degree of apoptotic induction.

2.6: Western Blotting

2.6.1: Sample preparation and protein extraction

Primary cells were seeded at 3 $x10^6$ per well in 1.5ml in six well plates for each of the relevant time points (1, 24 or 48 hours depending on experiment.) Varying concentrations of drug or DMSO vehicle control were added made up in IMDM to give a total volume of 3ml per well. The plates were incubated at 37°C, 5% CO_2 until time point reached, then harvested into a universal container with 10ml TBS and spun for 5 minutes at 1200rpm. The pellets were aspirated to dryness and snap frozen on dry ice and stored at -20°C until ready for protein extraction.

Western blot experiments were also set up to analyse the effect of stroma on downstream signalling. These experiments were set up in the usual way except that, together with standard IMDM culture, an additional stroma comparison arm was set up using 6 well

plates. After the required time point, cells from the MS5 plates were harvested in two separate fractions. Firstly the suspension cells were aspirated as in previous experiments, washed, spun and frozen. The adherent population (containing both MS5s and adherent AML cells) were removed by washing and trypsinisation, followed by the usual process of centrifugation and snap freezing.

Pellets were transferred on ice and spun briefly then thawed in the presence of 1ul DNase 1mg/ml + $CaCl_2$ for 5 minutes with occasional vortexing. The pellet was resuspended in 150ul complete homogenisation buffer and incubated on ice for 30 minutes during which time samples were vortexed and transferred to pre-chilled eppendorf tubes. The tubes were then centrifuged at 10,000 rpm for 5 minutes after which time the aspirated supernatant was transferred to fresh chilled eppendorf tubes and the total volume of extract recorded. Lysate samples were then stored at -80°C until ready for use.

2.6.2: Protein quantification

In a 96-well Maxisorp flat bottom plate, 10ul protein standards were prepared in advance using BSA and sterile water, then aliquoted in duplicate with 1ul complete added to each standard well to equalize standard curve and unknown readings. Primary samples were diluted at 1 in 10 using 3ul sample lysate + 27ul sterile water per well. Sample dilutions were then aliquoted 10ul each in duplicate and 190ul Bradford's working solution (Bradford's stock diluted 1:1 with SWFIrr) added to all wells. The absorbance of the solutions was measured by reading the plate on a spectrophotometer at 590nm. Samples were equalised for loading using 4x Loading buffer (Life Technologies), sterile water and sample reducing agent and 20 µl of this composite solution was added to each well at the time of running the gels.

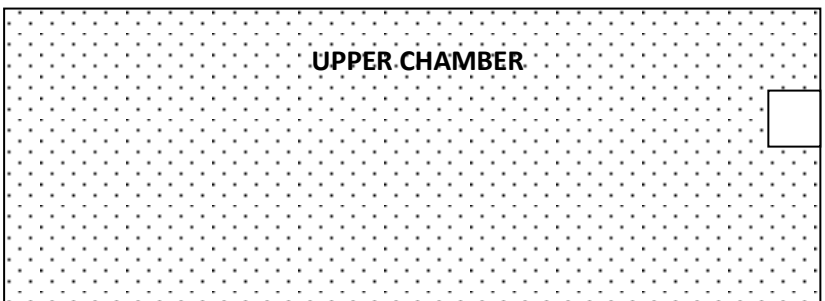
2.6.3: Western gel electrophoresis

Unless stated separately, all pre-prepared reagents and materials used in western blotting including NuPage gels, LDS loading buffer, sample reducing agent, antioxidant, 3-(N-morpholino) propane sulphonic acid (MOPS) running buffer, 20x transfer buffer, Magic Mark XP Western Protein Standards and polyvinylidine difluoride (PVDF) membranes were supplied by Invitrogen Ltd (Paisley, UK). XCell SureLock Mini-Cell electrophoresis tank and XCell II Blot Module were also supplied by Invitrogen.

Prior to loading, samples were incubated in a water bath at 70°C for 10 minutes then quenched on ice for 1 minute. Samples were loaded into pre-cast gels NuPage 4-12% Bis-tris gel (1mm 12 well) NP0322 (Life sciences) previously washed in sterile water and wells were washed and filled with running buffer with added antioxidant (229.425ml running buffer + 575ul NuPAGE antioxidant). Gels were run with MagicMan Marker(Life Sciences) as a running marker, at 200V constant for 50 minutes.

2.6.4: Western transfer

During electrophoresis a pre-cut nitrocellulosemembrane (0.45um pore size) and filter paper blotting sheets were pre-soaked in transfer buffer together with sufficient blotting pads to fill the transfer module. The gel was removed from the tank and after careful removal of the stacking gel a sandwich of blotting membrane and filter paper was constructed around the gel. This was surrounded by pre-soaked blotting pads and the blot module was filled with transfer buffer. The surrounding outer chamber was filled with de-iodinized water. The transfer was then run at 30V constant for 1 hour.



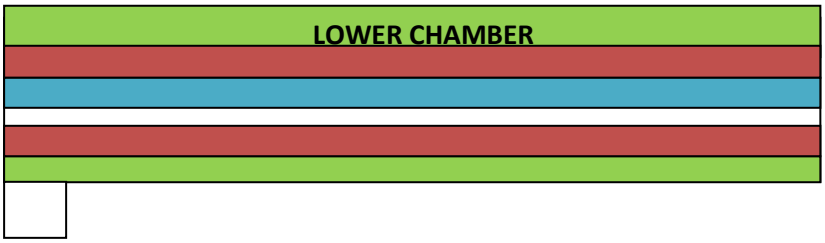


Fig 2.1: Western blotting module diagram: lower chamber contains sandwich of filter paper (red) gel (blue) and nitrocellulose membrane (white) filled with transfer buffer.

2.6.5: Immunodetection

At the end of transfer the gel was removed and washed twice in 20ml double distilled water for 5 minutes. It was then incubated in 10ml pre-made 5% ECL Advance Blocking Solution for 1 hour and then washed in TBST for 15 minutes followed by 3x 5minute washes. After this time the primary antibody was added, made up in 10ml 5% ECL Block and diluted, according to the manufacturer's instructions for the given antibody. The membrane was incubated overnight in primary antibody diluents at 2-8°C.

Following incubation, the membrane was rinsed twice in TBST and then washed for 15 minutes followed by three 5-minute washes. It was then incubated in secondary antibody (rabbit [1/25000] select or mouse [1/5000] prime, depending on the primary antibody used) in 2% ECL Advance Blocking Solution for 60 minutes. After this time the membrane was given a 15 minute wash followed by three further 5-minute TBST washes and then developed with Chemiluminescent Substrate (ECL Select or Prime depending on the antibody) for 5 minutes. Finally the membrane was processed on the LAS-3000 imager for a period of time determined by the strength of the signal ranging from 1-30 minutes.

2.7: Statistical analysis

Statistical significance of the difference in mean cell glo cytotoxicity responses between the entire cohort of FLT3 WT versus ITD mutated samples was determined by Students *t* test where p=<0.05 indicated significance. The data was further analysed at an individual dose level of pacritinib using the Kruskal-Wallis Test where initially the mean score for each dose was calculated for the ITD and WT cohorts separately using the Mann-Whitney U test and the difference between them used to generate a Chi-Square where a Pr>Chi-Square reading of <0.05 was taken as being statistically significant. The same test was used for the stroma cohort to examine the significance of the difference in EC_{50} in IMDM versus MS5 co-culture setting for the entire cohort of 7 samples analysed at each dose of pacritinib. The Mann-Whitney U test was also used, for example to compare the median EC_{50} between cohorts when the samples carrying FLT3 ITD mutations were stratified into low and high allelic ratio making the assumption that the values are normally distributed. This test compares the median EC_{50} to the range of low and high values within each cohort where the null hypothesis asserts that the medians of the two samples are identical and the alternative hypothesis states that a particular population tends to have larger values than the other.

Drug combination data were analysed using the median effect method of Chou and Talalay. The software package Calcusyn (Biosoft, Cambridge, United Kingdom) was used to perform linear regression analysis of dose-response data from each experiment and calculate a combination index (CI) for each individual patient for each drug combination. According to this method strong synergism was implied by a CI < 0.3 and lesser synergy by CI of 0.3-0.9 with a CI of 0.9-1.1 representing an additive effect.

Further statistical analysis was carried out looking at the relationship between clinical characteristics of the overall patient cohort such as age, presenting WBC etc and median

EC_{50} for each group. Given that these data were not normally distributed the Spearman correlation test was used to assess significance of any difference between groups.

Chapter 3

The *in vitro* cytotoxic effects of pacritinib

Chapter 3: The in-vitro cytotoxic effects of pacritinib

3.1 Introduction

FLT3 ITD mutated AML is a heterogeneous disorder which is very difficult to treat effectively. This is exemplified by the fact that despite 10 years of development, no single FLT3 inhibitor has been licensed for this purpose. Weak or transient inhibition of the target has been proposed as an explanation for this relative lack of efficacy. Potent inhibition of cellular FLT3 autophosphorylation and viability has been well described in cell lines (Hart S *et al*, 2011b) (Zarrinkar P *et al*, 2009) including the FLT3-ITD mutated cell line MV4-11 and evidence for translation of this property into therapeutic response in xenograft models has also been shown (Zarrinkar P *et al*, 2009) Several of the early clinical trials of tyrosine kinase inhibitors in AML have shown both improved efficacy in those patients with FLT3 ITD mutated disease and a correlation between achievement of drug plasma levels that are sufficient to produce comprehensive, sustained FLT3 inhibition *in vivo* (via the Plasma inhibitory assay (PIA) assay) and clinical response (Weisberg E *et al*, 2012). Using all these factors we may be able to maximise patient response to therapy provided that FLT3 inhibition and it's downstream effects play a major role in determining cell survival.

Regarding the use of any cytotoxic drug, there will always be a degree of disparity between the ability to establish that cell death has occurred, the level of confidence in the mechanism by which this effect has been produced and therefore, the ability to demonstrate that the pathway targeted by a given agent has been effectively modified to contribute to this effect. Cytotoxicity is a generic term for a destructive effect on living cells which interrupts their ability to divide and proliferate normally. There is a danger that this property will be incorrectly interpreted as being consistently associated with the establishment of cell death whereas many 'cytotoxicity' experiments lead to more of a 'cytostatic' effect where the cell can no longer divide but remains viable. Establishment of cell death is dependent on disruption of cell membrane integrity following cleavage of caspase family proteins which is associated with other morphological features such as membrane blebbing, cell shrinkage and condensation of nuclear chromatin (Zeestraten EC *et al*, 2013). Many high throughput viability based assays cannot distinguish between changes induced by a drug due to inhibition of cell cycle proliferative mechanisms or establishment of apoptosis.

Apoptosis or 'programmed cell death'; refers to a series of genetic events that lead to the controlled death of the cell. It is a normal part of mammalian physiology that is essential for development, tissue homeostasis and immunity. Apoptosis is also part of the internal quality control system that allows abnormal cells to be eliminated; it is therefore unsurprising that loss of apoptotic regulation has been shown to be associated with the development of a variety of malignancies and auto-immune diseases (Czabotar PE *et al*, 2014). In contrast, over-activity of the apoptotic pathway can result in worsening of ischaemic conditions and drive neurodegeneration (Czabotar PE *et al*, 2014). As described in chapter 1, FLT3-ITD mutations play a role in inhibition of apoptosis which is part of the mechanism which confers a survival advantage to the leukaemic clone.

3.2 Methods

3.2.1 Laboratory assays used to quantify cell viability

Several laboratory techniques may be used to measure and quantify the cytotoxic effect of drugs on leukaemia cells. These include colorimetric proliferation assays that measure change in NADPH metabolism as a surrogate marker of cell viability. Such assays incorporate compounds such as MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide or MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphenyl]-2H-tetrazolium, inner salt;MTS) when the tetrazolium salt MTT is reduced to formazan (Sims J & Plattner R, 2009). We and other authors (Savasan S *et al*, 2005) (Haselsberger K *et al*, 1996) have however, noted contradictory results to question the reliable use of this assay for drug screening due to several factors including: inhibitory effect of the drug itself on the tetrazolium salt, drug induced increased cell volume, mitochondrial number and activity (Pagliacci M *et al*, 1993), pH and glucose supply (Marshall N *et al*, 1995) and its inability to distinguish between malignant and normal cells all of which can be independently variable between cellular cohorts and culture conditions particularly in primary AML (Srinivasan D *et al*, 2008).

An alternative technique is the Cell Titer Glo assay (described in more detail below) which measures ATP generation by living cells regardless of metabolic turnover and this measurement is used to generate a reading of percentage viability compared to untreated or vehicle control wells. Using this technique, the same authors showed a reduction in luminescence consistent with the known growth kinetic of the experiment, summarised as a decrease in viability following treatment with imatinib which the MTT assay was unable to show.

The Cell Titer Glo luminescent cell viability assay (CellGlo, Promega, Madison WI) allows quantification of the number of viable cells in a culture system by measurement of the amount of adenyl-triphosphate (ATP) present. ATP is an energy bearing molecule found in all living cells. The energy released following hydrolysis of ATP to adenyl-diphosphate (ADP) is essential for a variety of metabolic reactions. The activity of luciferase enzymes is ATP dependent; therefore, the amount of luminescent signal is directly proportional to the amount of ATP present in the lysate which is, in itself, a reflection of the number of viable cells present. The cell glo reagent also simultaneously inhibits endogenous ATPase release during cell lysis which may otherwise negatively influence the amount of ATP measured.

The assay relies on the properties of a proprietary thermostable luciferase (Ultraglo Recombinant Luciferase) which generates a stable “glow-type” luminescent which is stable for up to five hours. This is another advantage over the MTS/MTT technique where plates must be ‘read’ at specified time points and a different result will be obtained outside of this time window as the colour intensity and therefore absorbance continues to saturate with time. All of these factors influenced the choice of Cell Glo in preference to MTS/MTT assay in attainment of the following results.

3.2.2 Cell Glo Assay method

According to the methods described in chapter 2, primary AML mononuclear cells were obtained, cryopreserved, thawed and cultured. Following the initial thaw a cell count and viability stain was undertaken using trypan blue exclusion (samples with greater than 80% viability were used in cytotoxicity experiments). Sample thawing and plate set up were always carried out within a maximum of 4 hours.

Cell Titer Glo cytotoxicity assay was performed using opaque 96 well plates and doubling serial dilutions as described in chapter 2. For each patient sample the cytotoxic response to pacritinib used at doubling concentrations ranging from 6.0nM to 3μM was assessed. AraC was used at concentrations ranging from 24nM to 3μM in AML cell lines and 180nM to 100μM in AML patient blasts due to decreased efficacy in primary samples. 20,000 cell line cells or 80,000 AML cells were placed in triplicate wells at each drug concentration and cultured for 48 hours at 37°C 5%CO_2 prior to the assessment of viability by the addition of Cell Glo reagent. Prior to harvesting for the Cell Glo assay, a viability assessment was made using one of the untreated AML cell wells and trypan blue exclusion. This was to confirm sufficient survival of untreated/vehicle treated wells, as Cell Glo technique may give a signal from only a few viable cells present. Again the minimum acceptable viability was 80%.

As pacritinib is dissolved in DMSO the cell viability at each drug concentration was expressed as a percentage of cell viability in a control well containing no drug, but at an equivalent concentration of DMSO.

3.2.3 Assays to measure induction of apoptosis

Induction of apoptosis can be demonstrated in the laboratory by a number of different methods including the annexin V/PI technique, measurement of PARP cleavage and induction of caspase-3 by western blotting. The AVB assay has previously been shown to demonstrate that FLT3 inhibitors

induce apoptosis in FLT3 dependent cell lines (e.g. MV4-11) and that this phenomenon correlates with inhibition of cell growth (O'Farrell AM *et al*, 2003).

3.2.4 Annexin V Binding Assay

During apoptosis the arrangement of the plasma membrane phospholipids is disrupted which results in phosphatidyl serine (PS) residues being externalised or 'flipped' from the inside to the outside of the membrane. Annexin V is a protein which binds to phospholipid in a calcium dependent manner; it has a high affinity for PS and can be used as a marker for PS exposure when added to non-fixed cells and analysed by flow cytometry (van Engeland M *et al*, 1997;van Engeland M *et al*, 1997). Since PS translocation is not confined to apoptosis and also occurs during necrosis, a cationic dye such as Propidium Iodide (PI) is added to distinguish the double positive population which have lost membrane integrity and are undergoing late stage apoptosis from those with an intact membrane (PI negative/Annexin V positive).

Annexin V binding assay was performed using 24 well plates and 5 different doses of Pacritinib ranging from 10-200nM together with an untreated well. The dose range was selected in light of the EC_{50} of pacritinib in MV4-11 cells (approximately 50nM) this being around the mid-point of the range. 200,000 viable cells were added to each well and cultured for between 24 and 72 hours at 37°C, 5% CO_2.

Following drug incubation, cells were stained with AnnexinV-FITC and analysed immediately by FLOW cytometry using the Accurri cytometer and C-Flow software as previously described.

3.3 Results: Cytotoxic effects of FLT3 inhibitory compounds

3.3.1 Comparison of FLT3 TKI activity in cell lines

Initially, the efficacy of three different FLT3 inhibitors was assessed in AML cell lines compared to conventional AraC dosing. The drugs tested were ponatanib, crenolanib and pacritinib.

Crenolanib is a highly selective, potent FLT3-inhibitory TKI with activity against FLT3 ITD mutant cells and treatment emergent FLT3 D835 point mutations (Galanis A *et al*, 2013;Smith C *et al*, 2014). The same authors showed that crenolanib has an EC_{50} of around 2nM for inhibition of FLT3 (autophosphorylation by immunoblot assay) and also an EC_{50} of less than 10nM in MV4-11 cell lines when using the MTT assay (similar to data shown in Figure 3.1). This correlates with findings in this study where crenolanib showed a mean EC_{50} of 7.78nM in MV4-11 cell lines, however NB4s were largely resistant to this drug (mean EC_{50} 1120nM)

Ponatanib is a novel, multi-targeted kinase inhibitor that potently inhibits native and mutant *BCR-ABL* at clinically achievable drug levels (Gozgit JM *et al*, 2011) and is also the first drug to suppress all *BCR-ABL* mutations including T315I (Rivera V, 2011). It also has in vitro inhibitory activity against a discrete set of other kinases including FLT3, KIT, FGRF1 and PDGFRα (Gozgit JM *et al*, 2011). Previous authors have shown that ponatanib shows potent activity against MV4-11 cell lines (EC_{50} 2nM) with lesser activity in RS4-11 cells (EC_{50} >100nM) which express wild type FLT3 (Gozgit JM *et al*, 11 A.D.).

Pacritinib is described previously in Chapter 1.10

The cell lines tested included those arising from three different FAB types of AML, two which express wild type FLT3 (NB4 (M3) and HL-60 (M2)) and also the MV4-11 (M4) cell line which carries a FLT3 ITD mutation.

Crenolanib showed good efficacy in MV4-11 cell lines but all others tested were resistant (NB4 and HL-60 data not shown) Figure 3.1. Ponatanib showed good efficacy in MV4-11 cell lines with less potent effect seen in NB4s and HL-60s (Figure 3.2 and Table 6). Pacritinib shows low nanomolar efficacy in MV4-11 cell lines with a less potent effect in NB4 and HL-60s Figure 3.3.

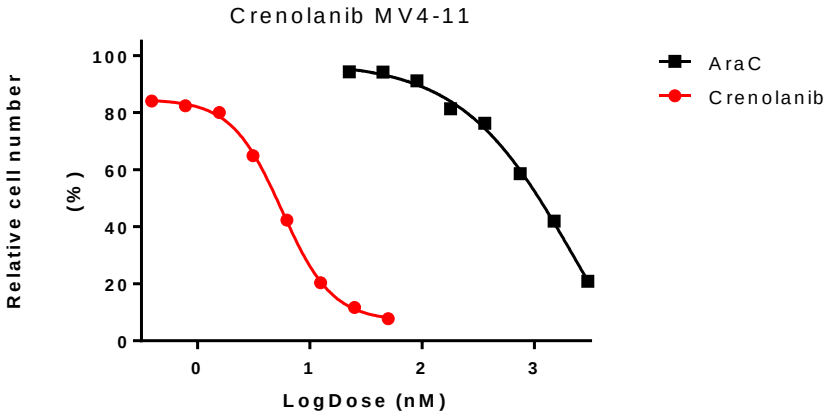
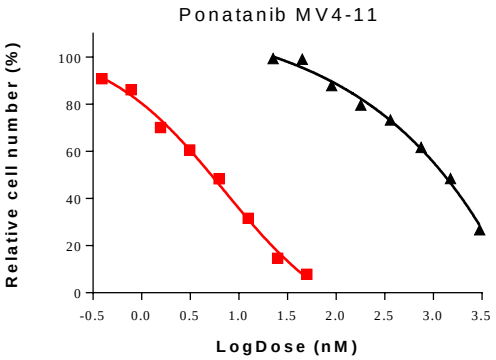


Fig 3.1: Mean cytotoxic dose response to crenolanib and AraC in MV4-11 cell lines



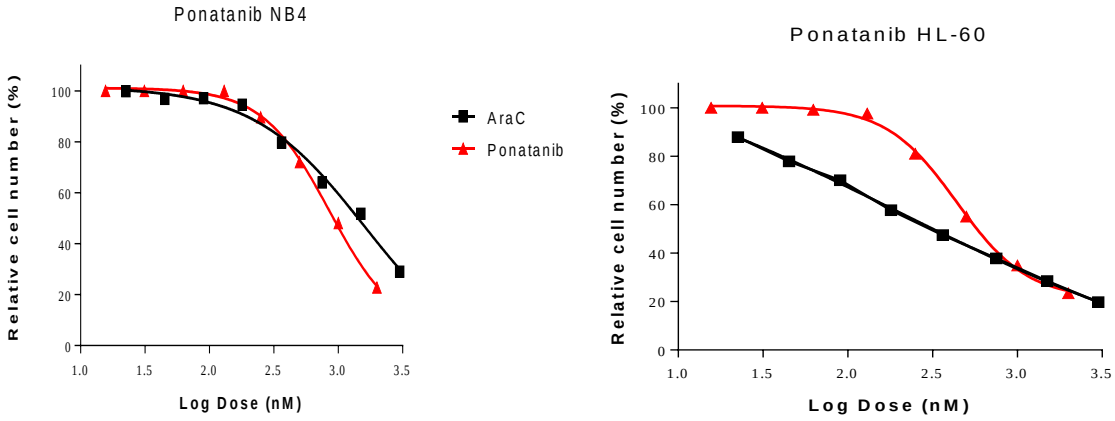


Figure 3.2: Mean cytotoxic dose response to ponatanib and AraC in cell lines.

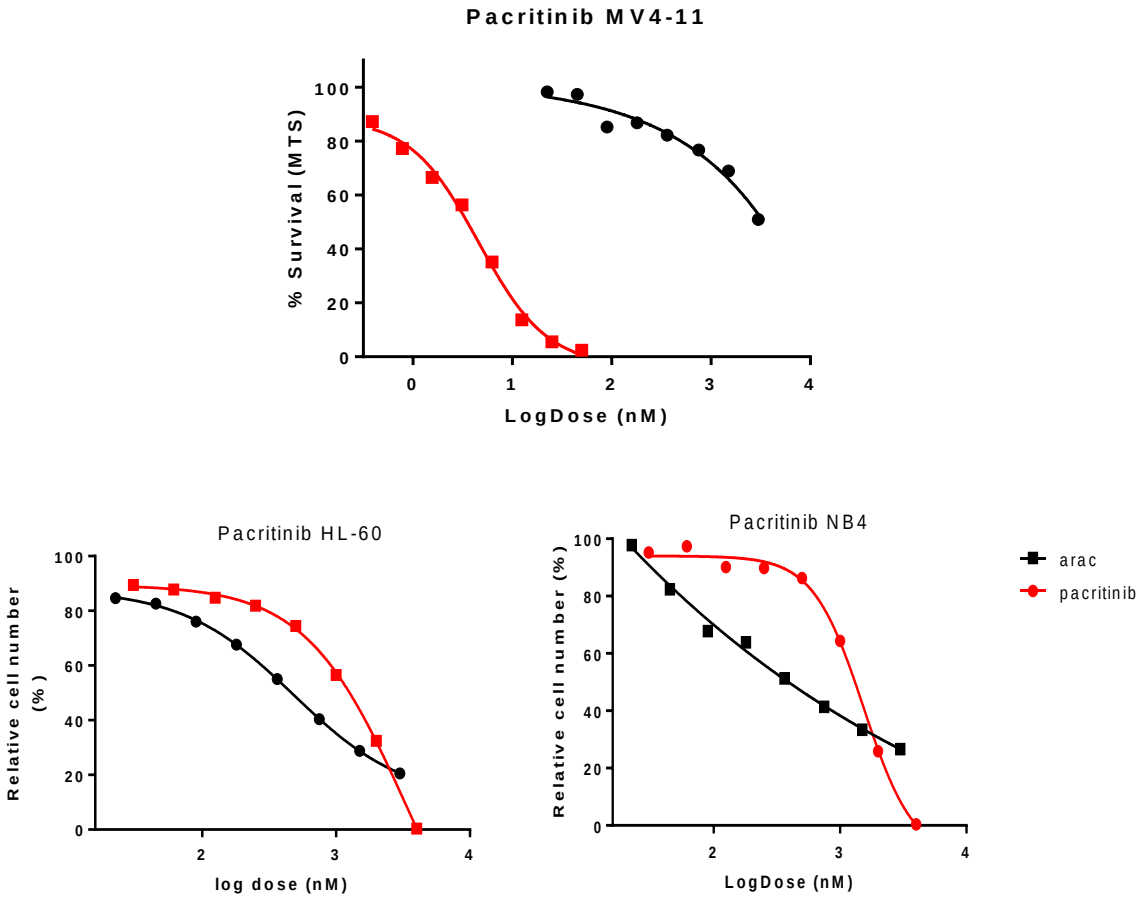


Figure 3.3: Mean cytotoxic dose response to pacritinib and AraC in cell lines (data from Mrs C Guy).

Drug	**EC_{50} NB4/nM**	**EC_{50} HL-60/nM**	**EC_{50} MV4-11/nM**
Pacritinib	1500 (+/-361)*	1433 (+/-230)*	54 (+/-13)*
Ponatanib	522 (+/-149)	715 (+/-26)	4.2 (+/-0.4)
Crenolanib	Resistant (>10µM)	Resistant (>10µM)	7.78 (+/-5.6)
AraC	492 (+/-132)	288 (+/-24)	961 (+/-88)

Table 6: Summary of EC_{50} for FLT3 tyrosine kinases inhibitors and AraC in cell lines; numbers are an average of three repetitions, values given are mean (standard deviation). Data from Mrs Carol Guy *.

In terms of potency, ponatanib appears superior to both crenolanib and pacritinib in MV4-11 cell lines with no other TKI showing considerable effect in either FLT3 WT cell line tested. Pacritinib was

also tested in 72 hour culture as well as 48 hour and the EC_{50}s were equivalent, hence 48 hour culture was taken forward into the primary AML sample experiments to maximise the number of samples surviving the assay.

3.3.2 Results of cytotoxicity experiments with pacritinib in primary AML cohort

Cell titer glo cytotoxicty assay was performed using cryopreserved primary AML mononuclear cells donated by a total of 74 patients enrolled in the MRC AML-15, 16, 17 and Li-1 studies. All samples had been screened for FLT3 activating mutations by other workers in the department using RT-PCR techniques. 63 patients had a confirmed FLT3 mutation status result and were considered eligible for inclusion within this study. Of the 63 eligible patients, 34 cases (52%) expressed only wild type FLT3, 19 (32%) harboured FLT3 ITD mutations ranging from 21 to 174 base pairs in size, 7 (11%) had FLT3 TKD point mutations and 3 (5%) were double-mutants with both ITD and TKD mutations (Table 7).

Characteristic	Number	Median EC50 (range)	p-value (relation between EC50 and characteristic)
All data	63	112 (11-12400)	
Trial‡			
AML15	37	111 (11-12400)	
AML16 (Int + non-Int)	2+2	282 (115-618)	
AML17	22	98 (11.6-1100)	0.2*
Treatment regimen			
Intensive chemo	61	111 (11-12400)	
Non-intensive	2	Values: 184, 380	
Age (years)			
0-29	10	99 (25-779)	
30-39	6	90 (19-361)	
40-49	16	93.5 (11-732)	
50-59	20	191.5 (16-2600)	

60+	11	64.5 (13-12400)	
Median (Range)	47 (0-70)		0.4**
Sex			
Male	31	127 (11-1100)	
Female	32	109.5 (11.6-12400)	0.6*
WBC			
0-9.9	2	Values: 415, 700	
10-49.9	24	121 (22-779)	
50-99.9	18	123.5 (13-12400)	
100+	19	56 (11-732)	
Median (Range)	61.4 (1.8-386.5)		0.005**
Type of AML			
De Novo	58	113.5 (11-12400)	
Secondary	5	111 (20-380)	0.6*
Cytogenetic group			
Favourable	9	133 (20-1100)	
Intermediate	43	111 (11.6-12400)	
Adverse	3	732 (361-779)	
Unknown	8	96.5 (11-2600)	0.3**
WHO Performance status†			
0	33	112 (11.6-2600)	
1	22	124 (13-12400)	
2	3	28 (11-380)	
3	4	85 (16-322)	0.6**
FLT3 status			0.2*
ITD wt	40	147 (19-2600)	
ITD mutant	23	85 (11-12400)	0.01*
TKD wt	51	108 (11-12400)	

TKD mutant	12	138 (24-1100)	0.3*
NPM1 status			
WT	41	115 (11.6-12400)	
Mutant	22	110.5 (11-2600)	0.6*

Table 7: Relationship between patient characteristics and Pacritinib EC50 * Wilcoxon Rank-Sum/Kruskal-Wallis test for difference between groups; **: Spearman correlation coefficient for continuous data/ordered groups; ǂ Trials AML15, 16 and 17 patients were treated intensively up to 2 rounds: ADE (Daunorubicin, Cytarabine, Etoposide), DA/DAT (Daunorubicin, Cytarabine/Daunorubicin, Cytarabine, Thioguanine), FLAG-Ida (Fludarabine, Cytarabine, Idarubicin, G-CSF) (follow-up complete to 1/1/2015). † 1 child completed play performance scale.

Results of initial cytotoxicity assays showed that pacritinib exerts a much greater cytotoxic effect than AraC in primary AML samples (Figure 3.4). Its activity is similar although superior to that of ponatanib and far superior to that of crenolanib (Figure 3.5).

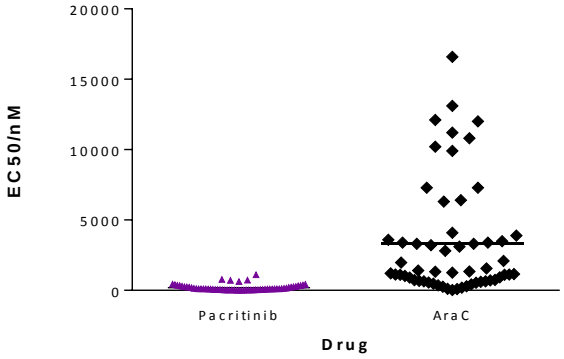


Fig 3.4: Scatter plot for EC_{50} of 63 primary AML samples following treatment with pacritinib and AraC. Mean EC_{50} pacritinib 191.6 nM AraC 3323 nM.

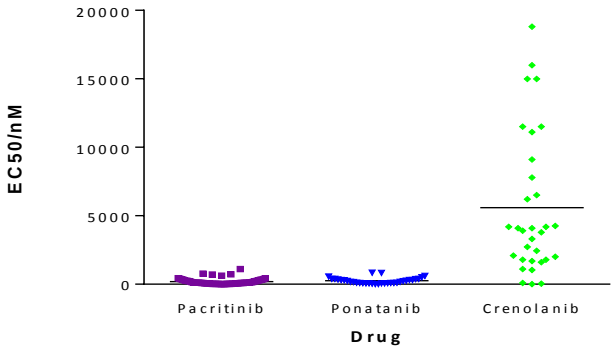


Fig 3.5: Scatter plot for EC_{50} of primary AML samples following treatment with three FLT3 TKIs. Pacritinib mean EC_{50} 191.6nM (n=63 samples), Ponatanib mean EC_{50} 253.8nM (n=34), Crenolanib mean EC_{50} 5588nM (n=32)

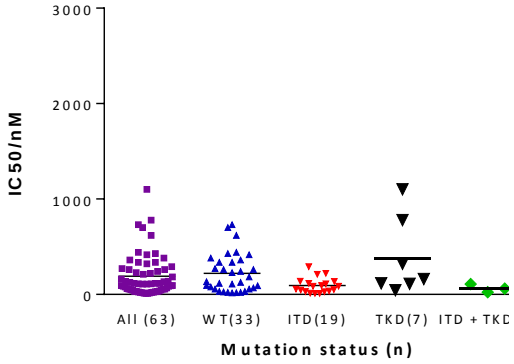


Fig 3.6: Scatter plot of EC_{50} for cytotoxic response to pacritinib in 63 primary samples, sub-divided according to FLT3 mutation status. Mean EC_{50} 191nM all samples, 221nM WT, 93nM ITD, 376nM TKD, 65 nM ITD+TKD. Mann Whitney U tests for EC_{50} < WT value p=0.01 ITD, p=0.5 TKD,p= 0.004 ITD+TKD (*p<0.05)

Using the mean EC_{50} for each cohort as a reference, pacritinib was more cytotoxic to primary AML blasts with FLT3 ITD mutations than those that expressed only wild type FLT3 (Mann Whitney U test p=0.01). This statement masks the fact however, that within each cohort there were individual samples showing markedly high or low sensitivity to pacritinib treatment as would be suggested by normal distribution such as is seen in Figure 3.6.

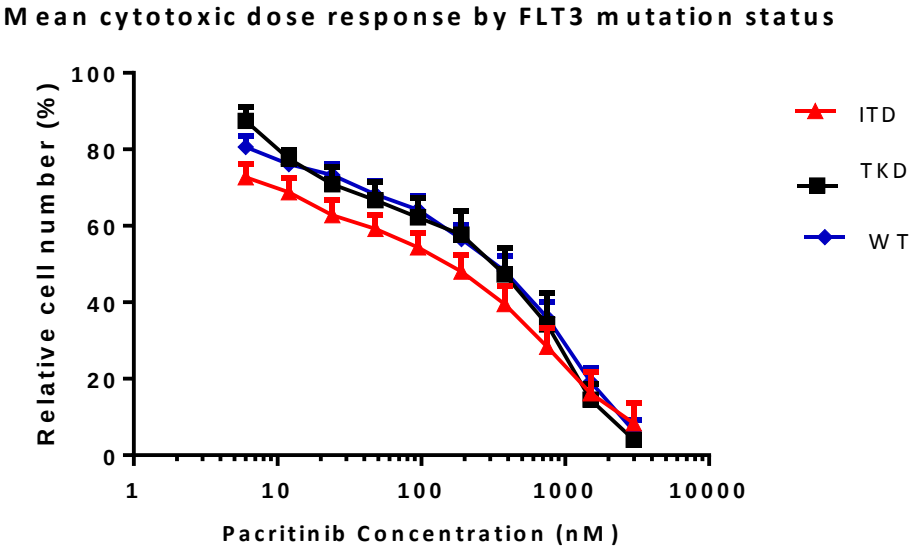


Fig 3.7: Mean cytotoxic dose response to pacritinib normalised to untreated control and grouped by FLT3 status for 60 primary samples, the 3 double mutant samples were excluded from this analysis

as numbers were small. Error bars represent standard error of the mean. Mann Whitney U test p=0.01 ITD vs WT, p=0.5 TKD for mean EC_{50} and for individual doses of pacritinib *=p<0.005 for ITD <WT.

Fig 3.7 shows a difference between the mean cytotoxic dose response as measured by mean % survival in cell glo experiment for primary AML mononuclear cells expressing a FLT3 ITD mutation as compared to WT samples or those with a FLT3 TKD mutation. This difference was significant at the lower end of the dose range from 5nM – 100nM suggesting that at low concentrations of the drug the presence of an ITD mutation can enhance sensitivity. Between the doses 95 and 190nM the significance of this difference disappears which coincides with the threshold of the mean EC_{50} for ITD mutated samples (93nM).

It is interesting to note that there is a significant p value for a lower mean EC_{50} in ITD vs WT (0.01) and also in double mutant ITK+TKD versus WT (0.004). The presence of a TKD mutation alone appeared to have no bearing on sensitivity to pacritinib with a mean EC_{50} greater than that for WT samples. To further assess the impact of the presence of ITD mutations on pacritinib it is rational to consider whether the allelic ratio or percentage mutation of a given sample has any bearing on drug sensitivity. Potentially those samples with a higher percentage mutation might be expected to show higher sensitivity (and hence lower EC_{50}) following treatment with a drug that is assumed to target the FLT3 ITD receptor pathway such as pacritinib. The ITD mutation data for the 19 ITD samples in summarised in table 8 below.

Sample	Age	ITD size	ITD %	source
1. 15-1997 (JH)	47	27	32	BM
2. 15-1979 (DH)	58	57	29	BM
3. 15-2913 (JB)	41	66	9	PB
4. 17-1904 (LS)	22	35/84	92/3	PB
5. 15-1718 (JO)	65	24/42	21/65	
6. 17-1689 (LVA)	61	81	19	BM
7. 15-2729	55	42	15	BM

(AC)				
8. 15-3358 (DP)	45	18/57	29/10	BM
9. 15-3135 (SL)	37	39/90/174	23/4/6	PB
10. 17-3400 (DK)	59	57	63	BM
11. 17-3549 (LH)	42	60	70	PB
12. 17-1629 (SG)	51	18	28	BM
13. 15-3389 (EL)	56	54	17	PB
14. 15-2917 (JB)	45	84	87	BM
15. 17-2093 (SFH)	40	66	62	BM
16. 17-3288 (AAG)	41	27	37	BM
17. 15-3063 (PH)	65	83	8.7	PB
18. 15-1323 (JB)	55	21	43	
19. 15-2098 (GH)	68	51	33	PB

Table 8: Mutation characteristics of ITD samples

Using these data, the samples were then stratified into two groups, either high (33-92%) level percentage mutation or low (9-32%), the top 10 samples were selected for the high ratio group and the remainder for the low ratio group and this data in reference to EC_{50} to pacritinib is presented in the Figure 3.8.

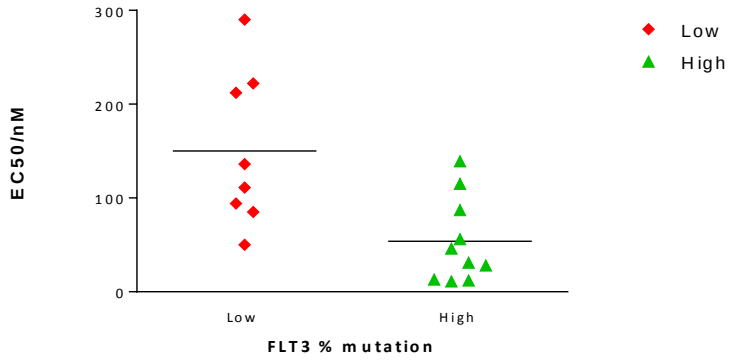


Fig 3.8: Scatter plot for 19 ITD samples EC_{50} stratified by ITD % mutation. Low ratio n=9 (range % mutation 9-32%) , High ratio n=10 (range % mutation 33-92%), p=0.02 (Mann-Whitney U test)

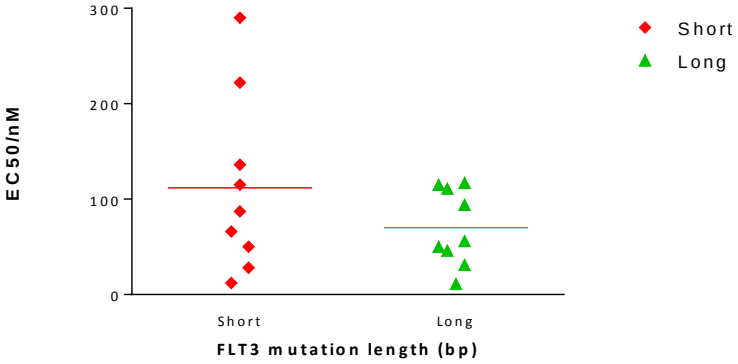


Fig 3.9: Scatter plot for 19 ITD samples EC_{50} stratified by ITD mutation length in base pairs. Short mutation n=9 (range 18-54), long mutation n=10 (range 60-303)

The data shown in Figure 3.8 suggest that a higher FLT3 % mutation is associated with a lower mean EC_{50} in primary AML samples that carry an ITD mutation. Interestingly, the mean EC_{50} is around three fold higher in the low ratio cohort compared to the high ratio cohort (mean 150nM low ratio versus 53.8nM high ratio) and this was a statistically significant difference p=0.02. Also there was a single sample with a very high EC_{50} of > 3000nM, one possible explanation for the resistance is that this sample also carried the lowest percentage mutation (8.7%) which may mean that it's phenotype is borderline and essentially that of a WT sample. There were 4/19 samples that carried two or three different length ITD mutations, in these cases the highest percentage level across all 2/3 mutations was used to stratify patients as either high or low. Similarly the data shown in Fig 3.9 suggests that a

longer length of FLT3 mutation in base pairs is associated with a lower EC_{50} following treatment with pacritinib (mean EC_{50} long mutation cohort 70nM vs 112nM short mutation cohort).

3.3.3 The effect of pacritinib on apoptosis

The data described above suggest that primary AML cells and certain cell lines are sensitive to pacritinib as measured by reduction in cell viability following treatment. It does not provide absolute confirmation that cell death has occurred and this question will now be addressed. As described above the annexin V binding assay can be used to measure apoptotic induction in cell lines and primary AML cells by tracking the percentage change in number of viable and apoptotic cells over time following drug treatment. Using combined staining with annexin V and propidium iodide this transit can be characterised as described in the example flow cytometry plots below.

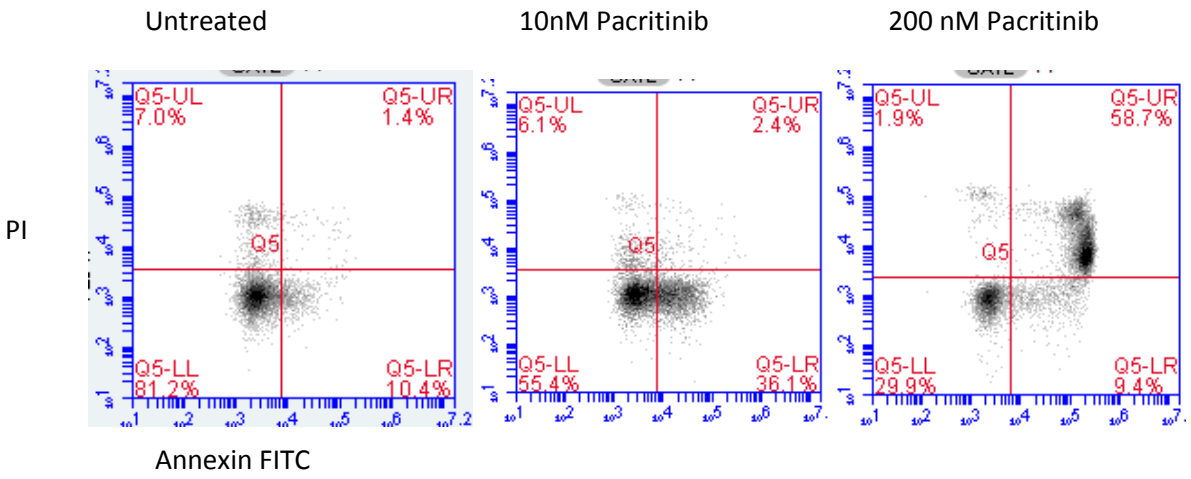


Fig 3.10: Example plots for Annexin V (AV) vs Propidium Iodide (PI) staining of MV4-11 cells at 72 hours showing transit from predominant viable (AV-PI-) population to early apoptotic (AV+PI-) and late apoptotic (AV+PI+) population.

Figure 3.11 below shows percentage late apoptosis induction (annexin V/PI ++) over time for untreated MV4-11 cells and 5 concentrations of pacritinib (10nM, 20nM, 50nM, 100nM, 200nM). The experiment was repeated in duplicate with different MV4-11 cell batches on different days.

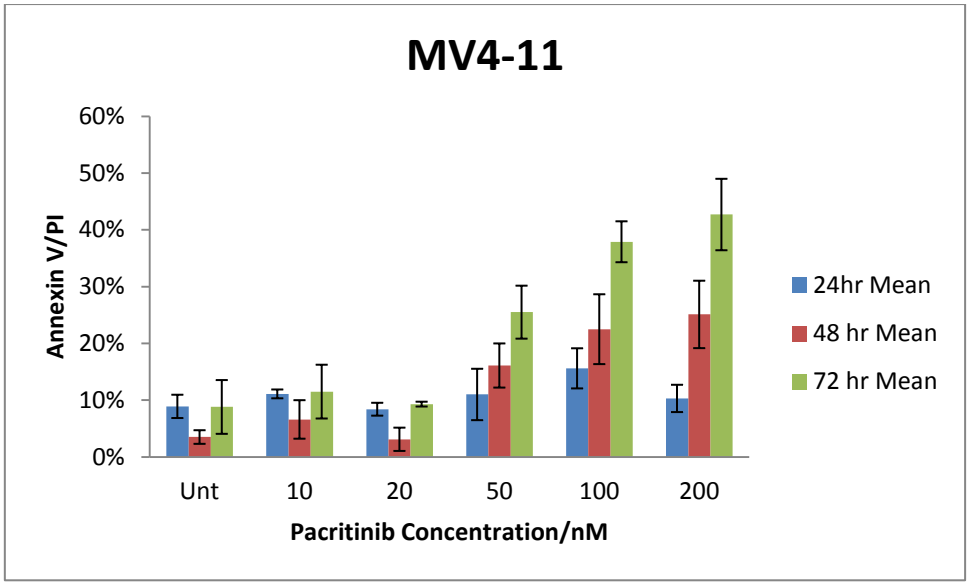


Fig 3.11: Percentage late apoptotic induction (annexin V+/PI +) over time for a range of doses of pacritinib from untreated to 200nM. Error bars represent standard deviation and n=2

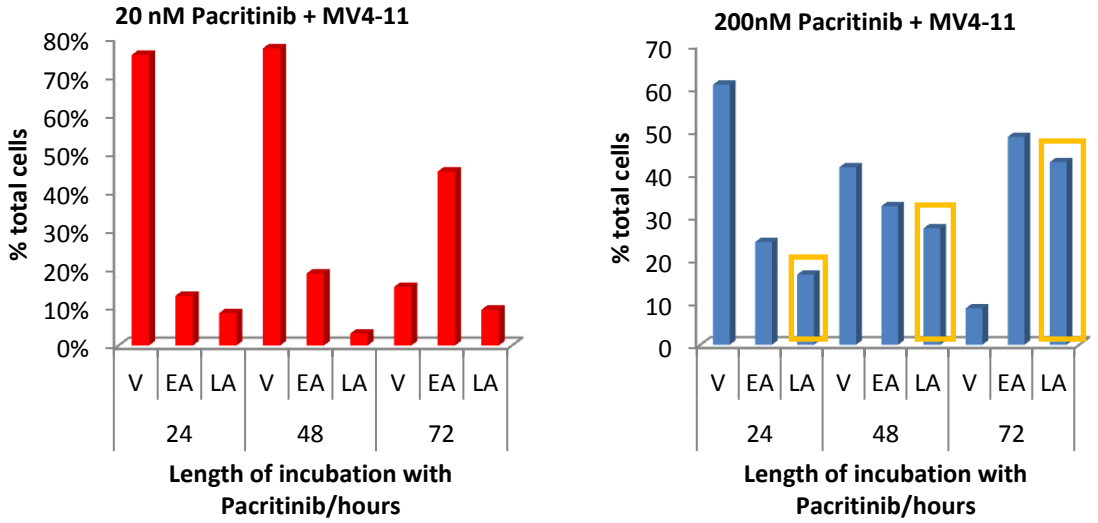


Fig 3.12: Change in response of MV4-11 cells to pacritinib over time for 2 concentrations (20nM and 200nM) V=Viable (AnnexinV-PI-) EA= (Early) Apoptotic (annexin+/PI-) LA= (Late Apoptotic) (++) Data are the mean of two independent determinations. Bars highlighted in yellow show increase in late apoptotic population size with time for 200nM dose.

Figure 3.12 above shows that there is a progressive induction of apoptosis with time which is also dose-dependent. There is a gradual increase in early and late apoptotic cell numbers with time more marked for the higher dose level, with numbers approaching 50% apoptotic induction only seen after 72 hours of incubation. Significant late apoptotic induction was only seen with the higher dose

level (200nM) when the effect was quite marked, but it was largely absent at the lower dose shown (20nM).

The annexin V binding assay was also carried out in primary AML mononuclear cells (3 samples all of which carried a FLT3 ITD mutation) following treatment with pacritinib and culture for 24,48,72 hours and also 7 days shown in figure 3.13.

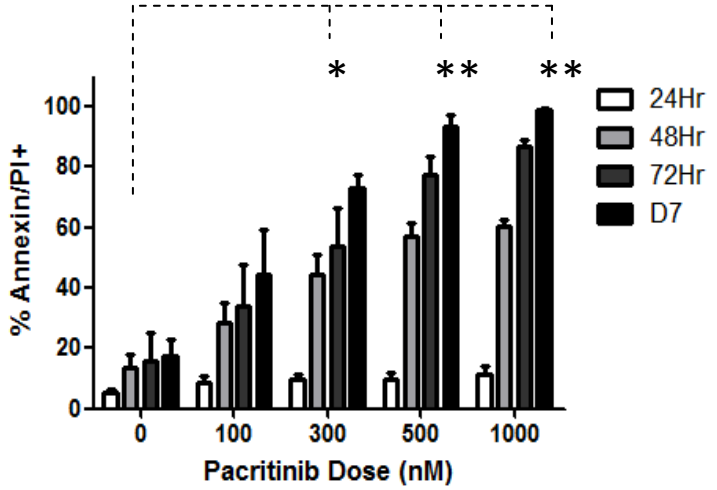


Fig 3.13: percentage late apoptotic induction (Annexin V+/PI+ cells) over time for 3 primary AML samples showing significant dose and time dependent response compared to untreated control . * p<0.001 ** p< 0.0001. Data from Mrs Michelle Lazenby.

These data show minimal late apoptotic induction in primary AML cells following 24 hours of culture with pacritinib with a failure to achieve a level of 20% Annexin V+/PI+ at any dose tested. There was however a markedly improved response after 48 hours with >50% achieved for 500nM and 1000nM dose and this trend continued up until 7 days of culture.

3.3.4 Caspase 3 induction

Generation of an apoptotic signal is followed by activation of a common final catalytic pathway involving the caspase and cysteine protease enzymes. This ultimately leads to cleavage of structural proteins and a loss of membrane integrity, (Czabotar PE *et al*, 2014).Western blotting experiments were carried out using MV4-11 cell lines and primary AML cells as described in detail in chapter 4.

The same membranes were subsequently re-probed using cleaved caspase 3 primary antibody with an example given below in figure 3.14 for sample 15-2913 (ITD mutated) and MV4-11.

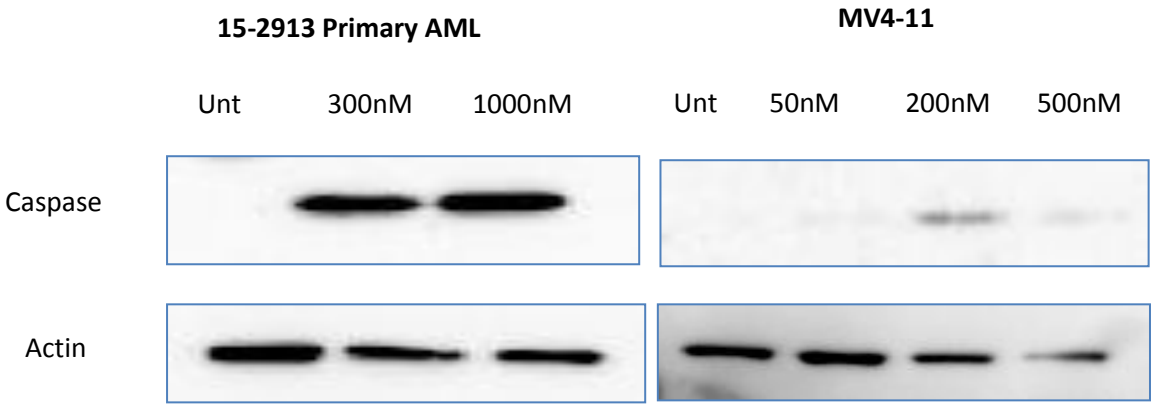


Fig 3.14: Cleaved Caspase 3 induction in primary AML sample and MV4-11 cell line at 24 hours

This experiment shows induction of caspase 3 protein in MV4-11 cell line and AML primary mononuclear cells (single example shown) following treatment with pacritinib which was not seen in the untreated cells. It correlates with the result of the AVB assay above and suggests that pacritinib can induce cell death via apoptosis.

3.4 Discussion

The results of the experiments described in this chapter have shown that pacritinib induces a cytotoxic response in AML primary mononuclear cells at 48 hours, whereas the effect in cell lines is more likely to be cytostatic. Although the 48 hour Cell Glo data in cell lines (fig 3.1 and table 1) suggest a cytotoxic effect, the evidence from the MV4-11 annexin experiments (figures 3.11 and 3.12) confirms that significant apoptotic induction (close to 50% late apoptotic cells) only occurs after 72 hours. This apoptotic blockade is probably related to cell cycle arrest at G2 phase given that MV4-11 cells carry a p53 mutation (Zauli G *et al*, 2012) which is therefore a reflection of the cellular processing mechanisms of the cell line rather than a deficiency in the effect of the drug. Most primary AML cells retain p53 activity and hence less limitation on apoptotic induction is seen in the AVB assay as shown in figure 3.13 where levels approaching 50% late apoptotic cells are seen at doses above 300nM from 48 hours onwards.

This highlights several of the limitations of the use of the Cell Glo assay in this study; firstly that it was only used to assess short term cytotoxicity to Pacritinib after 48hours of treatment. The rationale for using a 48 hour time point for analysis was derived from the observation that in MV4-11 cell lines the mean EC_{50} was similar at 48 and 72 hours of culture. Primary AML cells can be difficult to support beyond 48hours of culture without using supplemented media which would introduce additional confounding signalling effects so this aspect was not formally tested. Taking the data from the Cell Glo and AVB cytotoxicity experiments together however, we see significant Cell Glo efficacy with confirmatory evidence that apoptosis has occurred at 48 hours in primary cells which is not seen in cell lines until 72 hours. To resolve these conflicting results it would be necessary to carry out cell cycle analysis to establish whether cell cycle arrest is responsible in MV4-11 for the delayed apoptotic induction.

Cell lines which express FLT3 ITD mutations (MV4-11) are markedly more sensitive to pacritinib than AraC (mean EC_{50} 54 vs 961 nM) which is a more standard chemotherapeutic drug used to treat AML. There was less of a difference between sensitivity to these two drugs in cell lines which express WT FLT3 (NB4 and HL-60); this suggests that the drug is appropriate for FLT3 targeted therapy with a therapeutic dose which is low and clinically achievable given that in humans, pacritinib achieves steady state free drug levels of approximately 200nM at a standard 400mg daily dose (Singer J *et al*, 2014)

When comparing three different FLT3 TKIs however, although all three drugs preferentially target the ITD mutated cell line MV4-11, pacritinib showed marginally reduced efficacy in comparison to both ponatanib and crenolanib in this context. The MV4-11 cell line was established from the blast cells of a patient with biphenotypic B-myelomonocytic leukemia and although it carries a FLT3 ITD mutation, it's *in vitro* behaviour may not be a good predictor of *in vivo* drug performance. The differential response to pacritinib as compared to pontanib and crenolanib in MV4-11 cell lines may be a reflection of the fact that the latter two drugs rely on cell cycle interference whereas pacritinib

does not. Therefore the effects of ponatanib and crenolanib can overcome the presence of p53 mutation in MV4-11s by inhibition of cell cycle transition at alternative site, an effect which pacritinib is unlikely to induce. This hypothesis would need to be confirmed in future work by carrying out experiments specifically analysing the effect of pacritinib on the cell cycle. One other interesting observation is that pacritinib and crenolanib show virtually no activity in NB4 or HL-60 cell lines whereas ponatanib (and also AraC) performed better. Two possible explanations again relate to cell cycle activity given that NB4 and HL-60 carry un-mutated p53 so can proceed normally through apoptosis and, secondly, that this is probably triggered by off target effects of ponatanib and AraC (an S phase inhibitor) that are distinct from FLT3. This was not observed in primary culture where the cells cycle less rapidly than cell lines, suggesting good efficacy for pacritinib *in vivo* in AML patients.

We also observed a marked segregation in sensitivity to pacritinib by FLT3 status in primary blasts with the mean EC_{50} in FLT3 ITD mutated samples being 92.3nM vs 292.1nM for FLT3 WT samples p=0.01. This is similar to values described elsewhere in the literature (Hart S *et al*, 2011b)) . Although these corresponding data were taken from a smaller cohort of 14 primary samples (compared to 63 samples in this study), similarly increased sensitivity was seen amongst those samples harbouring a FLT3 ITD mutation. The 63 samples used in this study were analysed according to certain clinical characteristics and mutation analysis to determine whether any were significantly associated with in vitro sensitivity to pacritinib (Table 2). Of these only the presence of an ITD mutation and higher presenting WBC showed a significant positive correlation. In addition higher presenting WBC is known to associate with presence of a FLT3 ITD mutation itself due to a proliferative effect on the clone and the observation of these two factors together validates the ITD mutation as a driver of pacritinib sensitivity.

The experiments in this chapter have also analysed the impact of ITD mutation level on *in vitro* sensitivity to pacritinib both in terms of percentage mutation (figure 3.8) and mutation length in

base pairs (figure 3.9). Previous authors have suggested that there is no definite correlation between ITD mutation length (in base pairs) and response to inhibitors including AC220 (Zarrinkar P *et al*, 2009). There is a larger body of evidence to support the rationale that FLT3-ITD mutant load or % mutation is more predictive of response to therapy *in vitro* and in clinical studies (Gale R *et al*, 2008) with higher percentage mutation being associated with poorer prognosis due largely to a higher risk of relapse (Whitman SP *et al*, 2001). In this study there was a correlation between samples with a higher mutation percentage being more sensitive to the effects of pacritinib (mean EC_{50} low:high mutant groups = 150nM:53.8nM p=0.02) which not only adds weight to the evidence that pacritinib targets ITD mutated disease but also suggests a potential therapeutic benefit if the higher level percentage mutation phenotype is associated with an increased risk of relapse. The data also suggests that a larger ITD mutation size (in base pairs) may also be associated with increased sensitivity to pacritinib (mean EC_{50} long mutation 70nM vs 112nM short mutation). This is also encouraging, given that some previous authors have suggested that a longer mutation size is associated with a reduced remission rate and/or reduced overall survival (Breitenbuecher F *et al*, 2009). There is some evidence that the structural location of the mutation is critical depending on whether or not it involves the kinase domain or the juxtamembrane domain.

Interestingly, further sub analysis of the initial Cell Glo work shows that the difference between mean cytotoxic response for ITD mutated sample vs. WT was only significant at doses <100nM which suggests that the lower mean EC_{50} in ITD samples is correct and also that above this dose level the presence of an ITD mutation does not confer increased sensitivity to the drug, presumably due to loss of the negative effect on the constitutive activation of the FLT3 receptor (conferred by the ITD mutation) which drives a proliferative survival advantage in untreated cells. It also shows drug specificity in the sense that at a low dose (<100nM) pacritinib acts in a FLT3 specific manner and FLT3 ITD is directly targeted over WT FLT3. This is in keeping with previous *in vitro* kinome based data, as described in chapter 1 showing low nanomolar efficacy for inhibition of the FLT3 ITD target (Singer J *et al*, 2014). Given that pacritinib also shows reasonable efficacy in samples expressing WT

FLT3 (mean EC_{50} 292.1 nM), it seems logical to conclude that between doses of approximately 100 and 200nM, the power of an ITD mutation to impact on sensitivity is lost and potentially at higher doses the drug exerts additional effects on other signalling pathways distinct from FLT3 such as JAK. In general many FLT3 inhibitors which have previously been through clinical development show very modest cytotoxic effects against wild type samples for example lestaurtinib (Knapper S, 2011) which does not really justify their use outside patients who harbour a FLT3 ITD mutation. Given the poor prognosis of patients with this subtype of AML this suggests that pacritinib may have promising therapeutic potential as an additional treatment to complement conventional chemotherapy in patients who harbour a FLT3 ITD mutation.

Interestingly the mean EC_{50} for samples carrying a FLT3 TKD mutation was very similar to that for WT suggesting no clear therapeutic advantage for pacritinib in patients with FLT3-TKD mutations although sample numbers were small. Some of the newer compounds such as crenolanib have been developed by specifically targeting cell lines which have been engineered to be resistant to some of the earlier FLT3 TKIs such as was seen following long term exposure of Ba/F3-ITD to sorafenib (Zhang W *et al*, 2008;Zhang W *et al*, 2014) and pacritinib requires further testing to characterise its therapeutic potential in this field. These have been termed *treatment emergent* resistance mutations, many of which are TKD point mutations such as D835V (Smith C *et al*, 2014). This may explain why crenolanib performed so poorly in these experiments using primary samples from TKI naive patients taken at diagnosis and the drug may show improved efficacy in samples taken at relapse. Crenolanib showed relatively poor *in vitro* activity against AML primary samples in this work compared to pacritinib and ponatanib (Fig 3.6) and its efficacy was not improved when tested against TKD samples compared to any other FLT3 mutation subgroup. It has shown improved efficacy elsewhere in an *in vivo* mouse model (Zimmerman E *et al*, 2013).

By aiming to combat primary *environment mediated* resistance and target the low level stroma adherent population the aim of a drug such as pacritinib is to prevent the development of the

former mutations which are likely to persist due to continuous evolution of the resistance mechanism. The TKI development story in CML demonstrates this effect succinctly due to the need for sequential development of new TKIs in response to new mutations in the BCR-Abl target which, interestingly, are also often centred around the ATP binding pocket, similar to the situation with FLT3 ITD mutations.

Following on from the initial cytotoxicity experiments, the next step was to characterise the effect on the AML cells which underlies the observed response. Annexin V binding assay in the MV4-11 (ITD mutated) cell line shows induction of apoptosis at 72 hours of pacritinib treatment with a dose response effect (Fig 3.11). In primary AML cells apoptosis is induced more rapidly with a >50% response seen by 48 hours of treatment (3.13). Previous authors have described that FLT3 inhibitory compounds such as lestaurtinib (Knapper S *et al*, 2006a) are selectively cytotoxic to, and induce apoptosis in, leukaemia cell lines such as MV4-11 and Molm-13 which harbour FLT3-ITD mutations as well as primary AML blasts samples taken from patients diagnosed with FLT-3 ITD mutated acute myeloid leukaemia (Knapper S, 2011;Knapper S, 2011;Knapper S, 2011;Levis M & Allebach J, 2002;Knapper S *et al*, 2006b). The cytotoxicity experiments carried out by Knapper et al were processed at 72 hours using the MTS technique for lestaurtinib and PKC412 compared to 48 hours in this study. Although there is no published data of cell cycle analysis in the lestaurtinib study this suggests that the 48 hour time point was considered to be too early to permit confidence that apoptosis has occurred. The primary annexin data on pacritinib in this study (Fig 3.13) shows that at 48 hours 50% late apoptosis induction occurs between the 100-300nM doses. This is very close to the experimental EC_{50} from the Cell Glo experiments (92nM). The early apoptotic effect seen at lower doses could still contribute to the Cell Glo EC_{50} of 100nM or lower as seen in many samples. Therefore, the apparent 'cytotoxic' effect in the Cell Glo experiments requires further analysis in terms of the effect on downstream signalling which will be described in chapter 4.

The other technique used to assess induction of apoptosis was the effect on Cleaved Caspase 3 (CC3) protein levels measured by western blotting (Fig 3.14). Here we see dose dependent induction of CC3 in a single primary sample after 24 hours, with a less marked effect in the MV4-11 cell line after a similar period of time. The primary sample used (15-2913) was sensitive to pacritinib by Cell Glo (EC_{50} 94nM) and so the strong CC3 signal at 300nM and 1000nM dose is to be expected. The MV4-11 signal appears weak in comparison, which is partly explained by uneven loading most marked for the 500nM dose. In view of the cell line annexin data however, (figures 3.11 and 3.12) this is to be expected as minimal induction was seen with 100nM and 200nM doses at 24 hours.

Elsewhere it has been shown that pacritinib, in a dose-dependent fashion, increases cell populations in early and late stages of apoptosis, without inducing necrosis in MV4-11 cell lines treated for 72 hours with doses between 0.03 and 0.15uM (Hart S *et al*, 2011b;Hart S *et al*, 2011b). The same authors also showed activation of caspase-3/7 in a dose dependent manner following treatment with pacritinib. Also, following 24 hour exposure to pacritinib, there was cell cycle arrest at the G_1 phase and a reduction in the size of the S-phase population in MV4-11 and MOLM-13 cell lines (both of which carry an ITD mutation) (Hart S *et al*, 2011b). This correlates with the inferior apoptotic response observed here in MV4-11 cell lines at 24 hours (Figure 3.11). Interestingly, less effect was seen in FLT3-WT cell lines such as RS4;11 by a factor of 15-20 fold (EC_{50} RS4;11 cells 930nM vs 47nM MV4-11 and 67nM MOLM-13) consistent with our data .

Data from the Cell Glo work gives an indication of the *in vitro* sensitivity of primary AML mononuclear cells to pacritinib and there is evidence from several sources that this property can translate from the bench to the bedside. The experiments described above show that pacritinib reliably induces cytotoxicity in primary AML cells cultured for 48 hours in IMDM with a greater magnitude of effect in those harbouring a FLT3-ITD mutation. The AVB assay and caspase western blot experiment suggests that the mechanism of cell death is primarily via induction of apoptosis in primary material.

In vivo, pacritinib has shown good efficacy in an MV4-11 allograft and xenograft model with significant survival benefits shown at a tolerable dose (William AD *et al*, 2015). Clinical trial development to date has included 8 clinical trials: 5 have completed, 2 are still in progress and 1 was terminated (Hatzmichael E *et al*, 2014). Only 1/5 completed studies has included AML patients. Recently, however, pacritinib has been added as a treatment option in the NCRI AML-17 study for patients with relapsed, ITD mutated AML.

The following chapter will explore the effect of pacritinib on signalling pathways downstream of FLT3 to further analyse the mechanism of action and potential clinical application of the drug.

Chapter 4

The effect of pacritinib on FLT3 downstream signalling pathways

Chapter 4: The effect of Pacritinib on FLT3 downstream signalling pathways

4.1 Introduction

As described in chapter 3, primary AML mononuclear cells show a heterogeneous *in vitro* cytotoxic response to several different FLT3 inhibitors including pacritinib, crenolanib and ponatanib. Also, within the cohort of results obtained for each individual drug there remains a wide distribution of EC_{50} results (high and low) which does not strictly segregate according to FLT3 mutation status. Part of this diversity in response is due to different dependency on underlying signalling pathways within AMLs and in chapter 3 the relationship between FLT3 ITD: WT mutation 'allelic ratio' and cytotoxic response was explored. This factor, however, only analyses the reliance on FLT3 signalling at the cell surface through different levels of receptor activation which fails to address whether a greater degree of constitutive activation, through a higher 'allelic ratio' ultimately results in up-regulation of the secondary effects of increased FLT3-mediated drive such as proliferative response and evasion of apoptosis. This chapter will focus on the effects of pacritinib on downstream targets and FLT3 status, to address differential pacritinib sensitivity in this context.

4.1.1 The FLT3 signalling cascade

FLT3 has a crucial role in many of the regulatory and survival pathways of haematopoietic cells (Stirewalt DL & Radich JP, 2003). This has been previously described in figure 1.1 (chapter 1). Activation of FLT3 results in phosphorylation of these proteins with further downstream effects on PI3K/protein kinase B (Akt) and mitogen activated protein kinase (MAPK) pro-survival pathways. FLT3 is also physically associated with other aspects of phospholipid metabolism and proliferation such as SH2-containing sequence proteins (STAT3, STAT5, PI3K, SRC, ABL etc), the SH2 domain binds a phosphorylated tyrosine residue and thereby facilitates communication/activation between signalling proteins (Stirewalt DL & Radich JP, 2003). FLT3 also interacts with GTPase-activating

protein (Gap) which is a key regulator of the proliferative RAS-RAF-MEK-ERK pathway, many components of which are associated with FLT3 inhibitor resistance (Yang X *et al*, 2014).

4.1.2: FLT3 expression in primary AML blasts: quantification and *in vitro* relevance

As previously stated, around one in three patients with AML carry an activating mutation of the FLT3 receptor; most frequently of ITD subtype. It is well known, however, that patients expressing wild type FLT3 can show up-regulation of the receptor and it has also been reported that this may contribute to disease pathogenesis. Patients showing only wild type FLT3 expression may, therefore, be sensitive to the effects of a FLT3 inhibitor if the drug is able to down regulate signalling from an activated but structurally normal receptor. The data discussed in chapter 3 showed that a subset of FLT3 wild type patients still show sensitivity to pacritinib with a mean EC_{50} of 292nM in 33 patients studied which although nearly threefold higher than the average for ITD mutated samples (92nM), is still much lower than the same reading for many conventional cytotoxic agents such as AraC. As a follow up to this finding, a sample of AML patients included in the overall cohort was examined to specifically analyse whether the degree of FLT3 expression at the cell surface has any influence on pacritinib sensitivity as this is a factor that potentially negates the effect of whether or not the receptor is structurally normal.

Previous methods used to quantify FLT3 expression include Western blotting, autoradiography using ^{125}I-labelled FLT3 ligand (FL) and flow cytometry (Grafone T *et al*, 2008). Flow cytometry is a relatively quick and simple technique which can give rapid quantification of cell surface FLT3 expression (CD135) on both malignant blasts and normal haematopoietic cells. The antibody binds to FLT3 at the cell surface, the advantage of this technique over Western blot analysis is that it requires minimal cell numbers ($<1 \times 10^5$) from patient samples to generate a result, the technique is much less complex and time consuming as it can be completed in less than 3 hours, and sub-populations of cells can be distinguished by co-labelling with surface markers such as CD34 and CD45 (Grafone T *et al*, 2008;Grafone T *et al*, 2008).

In addition, one group has previously suggested that FLT3 phosphorylation status and its response to drugs can be monitored by intracellular flow cytometry techniques where results have been shown to correlate with western blot and colorimetric cytotoxicity assays (Grafone T *et al*, 2008) using both FLT3 WT (HL-60), ITD mutated (MV4-11) cell lines and also in primary AML samples. In this technique leukaemic cells are incubated with anti-CD135-PE antibody then washed with PBS and fixed with 100µl Fixative Reagent (BIO^{E} Laboratories). Cells were then washed again and permeabilised with 90% methanol to allow the antibody to Tyr591 P-FLT3 (CS) to be taken up intracellularly which potentially gives the advantage of measuring change in internal p-FLT3 status in response to drug treatment rather than that measured at the cell surface. Although this technique appears encouraging the data in the paper appears to be limited by the relatively low maximum signal intensity achieved (10^2 on log scale) which is similar to the background reading; also the lack of additional publications employing the same techniques supports the impression that the method is unreliable and in our experience would require $1x10^6$ cell load per condition which makes it prohibitive for drug readout.

4.2 Methods

4.2.1: Flow cytometric methods

Antibody staining was carried out as described in chapter 2 using primary AML mononuclear cells and anti-CD34 PE (BD 345802) IgG PE (BD 340270), anti-CD45 FITC (Biolegend 304038) IgG FITC Biolegend 400108 and anti-CD135 APC (Biolegend 313308) IgG APC Biolegend 400120. The gating strategy used was CD45/Side Scatter to separate the debris (CD45-/SSC-) and lymphocyte (CD45+/SSC-) populations from the AML mononuclear cells (CD45+/SSC+). Next 7-amino-actinomycin D (7-AAD which has high DNA binding constant and is excluded by cells which have an intact membrane) staining was used to assess viability with a maximum cut off of $<10^4$ to define the viable population. In addition the percentage of primitive blasts was analysed using the CD34/Forward scatter (FSC) gate and finally FLT3 surface expression using CD135/FSC.

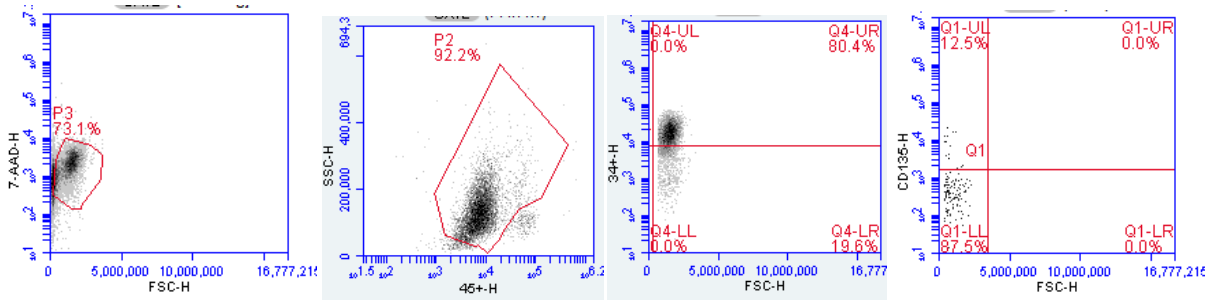


Figure 4.1: Gating strategy for primary AML mononuclear cells to determine percentage CD135 (surface FLT3) expression.

4.2.2 Western blotting methods

The effect of pacritinib on FLT3 signalling and downstream target activity was assessed using western blotting. As described in chapter 2, AML primary mononuclear cells or the MV4-11 cell line (which carries an ITD mutation) were suspended at 3 x10^6/ml (primary samples) and 8 x 10^5/ml per ml (cell lines) and treated with varying concentrations of pacritinib or DMSO as a vehicle control. For the cell line experiments the doses used were untreated (DMSO 0.01% as vehicle control), 50nM, 200nM and 500nM pacritinib. For the primary AML experiments the dose range was untreated (0.01% DMSO), 100nM, 300nM and 1000nM pacritinib. These ranges were designed to give a spread of doses above and below the mean EC_{50} for the given cell type (derived in Chapter 3) with the maximum dose being several fold higher to facilitate a maximal possible level of target inhibition. The plates were incubated 37°C, 5% CO_2 for 1, 24 or 48 hours.

Following electrophoresis and transfer, membranes were probed overnight with a range of different primary antibodies. Initially the MV4-11 gel was probed with rabbit anti-phosphoFLT3 (#3464 1/3000 dilution) followed by rabbit anti-total FLT3 (santa cruz S18 #480 1/5000 dilution) and mouse anti-beta actin (1/3000). Separate gels were then made for cell line and primary cell sample lysates and probed using rabbit anti-phospho STAT5 tyr694 (CST#C11C5 1/3000 dilution).These membranes were subsequently re-probed for the following antibodies anti-total STAT5 (Rabbit CST#9363P

1/5000 dilution), Rabbit anti-p42/44 MAPK (phospho-ERK CST#4370BC, 1/3000 dilution) and anti-total ERK (p44-42 MAPK CST#9102, 1/5000). Duplicate gels were run for phosphorylated and total proteins separately. Finally beta actin (1/30000) mouse monoclonal antibody was used to assess uniformity of loading.

4.3 Results of Flow Cytometry Experiments

4.3.1: FLT3 surface expression on AML blasts

Flow cytometric FLT3 surface expression assays were performed on cryopreserved mononuclear cells from a total of 13 AML patients, after thawing 8 of which showed sufficient viability and cell numbers to be included in the analysis. Among these patients, blast surface FLT3 expression ranged from 0%-20.5% (mean 8.5% Table 4.1) which refers to the percentage of blast cells in each given patient sample which gave a positive signal as measured by C-Flow software. The 8 cases examined included 3 with FLT3 ITD mutations ranging in size from 66 base pairs to one sample with 3 different ITD mutations of 21bp (36%), 132bp (9%) and 147bp (9%). The other 5 samples included expressed only WT FLT3 (by gene scanning). There was no significant difference between WT and ITD FLT3 CD135 expression: Mean Fluorescence Intensity WT 1127, ITD 731 (p=0.6 unpaired *t test*).

Sample	FLT3 status	CD45+ (%)	CD34+ (%)	CD135+ (%)	MFI	EC_{50}/nM
1. 15-0611	WT	80	85	4.8	629	25
2. 15-3067	WT	75	79	8.9	834	32
3. 15-3429	WT	74	55	0	0	415
4. 15-2913	ITD	90	40	0	0	94
5. 15-1299	WT	69	6	20.5	2826	336
6. 15-3223	ITD	84	74	16	1491	660
7. 16-5418	ITD	80	11.7	5.3	701	330
8. 16-1296	WT	72	85	12.5	1346	115

Table 9: Details of patients used in CD135 expression cohort, values for each cell marker quoted refer to % CD45+/FSC+ to identify the AML mononuclear cells, then % CD34+/FSC- to identify the primitive blast population and finally % CD135+/FSC- as a proportion of this population to give the level of surface FLT3 expression. MFI refers to Mean Fluorescence Intensity of the CD135+ population

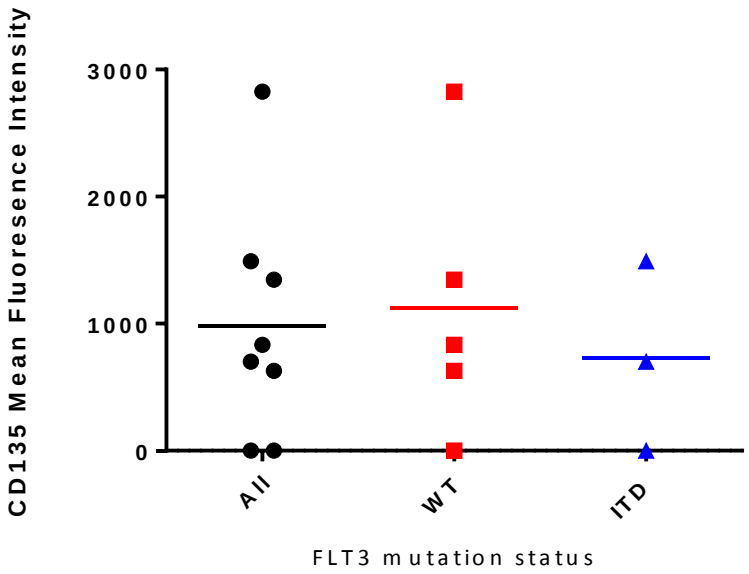


Figure 4.2: Scatterplot showing CD135 expression (Mean Fluorescence Intensity) in 8 primary AML samples analysed by Flow cytometry and stratified by FLT3 mutation status. Gating strategy: CD45+, CD135+ (FL-4 vs. FSC). MFI ITD mutated sample (n=3) 731 vs WT samples (n=5) 1127 p=0.6.

FLT3 ITD mutations are proposed to be preferentially linked to a primitive 'leukaemic stem cell' phenotype (Parmar A *et al*, 2011) and therefore, the association between CD34+ and CD135+ surface expression can be specifically analysed. The scatterplot below in figure 4.3 analyses the relationship between CD34+ and CD135+ expression which gives an indication of FLT3 receptor expression level at the surface of the AML blasts.

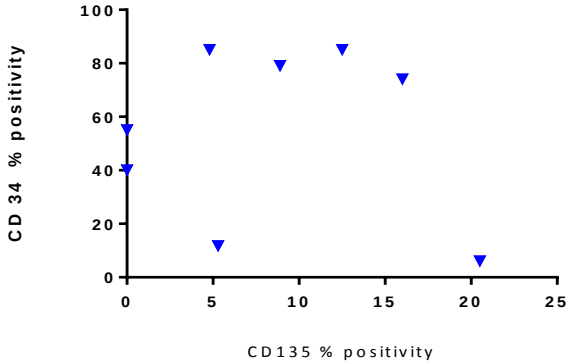


Fig 4.3: Scatterplot for CD135 vs CD34 percentage expression in 8 primary samples (Spearman correlation p=0.86)

The data shown in figure 4.2 shows that there was no significant correlation between CD34 and CD135 expression in the 8 samples tested (Spearman correlation p=0.86).

4.3.2: Correlation of FLT3 expression with FLT3 inhibitor response measured by *in vitro* cytotoxicity

Results from the Cell Glo cytotoxicity experiments using pacritinib treatment for the 8 patients were correlated with FLT3 percentage surface expression. Figure 4.4 below, illustrates the mean cytotoxic dose response as measured by EC_{50} versus CD135 Median Fluorescence Intensity.

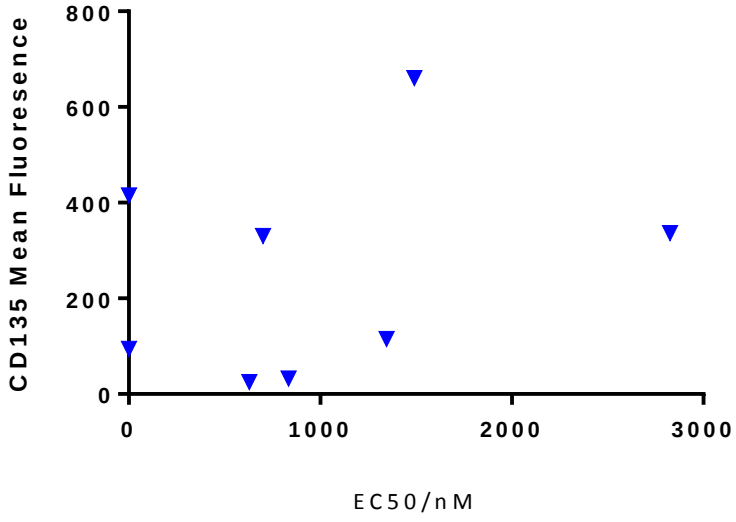


Figure 4.4: Pacritinib efficacy using cell glo technique for 8 samples included in analysis of CD135 expression.

The data shown in figure 4.4 shows that there was no significant correlation between CD135 expression and EC_{50} in the 8 samples tested (Spearman correlation p=0.9).

4.4: Results of Western Blotting Experiments

4.4.1: MV4-11 cell line FLT3 inhibition

To assess the in vitro effect of pacritinib on FLT3 receptor phosphorylation, MV4-11 cells were exposed to variable concentrations of pacritinib (as described above) for 1 & 24 hours and probed for phosphorylated FLT3 and total FLT3 protein.

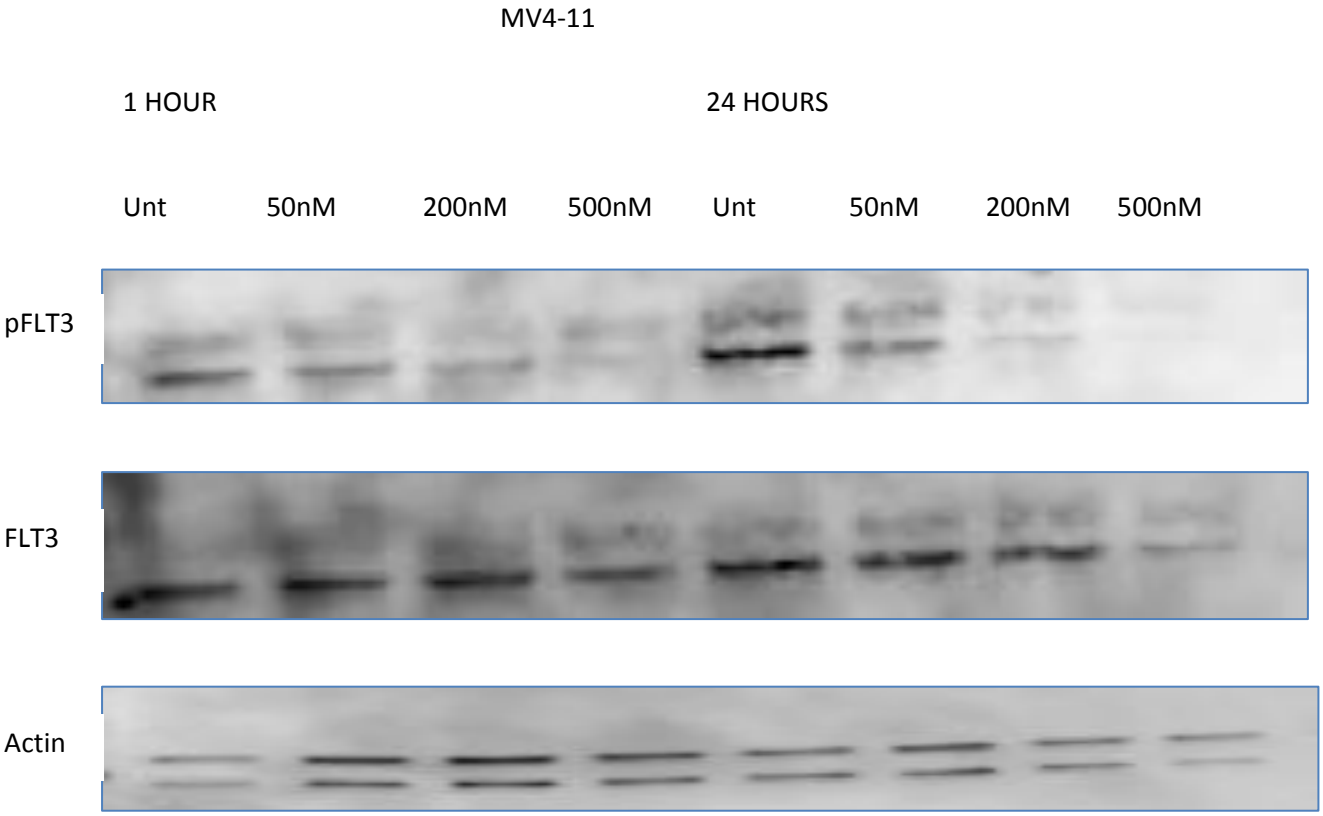


Fig 4.5: This shows dose dependent inhibition of phosphorylated FLT3 protein following treatment with pacritinib, with a more pronounced effect at 24 hours. There was no effect on FLT3 total

protein signal when inconsistency of loading in the outer two lanes (Unt 1 hour and 500nM 24 hours) is taken into consideration (gels run by conventional western blotting method).

4.4.2: MV4-11 cell line downstream pathways

As previously described, two of the main downstream signalling pathways of FLT3 are STAT5 and Raf/MEK/ERK. These were, therefore, specifically analysed in the western blotting experiments. Downstream of FLT3, the MEK/MAPK pathway plays a central role in regulation of cellular proliferation and differentiation (Swords R *et al*, 2012). There are in vitro data to show that inhibition of the MEK pathway leads to a reduction in growth of the FLT3-ITD mutated cell line MV4-11 and can also promote apoptosis in primary AML blasts. Further exploration of this effect has suggested that MEK inhibition allows these primitive cells to differentiate and thus become more sensitive to treatment with FLT3 inhibitors.

There was a dose dependent reduction in phospho-STAT5 signalling seen by 24 hours with a suggestion of inhibition of STAT5 total protein by 48 hours. Dose dependent reduction in phospho-ERK expression was seen at 1 hour and also at 48 hours although total ERK signal was largely sustained.

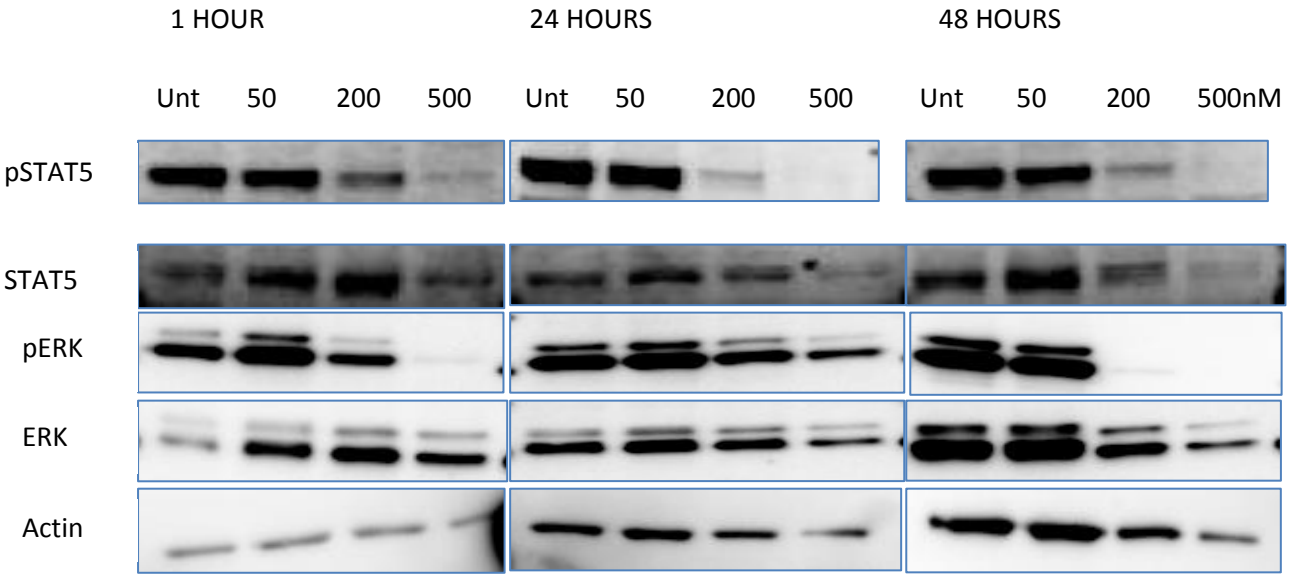


Figure 4.6: Effect of pacritinib on FLT3 downstream signalling in MV4-11 cell lines, doses are in nM.

The data show rapid inhibition of pSTAT5 signal from 1 hour onwards which is not unexpected, the relative decrease in total STAT5 signal is likely to be largely an artefact of uneven sample loading as shown by the actin result.

4.4.3: Primary AML samples downstream pathways

Similarly to MV4-11 cell lines there was rapid attenuation of phospho-STAT5 activity observed within 1 hour of treatment and sustained over the time course for both FLT3 ITD mutated and WT samples. Very little reduction in total STAT5 expression was seen following one hour of treatment with pacritinib.

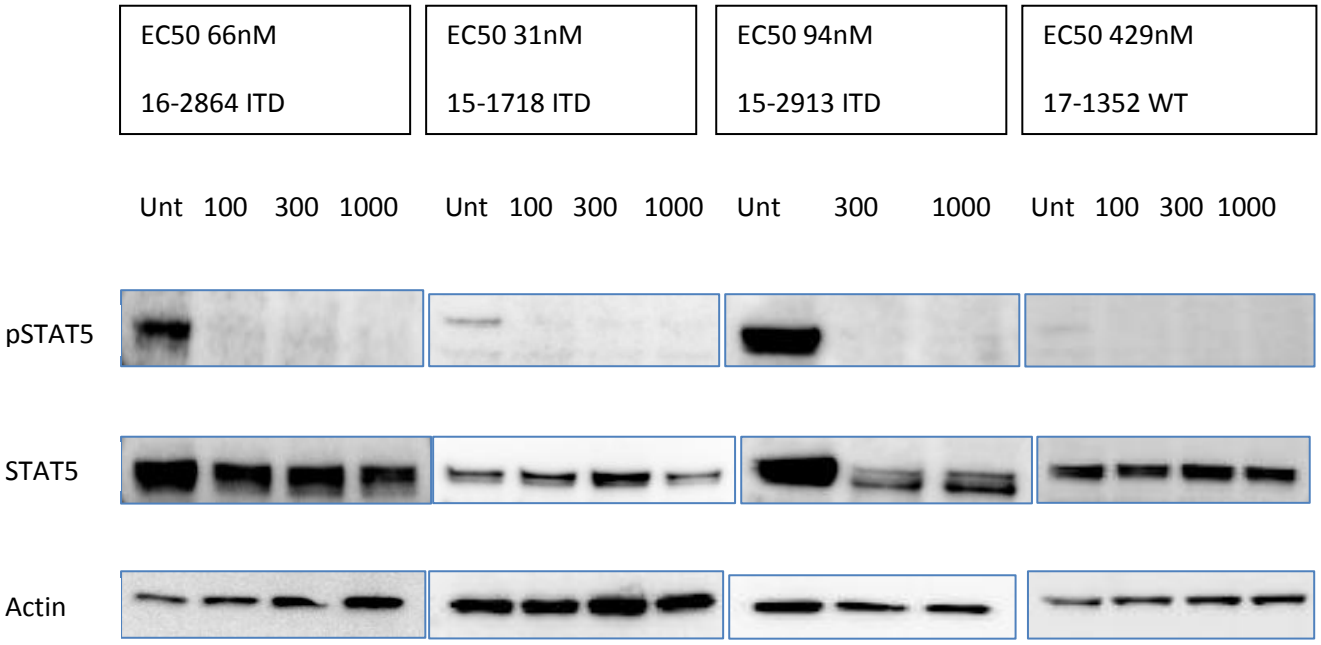


Figure 4.7: Four primary AML samples treated with Pacritinib for 1 hour. Dose range untreated, 100nM, 300nM, 1000nM (except 15-2913 which just shows untreated, 300nM, 1000nM).

Further western blotting experiments were subsequently carried out in the co-culture setting using a mouse stroma (MS-5) monolayer. These will be described in chapter 5.

4.5: Discussion

Flow cytometry experiments in this chapter have shown no evidence of increased CD135 expression in primary AML blasts which carry a FLT3 ITD mutation as compared to WT samples. This is not an

unexpected finding as it has been reported by other authors elsewhere (Knapper S *et al*, 2006b) . One possible explanation which has been proposed in the past is that the constitutive activation of the FLT3 receptor as a result of the ITD mutation leads to dimerisation and internalisation of the surface protein and therefore a reduction in the level of surface expression as measured by CD135 signal intensity. A cell with high surface FLT3 expression may, therefore, actually be less reliant on this pathway than a cell with a high proportion of activated FLT3 (such as those which harbour the constitutively activating FLT3-ITD mutation) which may have a reduced quantity of detectable membrane-bound FLT3. Antibody suitability is also an issue, with poor performance and signal strength limiting flow cytometry for use in activated/phosphor flt3 detection.

Surface expression in primary samples that have been previously frozen and thawed may also be affected by the temperature change which could lead to degradation of the protein in some of the receptors; this can possibly be reduced by adjusting the reagents included in the freezing medium (Sasnoor L *et al*, 2005), or using fresh primary AML samples. Further analysis of the cohort of samples used for the CD135 expression experiments has shown a small but non-significantly increased mean CD135 expression in the WT samples compared to ITD mutated samples (mean fluoresence 2826 WT vs 1491 ITD p=0.6 student's t test). This did not correlate with increased sensitivity to pacritinib. Rather the mean EC_{50} in the WT samples was higher than the ITD mutated samples using the Cell Glo technique amongst the cohort of 8 samples used for the CD135 flow cytometry experiments and also in the overall cohort as described in chapter 3. This suggests that the level of surface expression of the FLT3 receptor is not a major driver of sensitivity to the drug; binding and activity of the drug is not significantly affected by receptor frequency or density at the cell surface as expected. Pacritinib in common with most FLT3 TKIs targets the ATP binding pocket on the FLT3 molecule and it is known that FLT3 activating mutations exert a direct influence over the physical properties of this binding site (Pratz K & Levis M, 2008). The ITD mutation imparts constitutive activation of the receptor which leads to ligand independent activation, dimerisation and exposure of the ATP binding pocket (Pratz K & Levis M, 2008). It is logical to conclude that this is

a more significant driver of drug sensitivity than the magnitude of expression of a structurally normal (wild type) receptor.

As an alternative method, FLT3 RNA expression can be quantified by real time PCR in comparison to house- keeping genes *S14.* Previous authors have failed to show any significant difference (or trend) in FLT3 RNA expression between patients that harboured FLT3 ITD mutations and WT patients (Knapper S *et al*, 2006b). Further work in development of intracellular flow cytometry as a method to screen for phospho-FLT3 which is known to correlate with functional activity will be of interest to monitor drug responses (Grafone T *et al*, 2008). Intracellular flow cytometry was not carried out as part of this project but has been done by other members of the group in the setting of the medium term co-culture assay (Dr Gareth Edwards).

In summary more than one method of quantifying the level of FLT3 expression in primary AML samples have failed to provide a means to predict sensitivity to treatment with FLT3 inhibitors, which is likely to be a function of receptor activity rather than quantity. An alternative explanation is that it is the level of FLT3 inhibition induced by the drug which is more critical and this may be measured by means of the plasma inhibitory assay (PIA). This technique has shown a direct relationship between the degree of FLT3 inhibition (as measured by de-phosphorylation of the FLT3 protein) achieved and drug efficacy. Reduction of residual FLT3 phosphorylation below a threshold level of 15% compared to baseline correlates reliably with clinical response. Data presented in this chapter used western blotting as an alternative method to the PIA to examine downstream effect of FLT3 inhibition in response to pacritinib treatment. The result in MV4-11 cell lines showed dose dependent inhibition of phosphorylated FLT3 protein, most marked after 24 hours of culture. There was no effect on FLT3 total protein in the western blotting experiments as has been described elsewhere for other FLT3 TKIs such as SU5614 (Parmar A *et al*, 2011) and crenolanib (Zimmerman E *et al*, 2013).

In further western blotting experiments, inhibition of pSTAT5 signal was seen in MV4-11 cells following 24 hours of treatment with pacritinib and at doses above 200nM (fig 4.6). After one hour of treatment very little effect was observed and at the 50nM dose there was also minimal inhibition seen at any time point. This correlates with some previous cell line data where inhibition of pSTAT5 signal in MV4-11 cells was seen with a dose of 125nM pacritinib following 24 hours of treatment with no data shown for lower doses or shorter time points (Novotny-Diermayr V *et al*, 2012).This is likely to be due to the STAT5 pathway being less critical to survival of this cell line which has been artificially 'immortalised'. A redundant pathway may require a higher dose of drug to inhibit to the degree that cell survival is affected.

In contrast dose dependent, rapid and sustained inhibition of pSTAT5 signalling was more pronounced in primary AML samples. There was very little difference between the magnitude of the effect seen in the primary samples, with all 4 showing almost complete inhibition of pSTAT5 signal at the lowest dose of pacritinib tested (Fig 4.7). There was a suggestion of basal increase in pSTAT5 signal in the untreated lanes of 2/3 primary samples (16-2864 and 15-2913) both of which carried a FLT3 ITD mutation compared to the WT sample, supporting the observation that constitutive activation of the FLT3 receptor leads to increased signalling through this pathway. A previous similar study looking at the *in vitro* effects of treatment of 65 primary AML diagnostic samples with two other compounds CEP701 and PKC412 has suggested that cytotoxic efficacy correlates with inhibition of signalling through STAT5 (Knapper S *et al*, 2006b).

STAT5 is a cytoplasmic transcription factor which exists as two homologous proteins known as STAT5a and STAT5b (Stirewalt DL & Radich JP, 2003) . Activation of FLT3 up-regulates the activity of STAT5 which forms part of the proliferative response to FLT3 signalling in normal cells. Within the western blot readout the pSTAT5 antibody detects both STAT5a and STAT5b; some AMLs have more top band signal (STAT5a) which was seen most clearly for this cohort in sample 15-2913 (Figure 4.7). It is also known that STAT5 protein exerts differential DNA binding effects depending on whether it is

activated by WT FLT3 or ITD mutated FLT3 (Stirewalt DL & Radich JP, 2003). In addition, STAT5a and STAT5b isoforms exert different mechanisms to bind DNA; the former by means of a tetramer whereas STAT5b molecules form dimers (Lin J-X & Leonard W, 2000), this may also affect binding affinity. In reality, however, it is often difficult to distinguish the isoforms through separation on the gels although interestingly in this experiment sample 15-2913 appears to show more marked reduction in the STAT5a (top band) compared to STAT5b (lower band) (figure 4.7) suggesting that pacritinib can exert differential effects on the two isoforms in individual patients.

Cells harbouring FLT3-ITD mutations have a high level of STAT5 phosphorylation (correlating with the increased basal pSTAT5 signal seen here in 2/4 samples) and this is associated with the ability to bind to DNA (Stirewalt DL & Radich JP, 2003) and influence cellular growth and survival. Activation of STAT5 targeted genes has been shown to distinguish FLT3-ITD from FLT3-WT signalling in a mouse model (Kharazi S *et al*, 2011) although from the 4 examples seen in figure 4.7, pacritinib appears to exert equally potent inhibition of pSTAT5 in both FLT3 WT and ITD mutated samples, regardless of drug sensitivity as measured by EC_{50}.

A further knock on effect of activation of STAT5a via FLT3 is thought to be increased production of reactive oxygen species (ROS). These are unstable entities which can induce DNA double strand breaks (DSBs) and negatively impact on DNA repair mechanisms (Takahashi S, 2013), leading to genomic instability, and likely contributing to the overall poor prognosis of FLT3 ITD mutated AML. FLT3 activation via STAT5a also has an influence on the control of apoptosis as it prevents up-regulation and expression of genes that can induce apoptosis such as MCL1, cyclin D1, c-myc and p21 (Kindler T *et al*, 2010). FLT3, however, remains the proximal link in the chain and an ITD mutation therefore results in an enhanced ability for the cell to both proliferate and evade mechanisms of programmed cell death.

In contrast to the effect seen on phospho-STAT5 activity, there was a much weaker effect on total STAT5 signal seen in ¾ primary AML samples analysed (the exception being sample 15-2913),

although there was some inhibition seen in MV4-11 cells at 500nM dose (figure 4.6). This may also be an artefact given that MV4-11 cells are generally very sensitive to pacritinib. At the 500nM dose the majority of cells are dead and hence protein loading is compromised (also shown by actin result).

In contrast to the MV4-11 data shown in figure 4.6 following treatment with pacritinib, relative lack of inhibition of STAT5 total protein has been shown in response to other FLT3 TKIs in MV4-11 cell lines including ponatanib (Gozgit JM *et al*, 11 A.D.) and crenolanib (Galanis A *et al*, 2013) (Piloto O *et al*, 2007) showed a similar effect in both ITD mutated and FLT3 WT-expressing cell lines and suggested that this uncoupling of response to phosphorylated STAT5 and its total protein is observed as a result of other unidentified signalling proteins which may play a role in cell proliferation and survival through this pathway. Breakdown of total protein also requires activation of the proteosome and a degradation process which requires up to 72 hours for completion. This must also be preceded by inhibition of phosphorylated (active) protein. In addition, a more rapid response is associated with a phosphorylation site that is more essential to stability of the total protein; the above data suggest that pSTAT5 is essential for the stability of the MV4-11 cell line (hence additional total STAT5 response) to a greater degree than in primary AML cells.

Pacritinib has a dual inhibitory effect on both FLT3 and JAK2 mediated signalling, with both pathways converging on STAT5. Each of the four member proteins of the JAK family can recruit and phosphorylate (activate) STAT5 following cytokine engagement principally of the IL-2 family (Hatzmichael E *et al*, 2013;Lin J-X & Leonard W, 2000) which suggests that, to effectively silence this pathway, dual inhibition of both FLT3 and JAK2 may be required. Nevertheless, gain-of function mutations of JAK2 (V617F) which result in over-activation of the proliferative JAK2-STAT pathway are rare in AML although there is some association with one of the core binding factor mutations (t(8,21) (Dohner K *et al*, 2006). For this reason (and limitations with antibodies for these assays), the effect of pacritinib on JAK2 signalling was not specifically analysed as part of this project. Hart et al have previously shown that pacritinib can induce dose dependent inhibition of phosphorylation of

JAK2, STAT3 and STAT5 in 293T (human embryonic kidney cells) with inhibition of phosphorylation of STAT5 seen in murine Ba/F3-JAK2^{V617F} cells which are of B lymphoid origin (Hart S *et al*, 2011a). A reduction in pSTAT5 in SET2 and HEL92.1.7 cell lines, both of which carry a JAK2^{V617F} mutation was also seen.

The other main downstream effector of FLT3 analysed in this work was the MEK/ERK pathway. Here there was a similar effect on p-ERK signalling in cell lines and in a primary AML sample cultured alone in IMDM medium. In summary, inhibition of p-ERK signal was seen in MV4-11 cells at 1, 24 and 48 hours following treatment with 500nM pacritinib and a lesser effect with the 200nM dose (Fig 4.6). There was no real effect on total ERK signal in MV4-11s.

The work described in this chapter confirms that pacritinib is a drug which can inhibit phosphorylation of FLT3 in ITD mutated (MV4-11) cell lines and that this effect then translates into a knock on reduction in pSTAT5 and also pERK signalling. In primary AML samples, similar inhibition of pSTAT5 signal was seen. No correlation was seen between FLT3 expression, as quantified by cell surface expression, and pacritinib sensitivity which suggests that the mechanism of action of the drug is more complex than binding affinity for a normal receptor. The next step was to analyse how well pacritinib can perform in a co-culture setting, using a stroma monolayer since this property is likely to define its potential clinical superiority over and above that of existing FLT3 inhibitors.

Chapter 5

The *in vitro* effects of stroma on sensitivity to pacritinib in primary AML samples

Chapter 5: The in vitro effect of stroma on sensitivity to pacritinib in primary AML samples

5.1 Introduction:

The leukaemic clone is organised as a hierarchy (Parmar A *et al*, 2011), and leukaemic stem cell (LSC) behaviour is modulated by interactions and signals received within the BM microenvironment (Tabe Y & Konopleva M, 2014). This niche provides a supportive microenvironment (Parmar A *et al*, 2011) for normal and malignant haemopoeitic cells and regulates their survival via a cycle of proliferation and differentiation (Wilson A & Trumpp A, 2006). LSCs within the BM niche are felt to derive 'sanctuary' in this environment which allows them to evade death in response to conventional cytotoxic agents, in contrast to circulating myeloblasts. As described in chapter 1 there are a variety of characteristics of the bone marrow microenvironment in terms of its physical and chemical properties which govern how it interacts with normal and malignant myeloid progenitors. These will be briefly repeated in the introduction to this chapter but for a comprehensive description the reader is referred back to chapter 1 (1.8-1.10).

5.1.1 The difference between normal and malignant marrow stroma

In patients with AML the normal BM niche may be exploited by malignant progenitors as many of its components contribute to LSC engraftment, survival and drug resistance. Altered signalling within the leukaemic stroma, leads to suppression of re-growth of normal progenitors and the creation of leukaemia niches containing small numbers of quiescent LSCs. These mechanisms directly impact on patient outcome, for example via infective complications due to prolonged periods of cytopenia following induction chemotherapy which are a common cause of death. Brisk relapse following a period of very slow count recovery (also a common clinical feature) may reflect dual benefit from a return to 'stroma recovery' for both normal and malignant stem cells alike. Data from mouse models has shown that certain genetic mutations, such as *DNMT3a* mutations, provide a competitive repopulation advantage to pre-leukaemic haemopoeitic stem cells (HSCs) over non-mutated HSCs which allows them to expand in patients at diagnosis and during presumed periods of 'remission'

(Schlush L *et al*, 2014). This provides evidence that there is an ancestral clone of pre-leukaemic HSCs which can persist during remission and may therefore be resistant to induction chemotherapy (Schlush L *et al*, 2014). Such clones most probably reside within the bone marrow microenvironment in order to evade conventional cytotoxic agents and thus require specific targeting, with drugs that can overcome the protective effect of the stroma.

5.1.2: Use of therapeutic agents to overcome stroma-induced protection

As has been described above and previously in Chapter 1, the modification and exploitation of stromal signalling leads to persistence of LSCs in the bone marrow after chemotherapy and is thought to be a major factor in the dismally high level of relapse seen in patients with acute myeloid leukaemia (Alvares C *et al*, 201)) . The durability of this population within the bone marrow microenvironment makes the identification of agents which can overcome this stroma-induced protection of LSCs and increasingly popular area for therapeutic development. One such drug, which has been tested in vitro and in vivo in this context is plerixafor, a competitive antagonist of the CXCR4-CXCL12 axis (Uy G *et al*, 2012). Plerixafor has shown the ability to mobilise blasts into the peripheral blood in a mouse AML model and has also shown a similar effect in phase1/2 studies in humans with associated favourable remission rates. The combination of plerixafor with conventional induction chemotherapy was recently studied in the NCRI AML18 Pilot study, but the further clinical development of the agent in this clinical setting has recently been abandoned for commercial reasons.

FLT3 ITD mutations have been shown to be present in primitive human CD34+CD38- cells, including those with strong expression of PKH26 which are known to be non-cycling or quiescent (Alvares C et al. 457-65). This shows that the mutation can occur at a very primitive level of differentiation, within the haematopoietic stem cell compartment (Levis M *et al*, 2005) and in a paediatric setting it has been shown that detection of the FLT3 mutation within this sub-population of the disease clone

confers a poor prognosis. Targeting FLT3 ITD may, therefore, offer a means of eradicating this leukaemia stem cell population and thus reducing the risk of relapse.

The effect of FLT3 TKIs on the interaction between leukaemic FLT3 ITD+ stem/progenitors and the bone marrow niche has been previously investigated for several agents including SU5614 (Parmar A *et al*, 2011), Sorafenib and Quizartinib (Yang X *et al*, 2014) . The effectiveness of these drugs on AML cell lines or primary AML mononuclear cells co-cultured on murine stoma has been previously studied by these authors including the comparison of activity between FLT3-ITD mutated and FLT3-WT primary samples. Differential effects have been observed depending on FLT3 status, for example a lack of inhibition of pSTAT5 signal on stroma in FLT3-ITD + primary cells treated with SU5614 in comparison to FLT3-WT (Parmar A *et al*, 2011). Another observation noted by the same authors was an increased fraction of non-dividing cells following treatment with SU5614 regardless of FLT3 status, however, this effect was reversed by co-culture with EL08-1D2 stroma in FLT3 WT cells but not in FLT3 ITD mutated cells. This suggests the presence of a specific interaction between the FLT3 ITD mutated cells and the stroma which blocks cell division, or amplification of a pro-differentiation signal in the WT cells. Cell cycle analysis showed an increase in the fraction of non-dividing cells in response to drug treatment for both subgroups off stroma and, importantly, in the co-culture setting specifically for the FLT3 ITD mutated group; therefore cell cycle arrest was proposed as a possible mechanism of resistance.

An unexpected finding of the work by Parmar et al was that stroma support appeared to contribute to the maintenance of the primitive long term progenitors (LTC-CFCs) as measured in a 6 week colony formation assay where the number of colonies per 1000 CD34+ cells was seen to increase in the co-culture setting following treatment with SU5614 compared to DMSO vehicle control. The FLT3 status of these progenitors was confirmed using PCR and the same observation was not seen for FLT3 WT samples. Overall, the conclusion was that although inhibition of aberrant FLT3 signalling and disruption of downstream pathways may be achievable; this does not automatically correlate

with eradication of the earliest stem/progenitor cells responsible for disease propagation. This observation may be because this population is not dependent on mutant FLT3 for signalling and survival or because the potency of the drugs tested is inadequate to overcome enduring FLT3 activation (despite short term inhibition of pFLT3 being seen). One such explanation for this is that any drug which actively targets stages of the cell cycle (such as AraC which targets p21 induction) (Radosevic N *et al*, 2001) will inherently lack efficacy in non-cycling cells such as those which populate the stroma niche.

The differential effect seen in FLT3 ITD mutated and WT samples suggests the existence of some kind of specific survival or renewal signal emanating from the bone marrow niche which can only be utilised by ITD mutated cells. Although FLT3 WT cells may gain some benefit, Yang et al proposed that this may in part be related to exogenous production of FLT3 ligand (FL) to which FLT3 ITD+ progenitors are more sensitive and may impair the activity of FLT3 inhibitors (Yang X *et al*, 2014).

Sorafenib has a differential inhibitory effect to SU5614 and the expansion of malignant LTC-CFCs from the stroma niche was not seen following treatment with this drug using the same AML samples using a 6 week long term culture assay using culture on the FBMD-1 stromal cell line in the presence of TPO and FL (Parmar A *et al*, 2011). Using a different technique to assess potency, namely the MTT/proliferation assay, however, it has been shown that both stroma and FL conferred a protective effect against both sorafenib and quizartinib using FLT3-ITD mutated Molm14 cell lines cultured on human stroma cells donated by healthy volunteers (Yang X *et al*, 2014). An increase in the EC_{50} from 0.6nmol/l for cells in suspension to 4.8nmol/l for cells on stroma with the addition of FL was observed. What is clear from all of this earlier work is that even some of the most tested second generation FLT3 inhibitors appear to have questionable activity against FLT3-ITD+ progenitors within the bone marrow niche which justifies the exploration of this therapeutic target with emerging molecules such as pacritinib.

5.1.3: Rationale for the use of pacritinib against stroma induced protection

Intracellular signal transduction pathways are connected through a network of protein kinases and deregulation of their activity has been reported in the majority of cancers (Weisberg E *et al*, 2012). The pathways relevant to FLT3-ITD-derived survival advantage have been described in chapter 4 but can be summarised as the PI3K/AKT pathway, the RAS/MEK/ERK pathway and the JAK/STAT pathway. To further complicate matters, certain kinases can communicate or 'cross-talk' between pathways such as MAP kinase pathway, non-receptor TKIs (Src) and FLT3 itself to produce feedback in response to drug treatment and a complex interaction with the stroma niche which may warrant the use of multi-targeted agents to overcome both residual disease and drug-induced resistance (Weisberg E *et al*, 2012).

FLT3 ITD mutations lead to constitutive activation of STAT5 and this latter downstream target may also be activated by JAK. Since the end result of this pathway is enhanced cellular proliferation there is a strong rationale for combining the two targets via a drug which can inhibit both FLT3 and JAK. High levels of IL-6 secreted by stroma are known to activate JAK (Weisberg E *et al*, 2012;Novotny-Diermayr V *et al*, 2012) which may enhance the significance of this kinase within the bone marrow microenvironment. Combination of the FLT3 inhibitor PKC412 with several selective JAK inhibitors including ruxilotinib and AZD-1480 has been shown to induce synergy against primary AML cells cultured on HS-5 stroma and in the presence of stroma culture medium (SCM), although the activity was noted to be diminished against MV4-11 (ITD mutated) cells (Weisberg E *et al*, 2012). There are as yet minimal published data looking at pacritinib alone in this context which justifies exploration of this question as will be described below.

5.2 Methods

5.2.1 48 hour co-culture model using Cell Glo technique

In this experiment each individual primary AML patient sample was cultured with the same drug in three different conditions which included standard IMDM medium, cytokine enriched Long Term Culture Medium (LTC) medium and finally LTC medium in a 96-well plate previously seeded with a monolayer of mouse (MS5) stroma cells. The latter condition was used to represent an *in vitro* model of the bone marrow microenvironment to enable assessment of whether AML cells are similarly sensitive to pacritinib when supported by a stroma layer. MS-5 cells were chosen as they are robust and grow well in standard culture conditions. Their non-human origin also makes them easy to distinguish from human AML cells in co-culture experiments as they lack CD45 expression by FLOW cytometry. For experiments, cells were seeded in 96-well plates at a seeding density of 1500 cells/well (final volume 200ul/well), or at 7.6 x10^4 cells/well in 6 well plates (final volume 3ml/well) for western blot set up and incubated for 24 hours prior to the addition of AML primary cells.

As a control, additional plates were set up in parallel with each experiment containing just MS-5 cells and drug at the same concentration. This was necessary to assess the effect of the drugs on the MS-5 cells in isolation, for example a potent cytotoxic effect at 48 hours would suggest that the micro-environmental model was not robust. Also, by subtraction of the luminescence readings for MS5 cells alone from that of the combination of MS-5 and AML cells a reading of the signal relating to just the AML (and therefore their individual sensitivity on stroma) may be derived.

5.2.2 Patient characteristics of stroma cohort

Results pertaining to a total of 7 primary samples are available for analysis using this model. The patient characteristics of the 7 samples analysed and details of their presentation, disease phenotype and FLT3 status are detailed in the Table 5.1.

Sample	Age (years)	Gender	Cytogenetics	ITD	TKD	NPM1	De novo	CR post #1?
15-0611	17	M	Int	WT	WT	WT	Y	Y
15-2857	43	F	Int	Mut	Mut	WT	Y	Y
17-2661	27	M	Poor t(3,5)	Mut	WT	WT	Y	Y
15-3063	65	M	Int	Mut	WT	WT	Y	No Resistant disease
17-3549	42	F	Int	Mut	WT	Mut	Y	Y
17-3392	66	M	Int	Mut	Mut	Mut	Y	Y
17-3872	55	M	Int	WT	Mut	Mut	Y	No Allograft

5.3 Table 10: F=Female, M=Male, Int=Intermediate risk cytogenetics, Poor=Poor risk cytogenetics, WT=Wild type, Mut=Mutant, De novo=no prior MDS, AML or other malignancy, CR post #1=remission status post course 1 where CR = <5% blasts by morphology and no detectable MRD marker**Results**

5.3.1 *In-vitro* cytotoxic response according to culture medium

Mononuclear cells from the 7 evaluable patients were incubated for 48 hours with a range of concentrations of pacritinib as described above. Figure 5.1 shows the mean Cell Glo dose response curves for this cohort stratified by culture medium for all 7 samples. The AML only curve refers to cells cultured in bare 96-well plates suspended in IMDM. The MS5- curve represents the result for the value derived from subtracting the readings from the MS-5 and drug only plates from that for

the 'combination' plates (MS-5 monolayer plus AMLs). The MS5 plates were cultured in LTC and the effect of LTC vs IMDM in the absence of stroma is discussed later in this section.

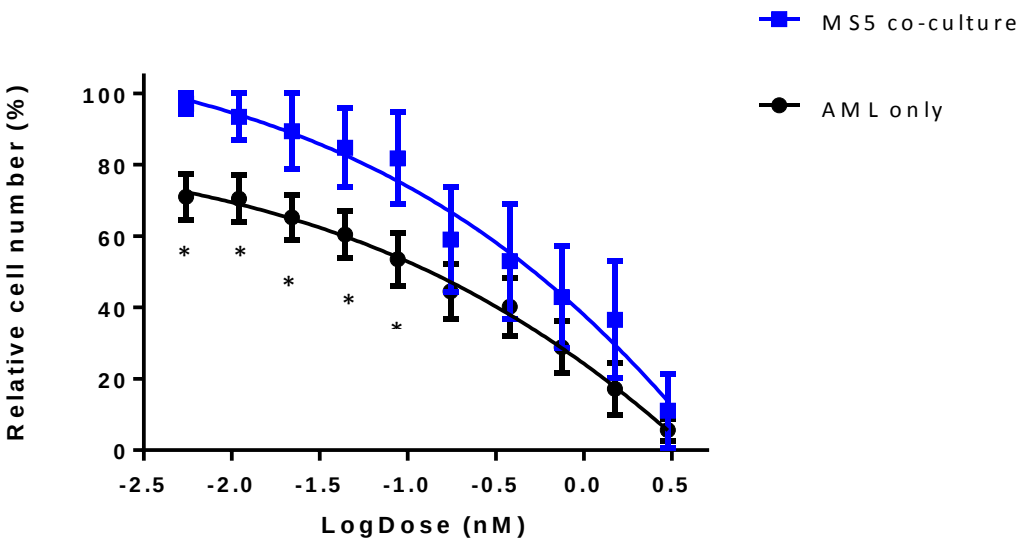


Figure 5.1: Mean cytotoxic dose response to pacritinib in 7 primary samples cultured independently (AML only) or in combination with MS-5 stroma cells. The graph shows a higher % survival in the co-culture setting compared to IMDM only which was statistically significant at low doses <95nM (*=p<0.05 Mann Whitney U test).

Further analysis of these data shows that, for doses of pacritinib <100nM, there is a statistically significantly increased cytotoxic response (measured as reduction in % cell survival) when cultured in IMDM compared to that seen on a MS5 stroma layer, suggesting a protective effect of the stroma. At doses of >100nM the difference in cytotoxic response was not statistically significant between IMDM and MS5 conditions which is in line with the mean EC_{50} for ITD mutated samples. Initially it was thought that this may be due to a toxic effect of pacritinib on the MS5 stroma at higher doses. Data from these experiments does not, however, support this hypothesis. Figure 5.2 shows the mean cytotoxic dose response for MS5 cells from 7 experiments and clearly shows that pacritinib has minimal effect on their survival.

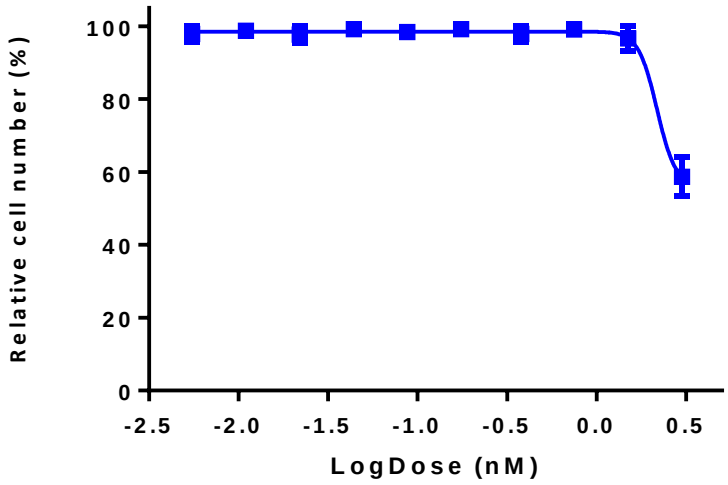


Figure 5.2: Mean cytotoxic dose response of MS5 cells to pacritinib treatment in 7 experiments showing no effect on survival below the maximum dose (3µM)

The histogram below (Fig 5.3) shows comparative EC_{50}s in individual patient samples cultured in the two culture conditions and allocated according to FLT3 mutation status.

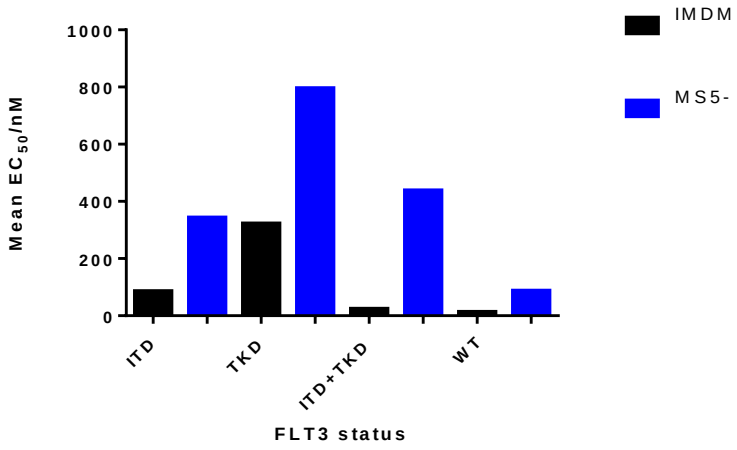


Figure 5.3: Histogram showing EC_{50} for AML primary samples cultured either in isolation or in combination with MS-5 stroma cells stratified by FLT3 mutation status. The data shows comparison for same sample cultured in isolation (IMDM) or on stromal layer (MS5-). ITD samples n=2 mean value given (sample 15-3063 excluded as resistant), WT n=1, TKD n=1. ITD+TKD n=2 (mean).

The data above suggest that the stroma layer supports the AML cells and makes them less sensitive to the effect of pacritinib, as shown by the consistently higher EC_{50} for culture on MS5 stroma

compared to IMDM medium alone. Suprisingly, the single WT sample appears to be more sensitive than any other subtype, but as a single example this likely represents individual variation and is sadly a reflection of the small sample size.

The next question to address is to what extent the composition of the culture medium itself impacts on sensitivity to pacritinib. As described in chapter 2 the LTC medium differs markedly from IMDM including the addition of cytokines (TPO, IL-3 and GCSF) and also a different base medium (α-MEM with supplements).

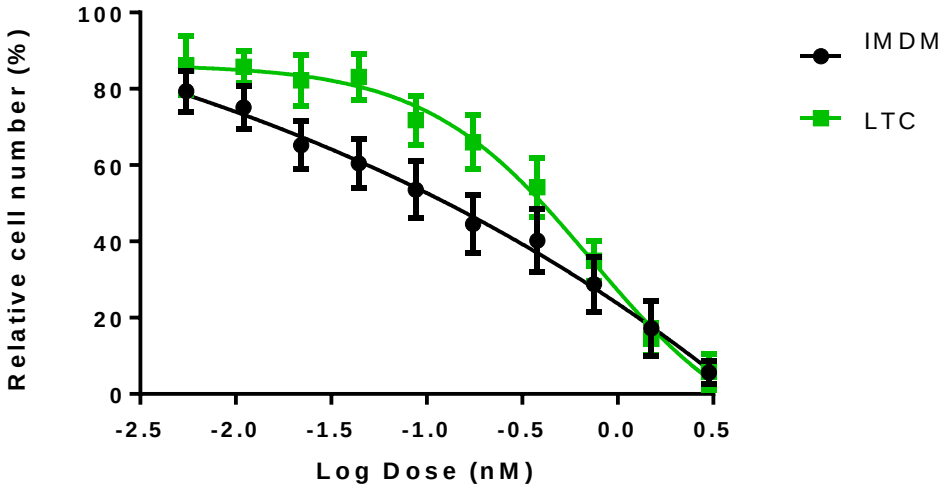


Fig 5.4: Mean dose response curve for 7 primary AML samples cultured in IMDM versus LTC medium. The graph shows higher survival in the LTC medium compared to IMDM which is most marked at low doses of pacritinib, the effect being absent at the 3μM and 1.5μM dose (lines converge). No significant differences found at any dose.

The results shown in Fig. 5.4 suggest that the LTC medium exerts a considerable positive impact on survival of AML cells in 48 hours culture even in the absence of stroma support. These 48 hour co-culture experiments were carried out using the Cell Glo technique. As discussed in chapter 3 the readout from cytotoxicity assays cannot be reliably interpreted as confirmation that cell death has occurred. Confirmation that living cells are no longer viable requires additional assays looking at induction of apoptosis such as the Annexin V Binding Assay (see chapter 3). This was carried out using AML mononuclear cells from 3 individual patients, all of whom harboured a FLT3 ITD mutation.

The primary cells were cultured on and off MS-5 stroma and the results of these experiments are shown in Figure 5.5 (48 hour culture) and 5.6 (7 day culture) below.

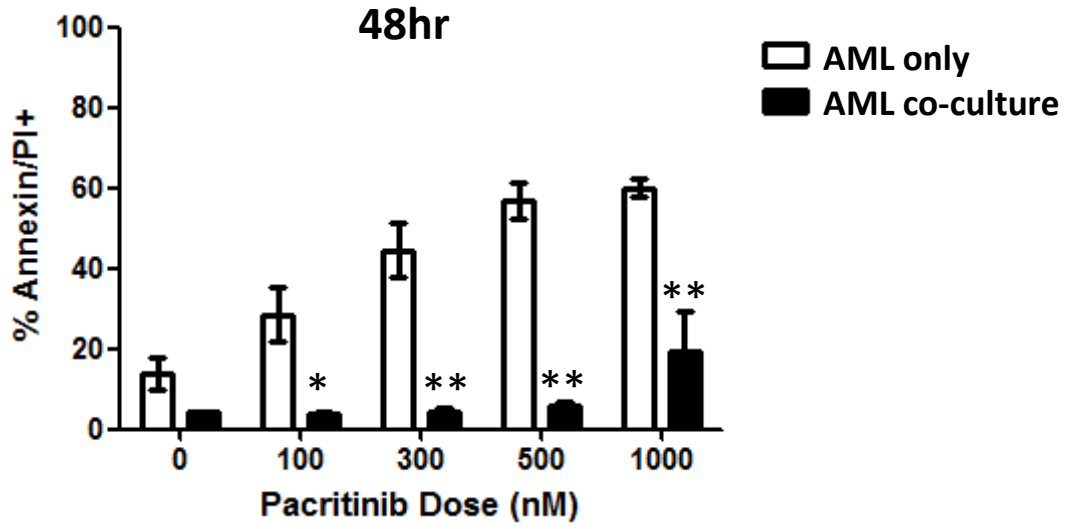


Figure 5.5: 48 hour Annexin V/PI response in primary AML cells treated with pacritinib or DMSO vehicle control in the presence and absence of MS-5 stroma (data from Mrs Michelle Lazenby) **p<0.001 *p<0.01

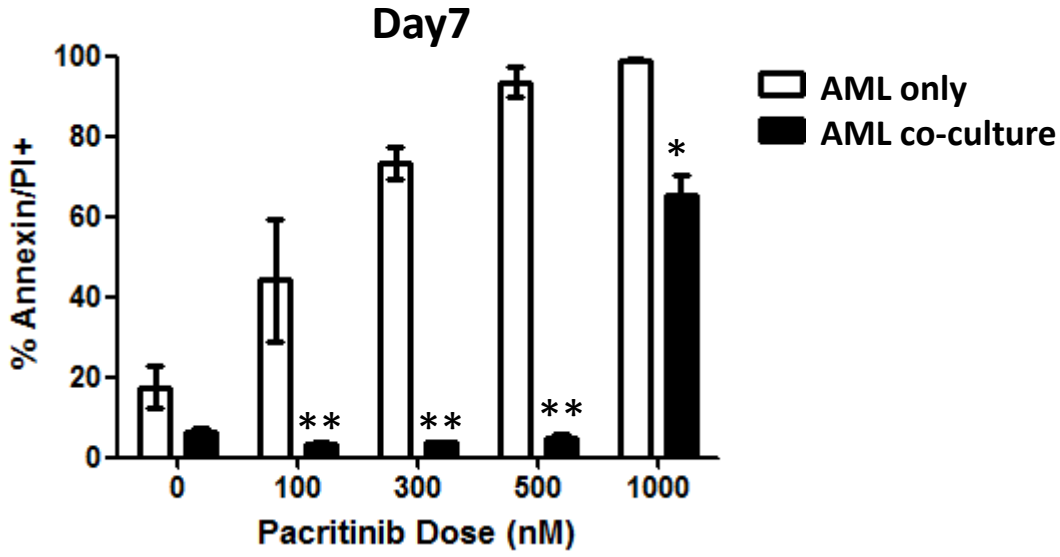


Figure 5.6: 7 day AnnexinV/PI response in primary AML cells treated with pacritinib of DMSO vehicle control in the presence and absence of MS-5 stroma (data from Mrs Michelle Lazenby) **p<0.001 *p<0.01

The results above show marked reduction in late apoptotic induction in primary AML cells cultured on stroma compared to IMDM culture medium. This was seen throughout the dose range at 48 hours. At 7 days a greater than 50% Annexin V/PI ++ response was only seen at the 1000nM dose in the co-culture setting.

5.3.2 Western blotting results: effect of stroma on downstream signalling

As described in chapter 4, pacritinib has a variable effect on signalling pathways downstream of the FLT3 receptor tyrosine kinase seen in *in vitro* experiments performed in the absence of a stroma monolayer. In order to explain why primary AML cells appear less sensitive to pacritinib in the co-culture setting it is necessary to analyse whether there is any differential effect on downstream signalling pathways when cells are cultured on stroma, which could potentially highlight a source of disease resistance.

Western blot experiments were carried out in the co-culture setting using six well plates pre-seeded with a MS-5 mono-layer. The aim of this was to analyse any difference in signalling response to pacritinib treatment when AML cells are cultured on stroma which is caused by the stroma itself. The six well plates were seeded with MS5 cells at a density of $7.5x10^5$ per well with AML cells and drug dilutions added following an initial 24 hour incubation. The samples were harvested after the desired time period and divided into two separate populations: suspension and adherent cells. The 'suspension' population contains those AML cells which are floating whereas the adherent population contains those cells that have attached to the stroma layer, these cells being removed by the addition of trypsin to each well after the suspension fraction had been removed. Results suggested that amongst the stroma adherent population there was a reduced effect on phospho-STAT5 signalling in comparison to that seen in the suspension fraction.

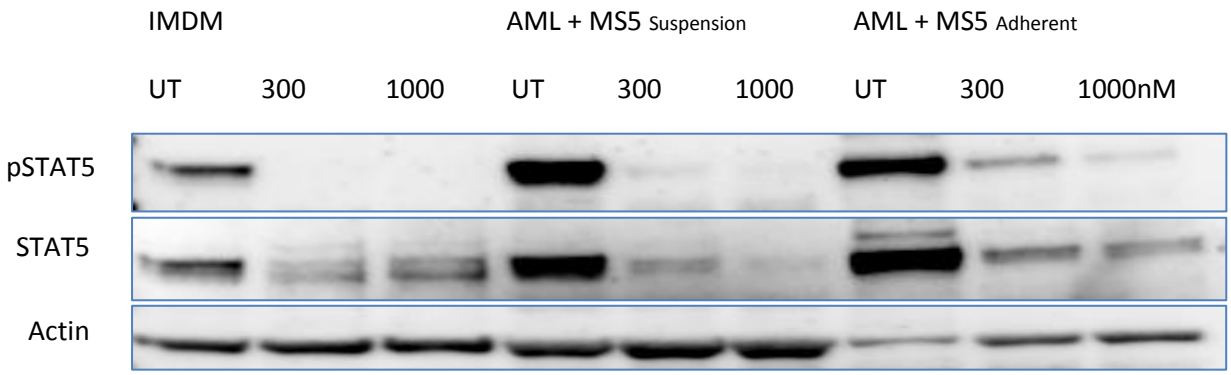


Figure 5.7 Sample 15-2913 (FLT3 ITD mutated) pSTAT5 and STAT5 signalling on and off stroma monolayer (loading adjusted in light of actin result) at 24 hours.

The blots above show similar levels of pSTAT5 inhibition in the stroma co-culture setting to those seen in AML cells cultured in isolation although the pSTAT5 signal was basally increased in co-culture and the effects of pacritinib were mildly attenuated amongst the adherent cell population. There was some inhibition of total STAT5 signal seen, more so than in the previous samples although this may be affected by the uneven actin loading. Interestingly however, the pSTAT5 signal was basally increased in co-culture setting and the effects of pacritinib were mildly attenuated amongst the adherent cell population. Also, the actin result shows uneven loading in the adherent cells for the untreated lane therefore if loading were equal, this effect may have been more marked. There was also a less pronounced effect on total STAT5 signal seen

As described, previous authors have shown that cytokine-activated ERK rather than STAT5 appears to be the most important downstream signalling protein mediating the protective effect on leukaemic blasts in a co-culture setting (Yang X *et al*, 2014). The blots from this experiment were, therefore, re-probed using anti-phosphoERK and anti-total ERK antibodies without the need for membrane stripping as the proteins have different molecular weights (pSTAT5 90kDa pERK 1+2 42/44 kDa doublet). Figure 5.8 shows the results of this same experiment using pERK/ERK antibodies.

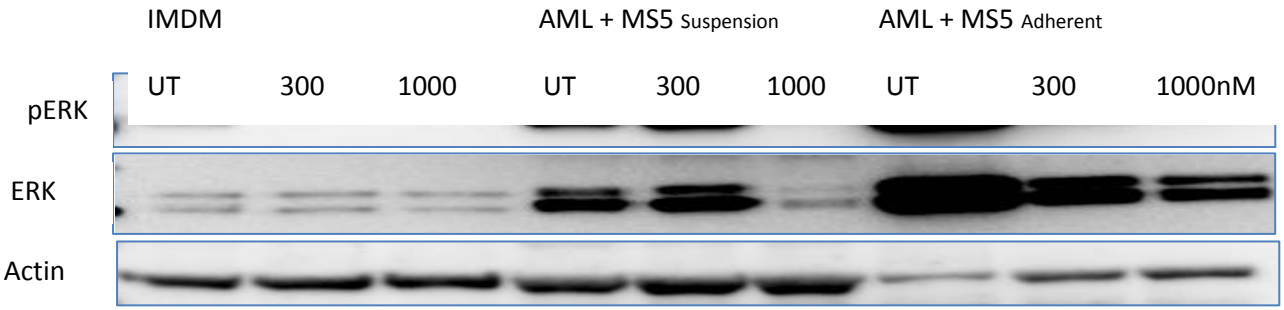


Figure 5.8: Sample 15-2913 (FLT3 ITD mutated) pERK and ERK signalling on and off stroma monolayer.

The result here shows striking up-regulation of pERK and ERK signalling amongst the stroma co-culture suspension and adherent cells in untreated samples. This was largely overcome by 1000nM pacritinib dose in the suspension cells but the adherent cell signal was effectively maintained even up to the maximum dose used. Off stroma there was inhibition of pERK signal seen with pacritinib treatment but no effect on total ERK.

5.4 Discussion

The experiments described in this chapter have shown that at 48 hours, AML cells are less sensitive to pacritinib if cultured on a monolayer of mouse stroma cells, using media enriched with cytokines compared to when they are grown in isolation using IMDM; a lower EC_{50} is seen for culture in IMDM compared to in the co-culture setting. This suggests that the stroma has an effect on survival, which may either be because it in some way inhibits the effect of the drug, or because it generates signals that can activate resistance pathways to allow the leukaemic cells to survive. This effect was statistically significant for doses of $\leq$95nM Pacritinib: using Wilcoxon Signed Rank test the p value for an increased cytotoxic response in IMDM as compared to culture on MS5 stromal cells was consistently <0.05. Another interesting observation from comparing the survival curves on and off stroma shown in Figure 5.1 is that the stroma appears to give a survival advantage to untreated cells given that the greatest divergence between the curves is seen at the lowest doses of the drug. This is likely be due to basal up regulation of pSTAT5 and pERK signalling on stroma as observed in the

western blotting experiments (Fig 5.7 and 5.8). The data also show that the enriched LTC medium confers a survival benefit even in the absence of stroma (Fig 5.4) which was most marked in the middle part of the dose range but seemed to be abrogated at very high or low doses of pacritinib. The maximum divergence of the curves across the middle part of the dose range seen in Fig 5.4 is likely to be due to the effect of cytokines in the medium which provide support and up-regulate pro-survival pathways sufficiently to improve survival around the threshold of the EC_{50} but cannot overcome the inhibitory effect of very high doses of pacritinib and is not required by effectively untreated cells at low doses in the absence of stroma. There was limited sensitivity to pacritinib seen in MS5 cells cultured in isolation (Fig 5.2) which suggests that they are not reliant on FLT3 signalling for survival.

When considering the 7 samples used for the stroma co-culture experiments, there were two samples with atypical results which effectively translated as resistance in both IMDM and MS5 conditions with no significant difference between the two. One of these patients (15-3063) carried a FLT3 ITD mutation. The other patient (17-3872) harboured a FLT3 TKD point mutation at diagnosis and went on to have clinically resistant disease, having failed to achieve remission following the first course of chemotherapy. These findings likely relate to a lack of reliance on FLT3 signalling in these samples despite the fact that both harboured a form of FLT3 mutation. This supports the theory that the link between the structure of the FLT3 receptor and sensitivity to FLT3 inhibitors is complex which limits the effectiveness of single agent treatment with pacritinib. Clearly with such small numbers it is difficult to draw meaningful conclusions and to improve the robustness of this data, additional samples would be required.

In addition to a reduction in the cytotoxic response to pacritinib seen when AML cells are cultured on stroma, the data described above suggest this effect is due to inhibition of apoptotic induction in this setting compared to culture in IMDM alone. Figures 5.5 and 5.6 show that >50% late apoptotic induction on stroma was only achieved following 7 days of culture in the 1000nM dose level. This

offers a plausible explanation for apparent resistance seen on stroma after 48 hours of culture using the Cell Glo technique. Outside of this dose and time point late apoptotic cell levels of <10% were seen and the difference between stroma and IMDM plates was highly significant ($p<0.0001$). The finding of potent FLT3 inhibition which does not result in clearance of bone marrow blasts has been described elsewhere (Yang X *et al*, 2014) and was previously attributed to the occurrence of cell cycle arrest rather than induction of apoptosis. Cytotoxicity assays which measure ATP generation are limited by the lack of ability to distinguish viable cells from 'static' cells.

When assessing the reliability of these findings it is prudent to analyse how well the experimental conditions reflect the climate *in vivo*. Clearly the use of a mouse stromal cell line (MS5) rather than human stroma immediately makes the culture model less akin to conditions within the patient's own microenvironment. Human stroma however, is often less robust and difficult to culture reliably in a high throughput drug assay. Also it takes around 6 weeks to grow human stroma to a level of confluence and it can only be passaged once which is limiting from a practical laboratory perspective.

The experiments above used different culture media, namely IMDM and LTC. The latter contains additional cytokines including IL-3, IL-6, Thrombopoeitin (TPO) and Granulocyte Colony Stimulating Factor (GCSF) which are of human origin and may be more advantageous in terms of leukaemic cell survival *in vitro* than the mouse stroma. All of these cytokines may independently affect response to the microenvironment; for example, IL-6 is known to activate STAT5 via JAK and this may confer additional resistance properties for the adherent/suspension primary AML mononuclear cell population which would not have been seen had the MS5 cells been maintained in IMDM or alpha-MEM without the addition of cytokines. In a study of other myeloid malignancies such as Chronic Myeloid Leukaemia and AML it has been shown that certain cytokines such as IL-6 and GM-CSF may play a role in stroma-mediated cytoprotection of TKI treated disease (Weisberg E *et al*, 2012).

The data comparing IMDM and LTC in the absence of stroma suggest that the enriched medium exerts a protective effect. The IL-3/6 data suggest that this is purely a beneficial influence; the role of G-CSF is, however, more controversial. Granulocyte Colony Stimulating Factor is a glycoprotein growth factor for myeloid cells which stimulates proliferation, differentiation of neutrophils and their precursors. The effect of G-CSF in a drug treatment model partly depends on the cell cycle effects of the drug(s) involved. G-CSF is, for example, commonly used as part of a standard chemotherapy regimen used to treat AML (Fludarabine-AraC-G-CSF FLAG) where it must be given 21 hours prior to the other two agents for maximal impact on leukaemic cell survival to be observed in an *in vitro* setting. In contrast sequential exposure to GCSF and fludarabine lead to improved survival suggesting a negative impact on drug efficacy. The effect of continuous exposure to G-CSF in a 48 hour *in vitro* model is, therefore, difficult to predict.

In addition, conventional cytotoxic drugs such as fludarabine and AraC exert their effect by means of 'bulk kill' with generic cytotoxic effects on all cells (e.g. interruption of base sequencing in DNA). Modern targeted agents such as FLT3 TKIs, however, have a more specific mode of action. In the context of FLT3 there is, as previously discussed, an association between FLT3 ITD expression and CD34+ phenotype especially within the non-cycling stroma-niche population. It has been shown that cytokine cocktails (IL-1beta, IL-3, IL-6, SCF, GM-CSF) can reduce the amount of CD34+ progenitors in acute myeloid leukemia (AML) (Braun SW *et al*, 2001). This was then shown to translate into a reduction in CD34+ colony formation which was also associated with a better clinical prognosis. There may, however, be a potential negative effect on the efficacy of a drug such as pacritinib as a result of cytokine induced differentiation, if the loss of CD34 expression is linked to a real time reduction in FLT3-ITD mutant phenotype and behaviour.

To formally answer the question regarding the presence of an independent effect of the medium it would have been necessary to compare LTC medium with and without cytokines to specifically analyse whether the cytokines provide a survival benefit to the AML cells. Once this has been

established it would then be possible to look at any additional benefit derived from the MS-5 cells, over and above the effect of the medium. This was beyond the scope of this work which was run over the course of one year, working part time in the laboratory and with some on-going clinical commitments. It would certainly form part of future work to be carried on in this project if time was available.

Overall these experiments suggest that a short term culture model and survival/resistance at 48 hours is probably less relevant to disease relapse than when a longer term culture model is used. Other members of this group have carried out additional medium and longer term culture experiments which suggest that pacritinib may be able to suppress leukaemic outgrowth from the stroma after 14 days of culture and suppresses colony formation after 5-6 weeks of growth using a cobblestone technique. I am grateful to Dr Gareth Edwards and Dr Caroline Alvares for their assistance with these techniques.

Reduction in the ability to target pathways downstream of FLT3 is part of the explanation for the lack of efficacy seen following treatment of AML cells on stroma with FLT3 TKIs. Previous work in the MV4-11 cell line using SU5614 was shown to reduce phosphorylation of FLT3 and its downstream markers STAT5 and ERK when co-cultured with the murine cell line EL08-1D2. The same authors found a lesser reduction in activated (phosphorylated-STAT5) in primary AML cells treated with SU5614 which was similar in both FLT3-ITD mutated and WT samples. Interestingly, uncoupling of FLT3-ITD from STAT5 signalling in primary CD34+FLT3-ITD+ cells in the presence of TKI was seen (Parmar A *et al*, 2011) and may be attributed to isolated inhibition of FLT3 at the plasma membrane level having less effect on intra-cytoplasmic signalling. This lends weight to the argument for a therapeutic role for agents which can target STAT5 via a dual-pronged approach.

The co-culture western blotting data presented here is limited to that derived from using a single primary AML sample, from a patient who harboured a FLT3 ITD mutation. Although the conclusions drawn from this work are interesting, it should be emphasised that this is still only a single example

and therefore further repetitions are required to corroborate the evidence presented here, given the heterogeneity of AML patients. This would have required significantly more time in the laboratory than was available within the constraints of this project in order to repeat the experiment in a minimum of 3, ideally 5, patient samples including a mixture if FLT3 ITD mutated and WT cases. This would however, form a large part of any future development of this project.

In the stroma co-culture setting, basal phosphorylated and total STAT5 signal was increased in both the suspension cells and in the adherent population (Fig. 5.7). In addition, there was some attenuation in the pSTAT5 response seen in the adherent AML cells compared to suspension cells or the same sample cultured off stroma (in IMDM).

Previous authors have looked at the relative importance of the STAT5 pathway in *in vitro* experiments looking at the efficacy of FLT3 inhibitor therapy on and off stroma (Yang X *et al*, 2014). Although its role is controversial (Parmar A *et al*, 2011) one interesting paper demonstrated that inhibition of STAT5 phosphorylation did not correlate with cytotoxicity in FLT3-ITD positive blasts on stroma using the MTT method (Yang X *et al*, 2014). Yang also confirmed that IL-3 activates STAT5 independently of FLT3 in Molm14 cells on or off stroma; this is relevant because the LTC medium used for the stroma plates in these experiments was supplemented with cytokines including IL-3, TPO and GCSF. This offers a possible explanation for the basal up-regulation of both p-STAT5 and the total STAT5 signal seen in these cells, but not seen in the IMDM culture conditions. Adherent fractions however, displayed increased p-STAT5 compared to suspension cells in the same medium which suggests that the increased signal seen on stroma is independent of the effect of the LTC medium.

The other main downstream effector of FLT3 analysed in this work was the MEK/ERK pathway which was also tested in the co-culture setting, albeit in a single patient sample. There was inhibition of ERK phosphorylation above the 300nM dose in the IMDM condition where as on stroma this effect was reduced. The adherent cells showed no inhibition of p-ERK signal even using the maximal

1000nM dose and the suspension cells were only sensitive at the 1000nM dose (Fig 5.8). This has also been reported elsewhere (Yang X *et al*, 2014) and, interestingly, this reduction in activity against components of the ERK pathway when AML cells are cultured on stroma suggests that this may be a mechanism of potential resistance to Pacritinib (and other TKIs) in the stroma-adherent population. Once again, phosphorylation of ERK (in a similar fashion to STAT5) appears to be basally increased in these experiments (Fig 5.8) in the co culture setting. This has been previously reported (Yang X *et al*, 2014) along with reports that ERK phosphorylation cannot be fully inhibited even at doses of other TKIs (quizartinib and sorafenib) which were sufficient to inhibit auto-phosphorylation of FLT3 using Molm14 cells cultured on human stroma. This is supported by the observation of a similar effect using primary AML mononuclear cells on mouse stroma (Fig 5.8). Yang et al hypothesise that this protective effect is due to multiple cytokines released by the stroma, to direct contact between the stroma and the AML cells and to the influence of FLT3 ligand. They showed that ERK remained phosphorylated despite inhibition of FLT3 autophosphorylation in cell lines and suggested that persistent activation of ERK confers resistance to FLT3 inhibitors.

In summary, there are some preliminary data here to suggest that attenuation of the pSTAT5 effect and minimal impact on ERK/MAPK pathway signalling may contribute significantly to the mechanism of resistance to pacritinib both on and off stroma. Although pacritinib can target pERK signalling in the absence of stroma, this effect appears to be reduced in the co-culture setting (Fig 5.8). The latter observation adds weight to the hypothesis that dual targeting of ERK using a MEK inhibitor in combination with pacritinib may enhance the cytotoxic effect. No previous studies have specifically looked at ERK signalling in the context of pacritinib treatment; however, this preliminary finding justifies the exploration of the synergistic relationship between Pacritinib and a MEK inhibitor (PD035901) as will be described in chapter 6.

Chapter 6

The role of synergy in overcoming stroma-derived protection of the leukaemic clone

Chapter 6: The role of synergy in overcoming stroma-derived protection of the leukaemic clone

6.1 Introduction:

Combination therapy is standard practice in the treatment of acute myeloid leukaemia and virtually all haematological malignancies. In the chemotherapy era anthracycline drugs (daunorubicin, idarubicin) in combination with cytosine arabinoside (AraC) have formed the backbone of treatment for many years. AraC is a cytotoxic antimetabolite with structural similarity to deoxycytidine that rapidly converts to cytosine arabinoside triphosphate in leukaemia cells. Cytosine is a base that normally combines with a different sugar, deoxyribose, to form deoxycytidine which is incorporated into DNA. The substitution of deoxycytidine for arabinose acts as a trigger for apoptosis via inhibition of the cell cycle S phase (synthesis of DNA). Clearly, any new drug intended for use in treating patients with AML must not hinder the effect of existing standard therapies such as AraC, as they are unlikely to be given as single agents, certainly in the setting of induction therapy.

A combination of agents is required to provide a multi-targeted attack against a leukaemic clone equipped with several innate and adaptive mechanisms to limit the effectiveness of drug therapy. In the more recent era of so called 'novel therapies', FLT3 inhibitors have been used in combination with standard chemotherapy in several large phase II and a smaller number of phase III studies (Knapper S, 2011) and in the laboratory they have been combined with a variety of other agents including MEK inhibitors (Yang X *et al*, 2014), HDAC inhibitors (Novotny-Diermayr V *et al*, 2012). Other authors have combined first and second generation FLT3 TKIs (Zhang W *et al*, 2014).

FLT3 is one of several mutationally-activated kinases which defines a clinically-validated target for cancer drug therapy (Wilson TR *et al*, 2012). Treatment of a variety of haematological and solid organ tumours with such kinase inhibitors has, however, been somewhat thwarted by the emergence of innate and acquired resistance mechanisms as barriers to effective therapy. One overriding theme of this process is that the cancer cell responds to blockade of a central receptor tyrosine kinase such as FLT3 by engaging other downstream pathways which may have been

previously redundant (Wilson TR *et al*, 2012) or quiescent in the context of the molecule involved. As illustrated in previous chapters, leukaemic blasts express multiple receptor tyrosine kinases which transduce signals via several key downstream 'hubs' crucial for survival of the cell. Notable examples include phosphatidylinositosol-3-OH kinase (Pi(3)k) and mitogen-activated protein-kinase (MAPK)/extracellular-signal-regulated kinase (ERK) (Stirewalt DL & Radich JP, 2003) see chapter 1 figure 1.1.

The relative up-regulation of ERK signalling in the mouse stroma cell (MS-5) co-culture model as opposed to culture in IMDM alone hypothesised from western blotting experiments in the previous chapter provided a rationale to explore potential combination effects between pacritinib and PD0325901, a small molecule inhibitor of MEK that has previously been investigated elsewhere in combination with alternative FLT3 TKIs with some encouraging results (Yang X *et al*, 2014). In essence, the ERK pathway is not thought to be critical to the enhancement of proliferative signals in normal myeloid progenitors or in leukaemic blasts cultured off stroma. In cell lines, however, AKT and/or MAPK signalling proteins remain phosphorylated following FLT3 TKI treatment in resistant lines even when FLT3 is inhibited (Piloto O *et al*, 2007); this is part of the mechanism that renders them FLT3 independent. As would be expected, in primary AML cells this effect is more variable and less predictable with some patient samples showing consistent activation of AKT etc and others not. In response to 'stress' from upstream inhibition of FLT3 the small population of leukaemic blasts within the bone marrow niche may up-regulate the activity of the FLT3 pathway, possibly through both soluble and membrane-bound factors secreted by the stroma which activate downstream ERK (Yang X *et al*, 2014). This may then act as an escape mechanism facilitating survival of a sub-population of cells within the bone marrow niche, justifying the testing of combination therapy to eradicate this 'minimal residual disease' population.

6.1.1: Previous experience with MEK inhibition in AML

Constitutive activation of the RAF/MEK/ERK pathway is frequently observed in haematological malignancies and is sufficient to transform mammalian cells and several upstream-acting oncogenes, leading to a change in cell phenotype (Ricciardi MR *et al*, 2014). RAF/MEK/ERK activation has been identified as a potential therapeutic target given that it has been shown to convey an adverse prognosis (Ricciardi MR *et al*, 2014) Altered regulation of MAPK has been shown to lead to an aggressive, therapy-resistant behaviour in acute leukaemias. PD0325901 is a small molecule, ATP-non-competitive, MEK inhibitor which has been previously studied in the treatment of human and murine acute myeloid leukaemia (Ricciardi MR *et al*, 2014) (Burgess MR *et al*, 2014) (Yang X *et al*, 2014). It inhibits phosphorylation of ERK (with no corresponding effect on FLT3) in Molm14 cells both in suspension culture and in co-culture with stroma (Yang X *et al*, 2014).

Treatment with PD0325901 in a primary mouse leukaemia model where the mice carry an induced NRAS mutation was shown to prolong their survival and reduce proliferation of the leukaemic clone. There was, however, no direct increase in apoptosis or differentiation seen (Burgess MR *et al*, 2014) Elsewhere it has been shown that in a cell line model PD0325901 produced marked inhibition of ERK phosphorylation and growth of AML cell lines and around 70% of primary AML samples as measured by inhibition of ERK phosphorylation by western blotting. Growth arrest was also seen, due to G_1-phase arrest and induction of apoptosis measured by AVB assay (Ricciardi MR *et al*, 2014;Piloto O *et al*, 2007) Piloto et al identified that activating RAS mutations were present in FLT3-TKI resistant cell lines and that treatment of these cells with MEK inhibitors and/or PI3k inhibitors combined with increasing concentrations of FLT3 inhibitors CEP-701 or CEP-5214 could restore sensitivity to the FLT3 TKIs in MTT assays (Piloto O *et al*, 2007). In a myeloma cell line model use of KRAS inhibition has been shown to target all cell lines regardless of RAS mutational status in contrast to the effect seen with isolated inhibition of ERK or MAPK within the same stroma co-culture setting (Aronson L, 2014). There was, however, considerable toxicity to the bone marrow stroma layer was seen which

suggests that selective targeting of RAS may be too fundamental to normal as well as malignant cells to be clinically feasible or safe. In contrast, ERK occupies a more distal position in the signalling cascade and also sits at a convergence point for several key pathways which makes it an attractive and more clinically tolerable therapeutic target.

6.1.2: Basic rules of drug synergy

The primary goals of drug combination regimens may be summarised as:

1. Achievement of synergistic therapeutic effect and avoidance of antagonism
2. Reduction of toxicity (often through dose reduction)
3. Prevention of development of drug resistance

It has been suggested that points 2 & 3 above may actually occur due to the effect of synergy alone(Chou T-C, 2010). There are many different definitions of synergism and methods for its determination, up to 13 having been quoted in one review from 1995 (Greco WR *et al*, 1995). Moreover, significant variation and disagreement between some models exists, which can lead to a lack of clarity regarding the evidence provided by clinical trials and scientific literature. This could ultimately compromise patient outcome and safety, given that this data is used to design the composition of drug regimens that must be both safe and effective.

The method of Chou & Talalay published in 1983 & 1984 which introduced the scientific term Combination Index (CI) to quantitatively depict synergism remains one of the most widely accepted methods to determine synergism. This model is based on the law of mass action where the median value serves as a link between single and multiple entities in the setting of first order or higher order dynamics (dose effect curve hyperbolic or sigmoidal respectively) (Chou T-C, 2010). This general equation is derived from several formulae including the Michaelis-Menten, Hill, Henderson-Hasselbach and Scatchcard equations in biochemistry and biophysics. Synergism is defined by CI<1, antagonism by CI>1 and if CI=1 this is known as an additive effect (although the range of this may be

extended to include CI values from 0.9-1.1). A CI value <0.3 is said to be strongly synergistic and >3.3 strongly antagonistic although, in reality, extreme values are generally thought to be unreliable.

6.1.3: Combination of drugs with differing modes of action

Drugs which have a mechanism of action which is totally independent of each other are known as 'mutually nonexclusive' and give a hyperbolic dose-effect curve when combined. An example of this would be the combination of a FLT3 inhibitor (pacritinib) with a cytotoxic antimetabolite (AraC). Under these conditions, the summation of effects of the two drugs can be calculated by multiplying the residual fraction of the activity unaffected by each drug individually.

For example:

Drug X inhibits a process by 20%

Fraction affected (f_a) = 0.2 Fraction unaffected (f_u) = 1 –f_a = 0.8

Drug Y inhibits same process by 40%

f_a = 0.4 f_u=0.6

According to the fractional product model, these two drugs would be called 'additive' if their combination leaves 48% (60% of 80%) unaffected.

(i.e.) $(f_u)_{X,Y} = (f_u)_X \times (f_u)_Y$

For non-mutually exclusive drugs with different modes of action, the CI is derived by:

$$CI = (D)_X / (D_x)_X + (D)_Y / (D_x)_Y + (D)_X(D)_Y / (D_x)_X (D_x)_Y$$

Where the numerator refers to combination dose of drug: denominator to single agent dose of drug that results in an inhibitory effect of x%.

6.1.4: Combination of drugs with identical modes of action

The method described above cannot be applied if the two drugs have the same mechanism of action, e.g. two different FLT3 inhibitors, even though it is logical to expect some potential synergy if the two drugs have subtly different modes of action through inhibition of different targets within the FLT3 molecule. An example of this would be the combination of a type I TKI such as crenolanib with a type II inhibitor such as sorafenib as previously studied (Zhang W *et al*, 2014). The reason the same principle cannot be applied is because, in this situation, the growth effect curve no longer follows the Michaelis-Menten (first order) kinetics/dynamics with *m*=1 and therefore both the potency (the D_m or EC_{50} value) and the shape of the curve (*m* value) must be taken into account (Chou T-C, 2010). All of the synergy experiments in this work used combinations of drugs with completely different mechanisms of action; therefore, the alternative situation will not be described in further detail here.

6.2 Results

6.2.1 Cytotoxicty of Pacritinib in combination with cytarabine

Cell Glo assessment of the combination of pacritinib and AraC was performed using 29 primary AML samples, including 10 from the initial cohort of 63 samples described in chapters 3&4 and an additional 19 samples processed by other members of the team (Mrs M Gilmour and Mrs C Guy). Three fixed ratios of pacritinib:AraC were chosen based on the previously-established pacritinib mean EC_{50} in primary samples of 92nM for ITD mutated samples and mean AraC EC_{50} of 3.3µM. Serial dilutions of these fixed ratios were set up; it was soon shown that the most effective pacritinib:AraC ratio was 1:100 and this was prioritised later in the experiment.

Figure 6.1 shows the variation in combination indices between pacritinib and AraC that were

obtained at each of the chosen fixed drug ratios.

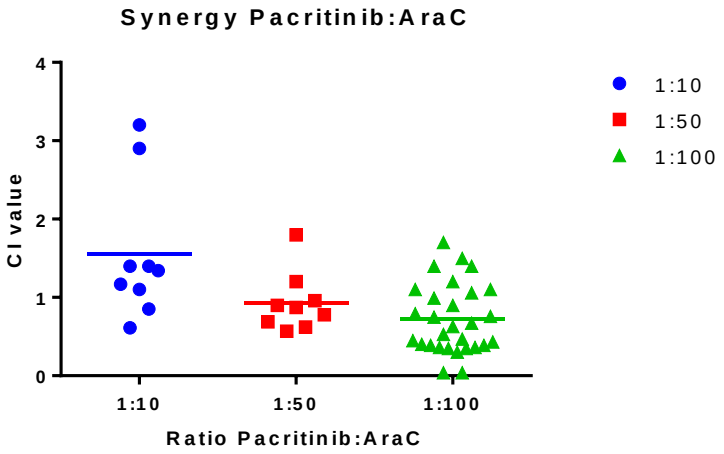


Figure 6.1: Scatter graph for CI values of combination of pacritinib with AraC at 3 different ratios in primary AML cells using cell glo technique: 1:10 n=9, 1:50 n=9, 1:100 n=29

Overall the results show moderate synergy between pacritinib and AraC. The median CI values for the 1:10, 1:50 and 1:100 ratios were 1.34, 0.87 and 0.63 respectively suggesting maximum synergy for the 1:100 ratio; with an antagonistic effect at a 10-fold lower ratio of 1:10. This lower ratio would not be feasible at a clinical level, given that AraC forms part of the therapeutic backbone of virtually all chemotherapy regimens used to treat AML and the pacritinib concentrations required would far exceed those clinically achievable. As expected for primary samples, the results show a relatively wide spread of high and low values for each ratio (for example 0.04-1.7 for 1:100 ratio) which reflects variable inter-patient sensitivity to both drugs- particularly AraC.

6.2.2 Cytotoxicity of pacritinib in combination with MEK inhibitor

As suggested in chapter 5, up-regulation of the ERK signalling pathway may be seen following treatment with pacritinib when AML cells are cultured in combination with MS5 stroma cells. This suggests that this pathway may be part of the mechanism of survival for the stroma-adherent AML population and may partly explain why these cells are more resistant to therapy than are AML cells in the peripheral circulation. This rationale was used to justify combination of pacritinib with a MEK

inhibitor PD0325901 which is a selective, non-ATP competitive inhibitor of MEK. Initially three combinations were tested (10:1, 5:1 and 2:1) which was based on the 10 fold higher EC50 in MV4-11 cell lines for pacritinib compared to PD0325901 (80nM vs 8nM). It soon became apparent that two of these combination ratios were more effective and therefore these were tested (5:1 and 2:1) in a total of 26 primary samples (2:1 n=21, 5:1 n=26) and the scatterplot results for the combination ratios are shown in figure 6.2.

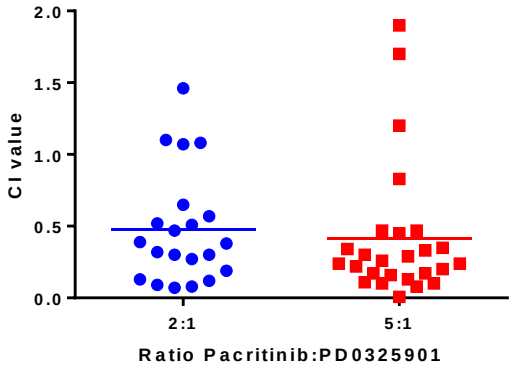


Figure 6.2: Scatterplot for CI value for pacritinib and PD0325901 in primary AML samples using cell glo technique

The median CIs were 0.38 and 0.25 for the 2:1 and 5:1 ratios respectively, the result for 5:1 is described as a good synergistic effect (median CI<0.3). There was a clustering of CI results <1.0 for both ratios, more marked for the 5:1 ratio where the range of CI values was from 0.007-1.9.

Figure 6.3 gives an example of the individual dose response to the combination of pacritinib and PD0325901 at the 5:1 ratio

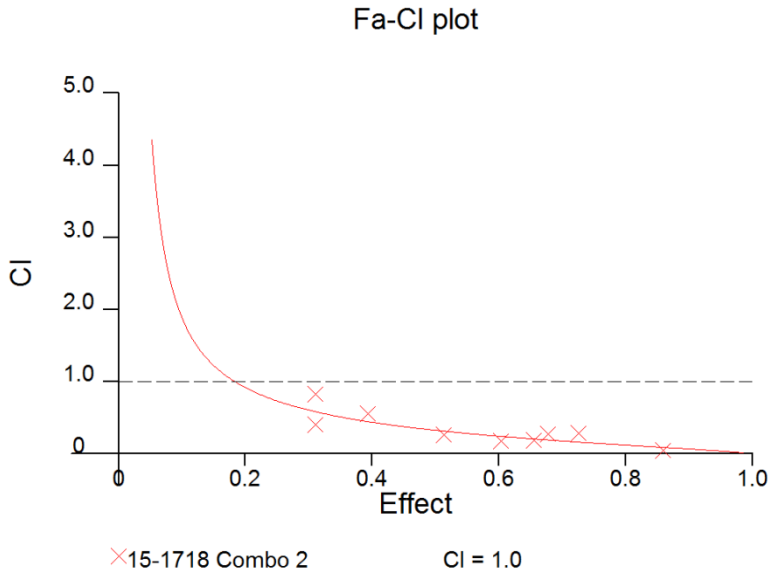


Figure 6.3: CI vs fa for sample 15-1718 showing good synergy (CI) with all points <1.0 for EC_{50}, EC_{75} and EC_{90}.

This shows that synergy is maintained across the dose range for the given combination of pacritinib and PD0325901.

6.4: Discussion

The experiments described in this chapter have analysed the impact of combining pacritinib with both conventional cytotoxic chemotherapy (AraC) and another novel agent (MEK inhibitor). This aspect of the drug's potential as a viable part of the therapeutic armamentarium against FLT3 ITD mutated AML is important to characterise for several reasons. Firstly it is not yet known where in the therapeutic algorithm FLT3 TKIs are best employed, for example, as part of induction chemotherapy or as part as salvage treatment for relapsed disease. In the former case pacritinib would inevitably be given at the same time as a drug such as AraC and therefore it is important to establish a 'non-antagonistic' effect when the two drugs are given in combination. Initially the effect of combining pacritinib with AraC was analysed and it was shown that a synergistic effect (defined by median CI <1.0) was shown for 2 ratios of 1:50 and 1:100 with the best result for the 1:100 ratio with a median CI of 0.63 (range 0.004-1.7 n=29). In these experiments the maximum doses of each drug used were

500nM for pacritinib and 50µM for AraC, the pacritinib dose being slightly higher than that which can be achieved *in vivo* with conventional drug dosing although in reality, given that the second and third doses of 250 and 125nM respectively still give a fraction affected of approximately 70%, synergy would still be observed if the starting dose was below the EC_{50}.

In the relapsed setting however, the biology of the FLT3 ITD mutated disease is likely to alter. We know that AML is a polyclonal disease and that potentially those clones which drive relapse may have acquired 'treatment emergent' changes either in the structure of the FLT3 receptor or in signalling pathway activation which can drive disease resistance. It was shown in chapter 4 that there is up-regulation of ERK signalling in AML blasts cultured on MS-5 stroma which was not fully overcome by pacritinib treatment alone especially in the stroma adherent population, suggesting that a combination of FLT3 TKI + MEK inhibitor may be able to overcome this effect. In this case it is important to establish whether the two drugs enhance cytotoxic efficacy in the non-co-culture setting first in order to justify further exploration in, for example, a phase 1 clinical trial. The data shown in figure 6.2 demonstrates a good synergistic effect for the combination of pacritinib with PD0325901 at 2 fixed ratios with the best result for the 5:1 ratio giving a mean CI of 0.25 with a range of 0.007-1.9. It is not unexpected to see a fairly broad range of CI results within a cohort of 26 samples tested as this will inevitably contain individual patient samples which are either strongly sensitive or resistant to either drug. Some authors have shown that the effect of MEK inhibition has been shown to be predominantly that of cell cycle arrest rather than significant increase in apoptotic induction (Piloto O *et al*, 2007). There was, however, a fairly marked segregation of results showing CI <1.0 for both ratios tested which was more prominent for the 5:1 ratio. There were two samples which were obvious outliers from this cluster. The first was an ITD mutated sample which had a low EC_{50} for pacritinib (46nM) but was much more resistant to PD0325901.

Again, the drug doses required for a synergistic effect were clinically acceptable, pacritinib as described above and for PD0325901 the maximum dose used for the 5:1 ratio was 400nM. A phase 1

study has shown that this level of PD0325901 is easily achievable at oral dosing above 2mg BD which was also well tolerated in patients with solid tumours including advanced breast cancer, colorectal cancer, non-small cell lung cancer or non-ocular melanoma refractory to standard therapy ((Yang X *et al*, 2014;LoRusso P *et al*, 2010).

In addition there is some rationale to support the hypothesis that combined, small molecule inhibitor therapy (such as FLT3 TKI + MEK inhibitor) if given at induction may be able to prevent the emergence of so called 'environment mediated' or 'treatment emergent' resistance mutations. Again it is necessary to fully establish the *in vitro* efficacy of such inhibitors used as a combination before this rationale can be tested in the clinical setting. It may ultimately also be necessary to carry out work looking at the triple combination of FLT3 TKI + MEK inhibitor + conventional chemotherapy (AraC) to first prove a non-antagonistic effect and then explore optimal synergistic ratios.

A previous study by Novotny-Diermayr et al (2012) looked at the combination of pacritinib with pracinostat (a pan-Histone-Deacetylase Inhibitor) in a variety of *in vitro* (cell line) and *in vivo* (mouse) models. HDACis alter the acetylation status of both histone and non-histone proteins, impacting on cellular processes such as activation of transcription, cell proliferation, survival and angiogenesis mechanisms. AML cells are among the most sensitive cancer cells to HDAC inhibition (Novotny-Diermayr V *et al*, 2012). Although the pathways involved are multiple, one common link between JAK2 signalling and FLT3 signalling is the heat shock protein 90 molecular chaperone protein. HDACis such as pracinostat in combination with JAK2 inhibitors have previously shown synergistic effects which were explained by a reduction in the chaperone function of HSP-90, leading to increased proteosomal degradation on the JAK2 protein (Wang Y *et al*, 2009). Also, in relation to FLT3 signalling the ITD mutated form of the receptor is known to be overly reliant on HSP-90 support compared to the WT counterpart (Oshikawa G *et al*, 2011) and it was therefore fitting that a reduction in FLT3/STAT5 protein levels was seen in the study by Novotny-Diermayr et all following

treatment with the combination of pacritinib and pracinostat in the FLT3 ITD mutated AML cells to a much greater extent than in WT samples.

One recurrent observation from clinical trials using FLT3 TKIs has been the achievement of relatively transient clinical responses. The inability of these drugs to sustain a cytotoxic effect on leukaemic blasts may be explained by the emergence of resistant cell populations due to mutations in the structure of the FLT3 receptor following prolonged exposure to FLT3 TKIs (Moore AS *et al*, 2012). An alternative explanation however, is that prolonged treatment leading to adequate suppression of FLT3 phosphorylation may lead to activation of alternative pathways related to, but not dependent on, FLT3 activation for their activity. Piloto et al tested this hypothesis by treating leukaemic cell lines known to be sensitive to FLT3 TKIs with increasing concentrations of CEP-5214 or CEP-701 for 2-4 months after which point they exhibited cross resistance. Western blot analysis showed that TKI treatment of these cells still resulted in adequate suppression of FLT3 phosphorylation suggesting that drug binding or uptake of drug across the plasma membrane was not the mechanism of resistance (Piloto O *et al*, 2007). Further western blot analysis of FLT3 TKI resistant cell lines and primary samples demonstrated that pathways such as PI3k/Akt and/or MEK/MAPK remained activated in this setting and that, if these pathways were inhibited, drug sensitivity was restored in many cases. The lack of a universal beneficial effect of combination treatment was explained by the occurrence of acquired N-Ras mutations in FLT3 TKI resistant cell lines that were not seen in the sensitive parent cells and N-Ras was the only mutant kinase or phosphatase found on screening cell lines and primary samples (Piloto O *et al*, 2007). Although N-Ras mutations are rare at diagnosis in AML, Ras activation is very common in all cancers and, given its upstream location, targeting Ras remains an attractive theroretical, if clinically difficult, therapeutic goal.

Finally it should be borne in mind that combination therapy may be more likely to be cytotoxic to normal cells that use these pathways for growth and survival (Piloto O *et al*, 2007) including bone marrow stroma cells. This becomes relevant when considering the degree to which normal or

malignant stroma function impacts on disease survival and recovery of normal haematopoeisis following treatment.

Chapter 7

Final Discussion

Chapter 7: General Discussion

Acute Myeloid Leukaemia remains a malignancy that is very difficult to treat effectively due largely to the heterogeneous nature of the disease and the lack of effective, targeted therapies. The finding that around 1/3 of patients harbour a mutation in the FLT3 receptor, most commonly of ITD subtype has fuelled the drive for over 10 years for the development of FLT3 tyrosine kinase inhibitors which has yielded sufficient data to justify the transition from bench to bedside. A variety of first and second generation drugs have been tested in vitro and also progressed as far as major international phase III clinical trials (Knapper S, 2011).

Although there has been considerable progress, this promise has yet to be delivered in terms of a recognised standard of care. No single agent has been licensed for this indication and emerging therapies seem to become rapidly dogged by treatment acquired resistance mutations, as has been the case in tyrosine kinase inhibitor development in other malignancies such as chronic myeloid leukaemia. One perpetual feature of early clinical trials of FLT3 inhibitors was the failure to achieve sustained remission at a bone marrow level, and following the observation that non-cycling CD34+ AML cells retain FLT3 mutations, occupy the bone marrow niche and show relative insensitivity to tyrosine kinase inhibitors, (Alvares C *et al*, 201;Parmar A *et al*, 2011) the search for agents which can target the stroma-adherent population has begun in earnest.

Pacritinib is a dual targeted TKI with equipotent inhibitory activity for FLT3 and JAK2 proteins. This makes it an attractive potential therapy for this indication given that constitutive activation of the FLT3 receptor (via an ITD mutation) generates cross talk between numerous protein kinases and suggests that a dual inhibitory agent will have a more potent knockdown effect on downstream targets. The ultimate aim of this is to overcome the proliferative advantage of the leukaemic clone carrying the ITD mutation, and drive the cell towards apoptosis. This advantage may be part of the explanation for why the stroma niche population has been shown to be less sensitive to

conventional or novel therapy to date, which has been described as 'environment mediated resistance'.

'Environment mediated resistance' refers to a form of *de novo* drug resistance which protects leukaemic cells from targeted therapy. Rather than being the result of inherent properties of the malignant clone, this is the result of features of the microenvironment which are either soluble (cytokines, chemokines and growth factors secreted by fibroblast-like tumour stroma cells) or mediated by cell adhesion mechanisms which govern the physical interaction between the tumour cell and extracellular matrix components (Meads MB *et al*, 2009). Previous authors have suggested that, in blasts on stroma, inhibition of FLT3 leads to cell cycle arrest rather than induction of apoptosis and that this effect is mediated through a combination of soluble factors and direct contact with the stroma niche cells (Yang X *et al*, 2014;Parmar A *et al*, 2011). Opinions are divided as to which pathway within the signalling cascade downstream of FLT3 is most responsible for this phenomenon and certainly there is a large degree of cross-talk between different links in the chain. It is known that many of the pathways downstream of FLT3 are up-regulated when myeloid blasts are in contact with stroma and this increased signal may form part of the mechanism of resistance seen in the stroma-adherent population which is not seen to the same extent amongst cells in the peripheral circulation (or, in *in-vitro* terms, in suspension). One of these key pathways is MAPK/ERK which is known to induce apoptotic resistance which can only be overcome with very high drug doses.

With this in mind, the initial aim of this study was to analyse whether pacritinib could effectively target FLT3 ITD mutated cells over WT samples in both cell lines and primary patient samples. This was clearly demonstrated as shown in chapter 3, with the observation of significant cytotoxic effect in MV4-11 cell lines in contrast to NB4 or HL-60 (which carry WT FLT3). Next, cytotoxic response to pacritinib was assessed in 63 primary samples and a clear differentiation in terms of increased sensitivity in ITD mutated samples vs WT was shown (mean EC_{50} 92.3 nM vs 292.1 nM p=0.01). This

confirms that pacritinib is a drug that can effectively target FLT3 mutated disease, and also that there is no real role for treatment in those who express WT FLT3.

The effect of FLT3 ITD to wild type allelic ratio on pacritinib sensitivity was also assessed, suggesting that the higher the mutated FLT3 alleleic ratio, the more effective the drug. This is in contrast to what has been shown with other FLT3 inhibitors e.g. AC220 (Zarrinkar P *et al*, 2009), although this may not be a fair comparison given that samples used in this in vitro diagnostic work were taken at diagnosis when dependence on FLT3 signalling may be lower than clinical studies in relapsed patients. If higher level mutant samples are more sensitive however, this is encouraging given that there is data to suggest that a higher allelic ratio is associated with a poorer clinical outcome due to an increased risk of relapse (Gale R *et al*, 2008).

In terms of clinical correlation, as well as presence of an ITD mutation, higher presenting WBC was associated with increased pacritinib sensitivity (median EC_{50} WBC >100 x 10^9/l = 56nM vs WBC<50x10^9/l = 121nM p=0.005). This is to be expected given that FLT3 ITD mutated disease is generally deemed to be more proliferative and often presents with a high white cell count compared to wild type patients. Interestingly, the majority of patients in this cohort had standard risk cytogenetics (n=43 median EC_{50}=111nM) which is a well known feature of FLT3 ITD mutated disease. Although the 3 cases where high risk features were present did show reduced pacritinib sensitivity (median EC_{50} 732nM high risk n=3 vs 133nM good risk n=9 p value not significant as numbers small).

Mechanism of cell death was assessed by means of the annexin V binding assay which suggested that in MV4-11 cell lines pacritinib exerts a cytostatic effect at 48 hours of treatment compared to a cytotoxic effect with more than 50% induction of apoptosis in FLT3 ITD mutated primary samples. Given that MV4-11 cell lines harbour a p53 mutation, apoptotic induction is delayed and not seen until 72 hours. This was not observed in primaries, which are heterogeneous and may retain p53 activity, and thus apoptotic induction was seen at an earlier time point. These data were supported

by the cleaved caspase 3 western blot experiment where induction of CC3 was seen at 24 hours in a primary sample and, to a much lesser extent, at the same time period in MV4-11 cell lines.

The results of this initial work support a link between FLT3 ITD mutation and increased pacritinib sensitivity. Nevertheless, the range of EC_{50} results was broad within both ITD and WT cohorts which may be explained by off target effects of the drug or up-regulation of signalling through a structurally normal receptor in wild type samples which leads to 'false sensitivity'. Also, given that FLT3 has a crucial role in many regulatory and survival pathways of haemopoeitic cells (Stirewalt DL & Radich JP, 2003) it is essential to assess the impact of altered signalling both in terms of inhibition of the receptor and impact on downstream pathways. The latter factor may vary between patients carrying ITD mutations due to cross talk between other pathways which again may vary at the individual patient level.

Two different techniques were used to directly analyse the relationship between pacritinib treatment and the FLT3 protein. Firstly flow cytometry was used to quantify the level of surface expression of the receptor, using CD135 expression. Secondly the western blotting technique was used to assess inhibition of phosphorylated (activated) FLT3 protein over time in response to different doses of pacritinib in MV4-11 cell lines which carry an ITD mutation. The flow cytometry data showed no difference between CD135 expression in ITD mutated or WT samples and no correlation between increased CD135 expression and pacritinib sensitivity (as measured by the EC_{50}). This has been reported elsewhere for other FLT3 inhibitors (Knapper S, 2004) and suggests that binding affinity of the drug is not directly related to the FLT3 receptor frequency or density.

The western blotting experiment using MV4-11 cell lines showed dose dependent inhibition of pFLT3 signalling following treatment with pacritinib, initially after 1 hour and increasing by 24 hours. There was no effect on total FLT3 protein level which suggests that the drug blocks or downregulates activity of the receptor but does not lead to its destruction in a short term model.

Two of the key signalling pathways downstream of FLT3 are the Raf/MEK/ERK and STAT5 pathways and therefore their activity was looked at in the development of this work. In MV4-11 cell lines and primary AML mononuclear cells there was inhibition of pSTAT5 signal in a dose dependent manner from 1 hour of treatment. The effect in primary cells was similar across the 4 samples tested which included FLT3 ITD mutated cases and a FLT3 WT patient. The pERK pathway was also analysed in the MV4-11 cell lines where inhibition was seen for higher doses of pacritinib.

This preliminary work established that pacritinib is a drug which can target FLT3 ITD mutated cells and that, in common with previously developed FLT3 TKIs, it exerts activity via inhibition of the major signalling pathways downstream of FLT3 such as STAT5. The next most important question which will likely define the future clinical effectiveness of FLT3 TKIs is whether these agents are able to target residual disease within the bone marrow microenvironment since it is known that the FLT3 ITD mutation is seen within the CD34+/38- primitive stroma-adherent population (Alvares C *et al*, 201) which is therefore a reservoir of resistant disease and may well be responsible for the high incidence of relapse. It is thought that signals derived from the stroma may block cell division (Parmar A *et al*, 2011) leading to their relative quiescence and this may underlie the relatively poor performance of drugs acting on the cell cycle (such as cytarabine) in targeting this population. In addition, although these cells may carry the FLT3 ITD mutation, they may be less dependent on FLT3 signalling for survival compared to those at other stages of the disease which may explain the poor efficacy of other FLT3 TKIs such as sorafenib and AC220 in this context (Yang X *et al*, 2014). Pacritinib is, however, a multi-targeted agent that can potentially overcome the protective effect of cross-talk between several different survival pathways (Weisberg E *et al*, 2012). Pacritinib can target STAT5 via both FLT3 and JAK inhibition and also via up-regulation of IL-6 on stroma which is known to be activated via JAK (Novotny-Diermayr V *et al*, 2012).

Initial results of short term cytotoxicity experiments using the Cell Glo technique and a mouse stroma layer (MS-5) showed that at 48 hours pacritinib could not overcome the protective effect of

the stroma. This was most marked for doses below 95nM where there was a greater cytotoxic response in the IMDM only culture setting compared to co-culture of AML with MS-5 cells; at higher doses this effect was not significant. For all samples tested the EC_{50} on stroma was higher than that seen when the cells were cultured in IMDM alone. Data also showed that pacritinib is not toxic to the stroma layer at doses tested which suggests that the MS-5 cells are not reliant on FLT3 signalling for survival at AML toxic doses. One major beneficial factor relevant to the co-culture setting which may significantly impact on improved survival of AML cells was that the LTC medium is enriched with cytokines. This medium appeared to give a survival advantage compared to IMDM, most marked in the middle of the dose range around the EC_{50}.

A plausible explanation for the lack of a comparable cytotoxic effect in the co-culture setting compared to IMDM comes from the annexin V binding experiment carried out on stroma. Here marked apoptotic block was seen in the MS-5 co-culture setting at 48 hours and also after 7 days of culture where >50% annexinV/PI positivity was only seen for the 1000nM dose.

Western blotting experiments were also carried out with the co-culture model using MS-5 stroma where, firstly, a basal increase in pSTAT5 signalling was seen, particularly amongst the stroma-adherent population. Secondly, there was relative attenuation of pSTAT5 inhibition on stroma, most marked in the adherent population compared to the suspension cells. STAT5 is a transcription factor known to be activated by FLT3 ITD mutations (Spiekermann K *et al*, 2003) and data here suggest this may be partly related to a specific signal enhanced by contact with stroma. An additional effect of STAT5 activation via FLT3 relates to the control of apoptosis as it prevents up-regulation and expression of genes that can induce apoptosis such as MCL1, cyclin D1, c-myc and p21 (Kindler T *et al*, 2010) which may again contribute to relative FLT3 TKI insensitivity amongst the stroma adherent population.

Similarly, the MEK/MAPK pathway plays a crucial role downstream of FLT3 in regulation of cellular proliferation and survival (Swords R *et al*, 2012) and there are data to show that inhibition of this

pathway leads to suppression of growth in MV4-11 cell lines and may promote apoptosis in primary cells. Western blotting experiments described in chapter 5 showed relative preservation of pERK signalling on stroma compared to culture of primary cells in IMDM only and also basal up-regulation of pERK signal, again more marked amongst the adherent population. Pacritinib was unable to inhibit pERK signalling in the stroma adherent population, even at the 1000nM dose and this has been reported elsewhere for other FLT3 TKIs such as quizartinib and sorafenib. Other authors suggest this may be due to protective signals emanating from the stroma by means of cytokines or increased FLT3 ligand levels (Yang X *et al*, 2014).

This observation lead to the rationale to combine pacritinib with a MEK inhibitor PD0325901 as described in chapter 6. A common observation from the study of many cancers is that blockade of a central receptor tyrosine kinase may lead to engagement of downstream pathways, previously redundant in the context of cell survival (Wilson TR *et al*, 2012). It has been observed that ERK signalling is not essential to enhancement of proliferation signals when AML cells are cultured off stroma. If FLT3 is blocked or down regulated on stroma, however, ERK signalling may become more critical to survival and may also induce more aggressive disease behaviour. PD0325901 is a small molecule MEK inhibitor which has been previously studied in murine and human AML (Riccardi MR, 2014;Burgess MR *et al*, 2014) Pacritinib was therefore combined with PD0325901 and a good synergistic effect was observed for two ratios of the drugs, the best result being median CI 0.25 for a 5:1 ratio. This synergistic effect was observed across the dose range which is important for therapeutic scheduling.

There are several experiments which would have been carried out as part of this project had further time been available and which would also form part of any future developmental work involving pacritinib in the laboratory. Firstly, in relation to the cytotoxicity experiments following the observation of a good cytotoxic response to pacritinib in MV4-11 cell lines at 48 hours with a lack of significant apoptotic induction as measured by AVB assay until 72 hours, we would carry out cell

cycle analysis experiments using MV4-11s and primary AML samples to look for any evidence of cell cycle arrest. There may also be some benefit in repeating the Cell Glo experiments in primary cells over a 72 hour culture period to see if the cytotoxic response is improved, however primary AML cells are difficult to support beyond 48 hours of culture. In regards to the cohort of 63 primary samples analysed, only 7 samples carried FLT3 TKD point mutations and 3 were ITD+TKD 'double mutants' so it would be prudent to increase the numbers in both these cohorts in order to strengthen the weight of any conclusions drawn over the efficacy of pacritinib in those subtypes of AML. Also, finally within the cytotoxicity chapter the number of primary AML samples for the cleaved caspase 3 western blotting experiment should be increased to at least 3 (from 1 at present).

The work in this project analysing the effect of pacritinib on signalling pathways downstream of FLT3 would be enhanced by firstly including more primary AML samples in the CD135 flow analysis; currently data from 8 samples is shown which would ideally be increased to 20-25 to make the conclusions more reliable. This is unlikely to show a different result to that which has been shown by this small sample cohort, that there is no clear association between FLT3 receptor expression at the cell surface and drug sensitivity but with such small numbers conclusions hold less statistical power. In addition, the western blotting gels looking at knock down of phosphorylated and total FLT3 protein were only run using MV4-11 cells and should be repeated using a cohort of 3-5 primary samples. The reason this work was not carried out was that initially this work was run using the immunoprecipitation technique and although these experiments were completed with further primary samples and showed similar results, the loading was poor and the results were not deemed to be reliable enough to present here. This unfortunately led to a lack of time to carry out primary experiments once a decision had been made to use the conventional western blotting technique. Further primary samples would also be run to analyse the effect of pacritinib on ERK signalling off stroma and in the co-culture western blotting experiment using MS-5 stroma to increase the number in this cohort from 1 at present to 5.

With regard to the efficacy of pacritinib in the stroma co-culture setting (as described in chapter 5), the number of samples analysed in the basic Cell Glo cohort would be increased from 7 to a minimum of 10 and it would also be helpful to analyse the effect of LTC medium in isolation further both by Cell Glo and western blotting. Finally, regarding the combination of pacritinib with the MEK inhibitor PD0325901 it would be logical to carry out experiments in a stroma co-culture setting to see if the combination of the two drugs can overcome the short term protection seen in the 48 hour Cell Glo experiments. Confirmation of any downstream effects by western blot would help to ascertain whether the cytotoxic effect seen if stroma and potentially on stroma is associated with knock down of presumed targets such as ERK, STAT5 and ideally JAK or more peripheral effectors such as AKt. As mentioned previously, the risk of combination therapy is that inhibition of multiple targets simply leads to up-regulation of additional signalling targets (such as PI3k or AKt in the case of pacritinib combined with a FLT3 inhibitor) which could therefore lead to a situation of diminishing returns.

In summary, AML is a heterogeneous, polyclonal disease which is still fatal in the majority of patients, outside of certain 'good risk' groups. The main thrust of the progress that has been made in treatment and survival of patients with AML over the last 10-15 years has been due to better supportive care and there remains a lack of effective targeted therapies to treat this disease. FLT3 remains an attractive therapeutic target given its place at the top of the signalling hierarchy from cell surface to the nucleus and the fact that it is commonly mutated and these mutations can be shown to persist from diagnosis to relapse. Although many of the early FLT3 TKIs have performed rather poorly in the clinic due to a lack of sustained remissions or impact at a bone marrow level, there remains scope for the development of drugs in this class which can potentially target the minimal residual disease population within the bone marrow microenvironment and therefore reduce the risk of relapse. This work has comprehensively analysed the in vitro cytotoxic effects of pacritinib and has shown that pacritinib actively targets FLT3 ITD mutated AML and exerts its effect by induction of apoptosis. Over a short term culture technique it was not able to overcome the

protective effect of a stroma model based on MS-5 fibroblasts and LTC medium. This was explained by enhancement of STAT5 and ERK signalling on stroma. Given that the drug combines effectively with a MEK inhibitor PD0325901 to give a good synergistic effect, it warrants further exploration with the aim of overcoming environment mediated resistance in FLT3 ITD mutated acute myeloid leukaemia and this justifies the development of such combinations in the clinical setting.

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Publications/Presentations as a result of this work:

- *Pacritinib suppresses Leukemic Outgrowth from FLT3-ITD Positive Stroma-Adherent Primary AML Cells*, Marrin CA, Edwards GOE, Knapper S, Burnett AK, Zabzkiewicz J, Alvares C, Oral Presentation #270, American Society of Hematology Annual Scientific meeting, San Francisco, December 2014

- *Pacritinib suppresses Leukemic Outgrowth from FLT3-ITD Positive Stroma-Adherent Primary AML Cells harbouring FLT3-ITD*, Edwards GOE, Marrin CA, Knapper S, Burnett AK, Zabkiewicz J, Alvares C, Oral Presentation #10, British Society for Haematology Annual Scientific Meeting, Edinburgh. April 2015

- Manuscript in preparation relating to the above, planned for submission to *Leukemia*