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FLT3 INHIBITION IN ACUTE MYELOID LEUKAEMIA

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SUMMARY

FLT3 is a receptor tyrosine kinase that appears to play a significant role in leukaemogenesis. Activating mutations of FLT3 are present in approximately one-third of AML patients and are associated with adverse clinical outcome, while many non-mutated cases also show evidence of FLT3 activation. FLT3 thus represents a potentially-exciting molecular therapeutic target. A number of small-molecule tyrosine kinase inhibitors with anti-FLT3 activity have been developed and several of these compounds have entered early phase clinical trials where clinical anti-leukaemic activity has been demonstrated. The depth and duration of clinical responses to FLT3 inhibitor monotherapy have been modest, however, and a number of mechanisms by which blasts may acquire resistance have been proposed. Based on pre-clinical evidence of synergy with conventional chemotherapy, several combination trials are now underway. FLT3 inhibition may also be effectively used in combination with other molecularly-targeted agents, in post-chemotherapy stem-cell-directed maintenance therapy and in MLL-rearranged infant ALL.

Keywords: FLT3, AML, acute myeloid leukaemia, inhibitors, tyrosine kinase

INTRODUCTION

Although gradual improvements in combination chemotherapy regimens and supportive care strategies have achieved a significant rise in cure rates in acute myeloid leukaemia (AML) over the last thirty years, the majority of patients continue to die from their disease. Only 35-45% of patients below the age of 60 years will be long-term survivors, and this figure falls to less than 15% in older patients (Lowenberg *et al*, 1999). Treatment with conventional cytotoxic agents may have reached its limits. Advances in the understanding of the heterogeneous molecular pathogenetic mechanisms underlying AML suggest that novel agents targeting individual molecular lesions, used either alone, in combination, or as an adjunct to conventional chemotherapy, hold much promise for further clinical improvement, with the drive toward such a targeted therapeutic approach being fuelled by the overwhelming success of the tyrosine kinase inhibitor imatinib mesylate in chronic myeloid leukaemia (CML)(Sawyers, 2002). The important role played by FMS-like tyrosine kinase 3 (FLT3) in the survival and proliferation of AML blasts, and its mutation and overexpression in large numbers of AML patients, make FLT3 a particularly attractive target. This review discusses the pre-clinical and early clinical development of small molecule inhibitors of FLT3 along with the ongoing translational research that will help to define their future clinical roles. Although some discussion of the biology of the FLT3 receptor itself is necessary, an extensive review of the structure, molecular function and signalling of FLT3 is beyond the scope of this article and the reader is referred to previous excellent reviews in this and other journals (Kottaridis *et al*, 2003;Levis & Small, 2003a;Stirewalt & Radich, 2003;Gilliland & Griffin, 2002).

THE FLT3 RECEPTOR – NORMAL BIOLOGY AND FUNCTION

FLT3, also known as fetal liver kinase-2 (FLK-2) or stem cell kinase 1 (STK-1), is encoded by a 24-exon gene located on chromosome 13q12 (**Rosnet et al, 1993;Small et al, 1994**). It shares the same structural features as other members of the type III receptor tyrosine kinase family such as FMS, KIT and PDGFR α and β , namely an

extracellular ligand-binding region with 5 immunoglobulin-like domains, a cell membrane-spanning domain, and a cytoplasmic portion comprising a juxtamembrane region and split tyrosine kinase motifs. FLT3 is expressed on early haematopoietic progenitor cells with expression normally being lost as differentiation occurs (**Gotze et al, 1998**). The ligand for FLT3 (FLT3 ligand or FL) is expressed by virtually all cell types and appears to act in synergy with other cytokines in promoting haematopoietic expansion (**Rusten et al, 1996**). Upon stimulation with FL, FLT3 dimerises and undergoes autophosphorylation, upregulating its tyrosine kinase activity and triggering signalling through an array of downstream pathways that promote cell **proliferation** and inhibit apoptosis including PI-3 kinase/AKT **and** RAS/MAPK **and** ~~STAT5~~ (**Lavagna-Sevenier et al, 1998; Marchetto et al, 1999**). Targeted disruption of FLT3 or FL in murine gene knockout models results in viable mice with subtly reduced numbers of haematopoietic progenitors, particularly of B-lymphoid lineage (McKenna et al, 2000).

FLT3 IN ACUTE LEUKAEMIA

The FLT3 receptor is expressed at high levels in 70-100% of cases of AML and virtually all cases of B-lineage acute lymphoblastic leukaemia (ALL) (Rosnet et al, 1996; Birg et al, 1992; Carow et al, 1996). FLT3 emerged as a potential therapeutic target largely following the discovery of activating mutations of FLT3 that occur in approximately one-third of AML patients and fall into two main classes. Internal tandem duplications (FLT3-ITDs), first described by Nakao and colleagues in 1996, consist of in-frame duplications of variable lengths of the juxtamembrane domain-encoding sequence of exons 14 and 15 (Nakao et al, 1996). Pooled data from over 5,000 AML patients show an overall FLT3-ITD incidence of 23% in newly diagnosed patients, with a lower incidence in children (Levis & Small, 2003a). ITDs are associated with leucocytosis at presentation and, with the exception of acute promyelocytic leukaemia (APL) where they occur commonly, are seen predominantly in normal karyotype AML. It is now well documented that FLT3-ITD mutations are associated with poor prognosis, in terms of increased relapse rate and reduced overall survival (Kottaridis et al, 2001; Meshinchi et al, 2001). Patients with a high mutant-to-

wild type FLT3 allelic ratio have a particularly dismal outlook (Whitman *et al*, 2001;Thiede *et al*, 2002) and ITD size may also have prognostic significance (Stirewalt *et al*, 2006). A further 8-12% of AML patients have single base substitutions, small deletions or insertions in the activation loop of the tyrosine kinase domain (FLT3-TKDs), most frequently involving substitution of aspartic acid 835 with tyrosine (D835Y) (Yamamoto *et al*, 2001). FLT3-TKD mutations do not appear to have the same adverse prognostic implications as FLT3-ITDs although complete consensus has not yet been reached on this issue and a favourable prognostic association has even been proposed (Mead *et al*, 2005).

Both types of FLT3 mutation result in constitutive activation of the receptor in the absence of ligand, FLT3-ITDs through interference with the negative regulatory function of the juxtamembrane domain (Griffith *et al*, 2004), and FLT3-TKDs probably through stabilisation of the activation loop in the open ATP-binding configuration. Mutations of FLT3 are examples of so-called ‘class II’ leukaemic mutations, increasing cell proliferation and survival but, unlike class I mutations, not effecting haematopoietic differentiation (Gilliland & Griffin, 2002): FLT3-ITDs induce a fatal myeloproliferative disease-like phenotype in mouse bone marrow transplant models, but are not sufficient to cause overt AML (Kelly *et al*, 2002b). When **FLT3-ITD** mutations are combined with other genetic alterations, however, transformation to leukaemia does occur: in experiments with promyelocytic leukaemia-retinoic acid receptor α (PML-RAR α) transgenic bone marrow, FLT3-ITD strongly increases the penetrance and decreases the latency period for the development of APL (Kelly *et al*, 2002a). **FLT3-TKD mutations, although also associated with activation of the FLT3 receptor appear to have subtly different biological consequences to ITDs, causing less activation of signal transducer and activation of transcription (STAT) proteins (Choudhary *et al*, 2005) and inducing an oligoclonal lymphoproliferative rather than myeloproliferative phenotype in a murine bone marrow transplant model (Grundler *et al*, 2005).**

The large body of data that has now been assembled regarding the incidence and prognostic impact of FLT3 mutations, has established the **ITD-mutated** FLT3 receptor as a worthwhile potential therapeutic target, **although the rationale for targeting TKD-mutated FLT3 remains less clear**. Whether overexpressed non-

mutated wild-type (WT) FLT3 plays a significant role in the pathogenesis of AML and should also be targeted remains a matter of debate. Leukaemic blasts frequently co-express FLT3 and FL, creating autocrine and paracrine signalling loops that cause constitutive activation of WT FLT3 (Zheng *et al*, 2004;Drexler, 1996) and there is evidence from murine models that constitutively activated WT FLT3 can contribute to leukaemogenesis: lethally irradiated mice transplanted with WT FLT3-expressing stem cells retrovirally transduced to coexpress FL develop either myeloid or lymphoid leukaemia after a long lag period (Hawley *et al*, 1998). While the question of the role of WT FLT3 remains open, there remains scientific rationale for the inclusion of both mutated and WT FLT3 in the laboratory assessment and early clinical study of FLT3-selective tyrosine kinase inhibitors.

FLT3 INHIBITORS IN AML

The successes of imatinib mesylate (Glivec) in CML and trastuzimab (Herceptin) in breast cancer have provided a tangible glimpse of the clinical gains that may be achievable through targeting dysregulated tyrosine kinase activity in cancer. Imatinib mesylate monotherapy induces haematological and cytogenetic remissions in the majority of patients with chronic phase CML (Kantarjian *et al*, 2002). In AML, however, FLT3 is only one among several genetic ‘hits’ that combine to cause the disease; more analogous to BCR/ABL in CML blast crisis / Philadelphia positive ALL, or HER2, a member of the ErbB receptor tyrosine kinase family that is overexpressed in 20-30% of breast cancer patients. In these situations imatinib and trastuzimab, a monoclonal antibody directed against HER2, produce favourable clinical responses, but not outright cures (Druker *et al*, 2001;Hortobagyi, 2001).

Large drug discovery programmes in the pharmaceutical industry have identified a range of potential small molecule tyrosine kinase inhibitors with varying degrees of specificity towards FLT3. Candidate compounds tend to initially move through a well-established series of *in vitro* laboratory investigations (Levis & Small, 2003b). FLT3-inhibitory activity is determined either through *in vitro* kinase assays which involve purifying FLT3 and then testing the ability of the inhibitor to prevent FLT3-mediated phosphorylation of a defined substrate such as a peptide-coated ELISA well,

or, perhaps more meaningfully, by cell-based phosphorylation assays in which intact FLT3-expressing cells are exposed to the inhibitor, followed by lysis, immunoprecipitation and immunoblotting with anti-phosphotyrosine antibodies. The relationship between FLT3-inhibition and the ability of an agent to induce cytotoxicity is then assessed by incubating FLT3-expressing cell lines or primary patient samples with a range of concentrations of the compound before measuring cell viability using techniques such as MTS (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulphophenyl]-2H-tetrazolium) assay or assessment of annexin V binding. Murine models of FLT3-dependent leukaemia may be used to demonstrate prolongation of survival in animals treated with the inhibitor, with successful compounds moving forward to animal toxicity studies and early phase human trials. Most candidate FLT3-inhibitory molecules are hydrophobic and highly protein-bound: only the minority of drug that remains free in the plasma is expected to show clinical activity and this limits direct extrapolation of results obtained during *in vitro* studies performed in aqueous culture medium into the clinical setting. Once a promising agent has entered the clinic, adjunctive laboratory studies are essential to allow correlation of patient response with the degree and duration of *in vivo* FLT3 inhibition achieved.

INDIRECT INHIBITORS OF FLT3 – HSP-90 INHIBITORS

Inhibitors of the molecular chaperone Heat shock protein 90 (Hsp-90) were the first class of compounds described to have inhibitory effects on FLT3 (**Figure 1**). Herbimycin A, a naturally occurring product of *Streptomyces sp.* was found to both inhibit the *in vitro* growth of FLT3-ITD transformed cells and reduce tumour size in a FLT3-ITD-driven mouse tumour model (Zhao *et al*, 2000). FLT3-ITD appears to require Hsp-90 for proper folding and stabilisation: the **anti-FLT3** effect of herbimycin A occurs indirectly through dissociation of the complex formed between FLT3-ITD and Hsp-90. WT FLT3 does not associate with Hsp-90 and is thus not affected by Herbimycin A (Minami *et al*, 2002). Another Hsp-90 inhibitor, the geldanamycin analogue 17-N-allylamino-17-demethoxyl geldanamycin (17-AAG) has shown promising *in vitro* activity against FLT3-ITD AML (George *et al*, 2004).

No reports regarding the clinical development of these compounds have yet been published.

DIRECT FLT3 INHIBITORS

Although the other small molecules that are currently in development as FLT3 inhibitors belong to a variety of chemical classes (Table 1) they are all heterocyclic compounds that directly inhibit FLT3 by mimicking the purine ring structure of adenosine and thus competing with ATP for binding to the ATP-binding pocket of the kinase domain of the FLT3 receptor. These agents may bind to the active, inactive or transitional state of FLT3, with their selectivity being greatly influenced by changes in the tertiary structure of the binding pocket that result from single amino acid changes such as those caused by FLT3-TKD mutations (Grundler *et al*, 2003; Clark *et al*, 2004). The pre-clinical and clinical development of each class of compound will be discussed in turn. An overview of the current published clinical trial literature is given in Table 2.

Quinoxalines

The first direct FLT3 inhibitors to be described were the bicyclic quinoxalines AG1295 and AG1296 (**Figure 2**) which were both originally described as PDGF-R β inhibitors but subsequently found to inhibit FLT3-ITD and WT FLT3 (but not FLT3-TKD) with a 50% inhibitory concentration (IC₅₀) of approximately 300nM. Due to their hydrophobicity, low bioavailability and relatively low potency these compounds are useful only as research tools but provided important validation of FLT3 as a therapeutic target. AG1295 was found to be selectively cytotoxic to primary AML blasts harbouring FLT3 mutations (Levis *et al*, 2001) while, in both Ba/F3 cells expressing FLT3-ITD and primary AML blasts with FLT3-ITD mutations, AG1296 treatment caused reduction in the phosphorylation of signalling proteins downstream of FLT3, including STAT5 and ERK (Tse *et al*, 2002). The more water-soluble tricyclic quinoxalines AGL2033 and AGL2043, similarly developed initially as PDGF-R β and KIT inhibitors also inhibit FLT3 and may be better candidates for clinical development (Gazit *et al*, 2003).

Indolinones

A number of 3-substituted indolin-2-one compounds have been studied (**Figure 3**) with two agents SU5416 (Semaxinib) and SU11248 (Sunitinib) reaching early phase clinical trials. These compounds are relatively 'broad spectrum' tyrosine kinase inhibitors, developed primarily as angiogenesis inhibitors in view of their activity against the vascular endothelial growth factor (VEGF) receptor family, but subsequently found to potently inhibit FLT3, KIT and PDGF-R β . SU5416, SU5614 and the 'second generation' indolinone compound SU11248 show selective cytotoxic effects in human leukaemic cell lines expressing activated FLT3 (Yee *et al*, 2002; O'Farrell *et al*, 2003a). SU11248 in particular causes significant *in vitro* cytotoxicity at concentrations much lower than those required for maximal FLT3 inhibition suggesting that its effects may arise from the simultaneous inhibition of several different kinases.

SU5416 is relatively hydrophobic and highly protein bound in plasma. It was the first FLT3 inhibitor to reach the clinic: two separate phase 2 studies were conducted in AML based on its anti-angiogenic potential before its activity against FLT3 had been documented. In one study, 42 elderly or refractory AML patients were treated with twice-weekly intravenous infusions of SU5416 in four-week cycles (Fiedler *et al*, 2003). One patient had a reduction in bone marrow blasts to <5% without neutrophil or platelet recovery while 7 other patients achieved a >50% reduction in bone marrow blasts, responses lasting between 1 and 5 months. Retrospective analysis showed that none of the responding patients harboured FLT3 mutations. Patients with high levels of bone marrow VEGF mRNA expression had a higher clinical response rate and reduction in marrow microvessel density than patients with low VEGF expression. In the second study, only 3 partial responses and 1 haematological improvement were documented amongst 33 AML and 22 MDS patients treated with SU5416 (Giles *et al*, 2003). Results from a subsequent pharmacokinetic and pharmacodynamic study of SU5416 suggested that, due to its short half life and high protein binding, SU5416 was likely to have inhibited FLT3 only briefly if at all in the above studies, possibly explaining the modest clinical efficacy (O'Farrell *et al*, 2004). This drug has now been withdrawn from further study.

Although also highly protein bound, SU11248 is orally-bioavailable and more hydrophilic than SU5416. In a single-dose phase 1 study, *in vivo* inhibition of FLT3 phosphorylation was assessed at different time points in 29 patients (O'Farrell *et al*, 2003b). FLT3 was inhibited in all 5 patients that harboured FLT3-activating mutations but only half of WT FLT3 cases. In a second phase 1 study, 16 patients with refractory AML were treated orally with SU11248 at a 50 or 75mg daily dose using a 4 weeks on, 1-2 weeks off schedule (Fiedler *et al*, 2005). Reductions in peripheral blood or bone marrow blasts were seen in 6 patients including all 4 with FLT3 mutations. Significant toxicity in the form of fatigue and hypertension, **possibly** due to the inhibition of multiple targets by this agent, was observed at the higher dose level. Although intermittent dosing has the potential to alleviate toxicity problems this may compromise target inhibition: in 4 cases rises in blast numbers were observed during the drug washout period. SU11248 is currently approved in the US for treatment of renal cell carcinoma and imatinib-refractory gastro-intestinal stromal tumours but, due to the pharmacokinetic difficulties in maintaining a FLT3-inhibitory concentration while avoiding toxicity, its future use in AML is uncertain.

Indolocarbazoles

Indolocarbazole FLT3 inhibitors (**Figure 4**) are alkaloid compounds that are derived from parent compounds of microbial origin. The two main agents in this class are CEP701 (Lestaurtinib) and PKC412, which are synthetically derived respectively from K-252a (the fermentation product of *Nonomurea longicatena*) and staurosporine.

CEP701 was initially identified as an inhibitor of TrkA, a member of the nerve growth factor receptor subfamily, but was subsequently found to also inhibit FLT3. CEP701 is highly selective, potently inhibiting FLT3 and TrkA with respective IC₅₀s of 3nM and 3.7nM but only inhibiting other type III RTKs such as KIT, PDGF-R β and FMS at concentrations above 500nM. Although the drug is preferentially cytotoxic to FLT3-ITD expressing cell lines and primary AML samples it also displays activity against WT FLT3 (Levis *et al*, 2002; Brown *et al*, 2004; Knapper *et al*, 2006b). CEP701 prolongs survival in a mouse model of FLT3-ITD leukaemia (Levis *et al*, 2002).

CEP701 has good oral bioavailability and was well tolerated in two early phase clinical trials, the commonest reported toxicities being mild nausea and diarrhoea. In a US phase 1/2 trial of this agent, 14 heavily pretreated AML patients with FLT3 activating mutations were treated with CEP701 60mg twice daily, with dose escalation to 80mg in 3 of the later patients after completion of 28 days of treatment (Smith *et al*, 2004). 5 patients showed a reduction in peripheral blood blasts and 1 of these patients showed a decrease in bone marrow blasts to <5%. All responses were short-lived, ranging from 2 weeks to 3 months in duration. In a second phase 2 trial of CEP701 monotherapy 29 previously-untreated elderly AML patients were treated at a twice-daily dose of 60mg for 8 weeks, with dose escalation to 80mg if well-tolerated (Knapper *et al*, 2006a). Clinical activity, manifest as transient reductions in bone marrow or peripheral blood blasts or longer periods of transfusion independence was seen in 3 of 5 evaluable patients with mutated FLT3 and 5 of 22 WT FLT3 patients. Both of these trials included detailed correlative laboratory analysis that confirmed that if a patient's blasts were inherently sensitive to the FLT3 inhibitor as determined by *in vitro* cytotoxicity assay, and that the patient maintained a plasma CEP701 level sufficient for sustained inhibition of FLT3 to less than 10-15% of baseline activity, then a clinical response would be seen.

PKC412 (N-benzoylstaurosporine) was originally developed as an inhibitor of protein kinase C, but has only relatively modest potency towards this enzyme. Like CEP701, PKC412 is a potent FLT3 inhibitor (IC₅₀ approximately 10nM), but is slightly less selective than CEP701 amongst other kinases, inhibiting PDGF-R β with an IC₅₀ of 80nM and KIT at doses above 500nM. PKC412 has been shown to inhibit FLT3 phosphorylation in mutant and WT FLT3-transfected Ba/F3 cells. It inhibits the proliferation of FLT3-ITD and FLT3-TKD-expressing cell lines by causing cell cycle arrest and inducing apoptosis (Weisberg *et al*, 2002). Orally administered PKC412 prolongs survival and prevents the development of splenomegaly in mice with lethal activated-FLT3-induced myeloproliferative syndrome (Kelly *et al*, 2002b; Weisberg *et al*, 2002).

PKC412 also displays good oral bioavailability and, in a phase 2 study, was administered at a dose of 75mg three times daily to 20 patients with mutated FLT3

and either relapsed/refractory AML or high-risk myelodysplastic syndrome (Stone *et al*, 2005b). 14 patients showed a greater than 50% reduction in peripheral blood blast count, this being accompanied by a reduction in bone marrow blasts in 6 cases, with blasts falling to less than 5% (without blood count recovery) in 2 cases. Median clinical response duration was 13 weeks. Correlative studies showed that FLT3 phosphorylation was inhibited in most responding patients. Interestingly, pharmacokinetic data from this trial showed that, following an initial peak, plasma concentrations of PKC412 fell by 70% at steady state, with levels of its active metabolite CGP52541 gradually rising and reaching a plateau after 28 days. CGP52541 has a broader spectrum of kinase inhibitory activity than PKC412 and is more cytotoxic to primary blast samples (Levis *et al*, 2006). It appears likely that much of PKC412's clinical activity is actually attributable to CGP52541.

MLN-518

The piperazinyl quinazoline compound MLN-518 (Tandutinib, formerly known as CT-53518) (**Figure 5**) is another orally-bioavailable agent that, similar to the indolinone molecules, shows a relatively broad spectrum of kinase inhibition. Studies with a FLT3-ITD-expressing cell line yielded an IC₅₀ of 30nM for FLT3 inhibition, but only 10nM for inhibition of proliferation, suggesting a possible multi-targeted effect. MLN-518 prolonged survival in a FLT3-ITD murine myeloproliferative model (Kelly *et al*, 2002c). Interestingly D835Y, the commonest FLT3-TKD mutation, confers *in vitro* resistance to this compound, which may limit its application (Clark *et al*, 2004).

In a phase 1 study, 40 patients with AML or high risk myelodysplastic syndrome were treated with escalating doses of MLN-518 (DeAngelo *et al*, 2006). Dose limiting toxicity was muscular weakness or fatigue which occurred at doses above 525mg and was attributed to off-target binding of MLN-518 to a nicotinic receptor at the neuromuscular junction. Preliminary results from a phase 2 trial of FLT3-ITD positive patients treated with a twice-daily dose of 525mg demonstrated a clinical response in 6 out of 15 evaluable patients with decreases in both peripheral blood and bone marrow blasts of 1-3 months duration (DeAngelo *et al*, 2004).

CHIR-258

The recently-reported benzimidazole-quinolinone compound CHIR-258 (**Figure 5**) is another multi-targeted inhibitor with high *in vitro* potency against FLT3, KIT, VEGF 1-3, PDGF-R β and fibroblast growth factor. It displays selective cytotoxicity in FLT3-ITD-dependent cell lines and prolongs survival in a murine FLT3-ITD model (Lopes de Menezes *et al*, 2005). Preliminary phase 1 data in 8 relapsed/refractory AML patients showed good tolerability (Morgan *et al*, 2005). The 1 patient that harboured a FLT3-ITD mutation had a near-complete clearance of blasts from both peripheral blood and bone marrow. Early phase clinical studies continue.

Other compounds

A number of other candidate molecules are at various stages of pre-clinical testing or early clinical development (**Figure 5**). Bay 43-9006 (Sorafenib), a urea derivative used in the treatment of renal cell carcinoma was initially developed to inhibit the serine threonine kinase Raf-1 but has recently been seen to inhibit class III receptor tyrosine kinases including FLT3, VEGF, KIT and PDGFR- β , and show selective cytotoxicity in FLT3-ITD expressing cell lines (Lierman *et al*, 2007). ABT-869, another urea-derivative displays multi-targeted receptor tyrosine kinase inhibition and, after evidence of *in vitro* cytotoxicity was seen in leukaemia cell lines and primary samples along with increased survival in a murine FLT3-ITD AML model, has entered phase 1 clinical development (Shankar *et al*, 2007). The *in vitro* efficacy of a third urea-derivative Ki23819 has previously been reported (Komeno *et al*, 2005) while a phase 1 trial of the compound KW-2449 is also underway.

CHALLENGES IN THE FUTURE DEVELOPMENT OF FLT3 INHIBITORS

Based on the data from early phase clinical trials it appears that inhibition of FLT3 is, in general, safe and well tolerated. The levels of clinical response seen have been broadly similar and limited to transient clearances of blasts from the peripheral blood, reductions in bone marrow blasts or periods of transfusion independence that have lasted, at best, for only a few months. Even though the heavily pre-treated or elderly

populations studied to date have a notoriously poor prognosis, the results with FLT3-inhibitor monotherapy fall some way short of those seen with imatinib in CML blast crisis. These trials and their correlative laboratory results have, however, provided good evidence linking sustained inhibition of FLT3 with clinical response while highlighting a number of issues that must be considered, both in the laboratory and in the future clinical development of these agents.

On- target versus off- target activity

As described above, FLT3-inhibitory compounds vary considerably in their potency and specificity. Although, at least initially, more is likely to be learned about the effects of FLT3 inhibition on AML pathophysiology by using ‘cleaner’ more FLT3-selective agents, AML is a complex multigenetic disease and the simultaneous inhibition of other important tyrosine kinases including those that inhibit angiogenesis or even kinases from other families may be therapeutically advantageous. Broader spectrum kinase inhibition may come at a price, however, and there is some early clinical suggestion of increased toxicity with agents such as SU11248 and MLN-518. Concerns about the potentially potent myelosuppressive effects of dual FLT3 and KIT inhibition were somewhat alleviated by *in vitro* data showing that MLN-518, which inhibits both kinases with approximately equal potency had no effects on normal haematopoiesis in mice (Griswold *et al*, 2004).

Protein binding and pharmacokinetics

Pre-clinical data suggest that a cytotoxic effect is only seen in leukaemia cells when FLT3 phosphorylation is reduced in a sustained fashion to less than 10-15% of its baseline level (Levis *et al*, 2002). In clinical studies any benefit to the patient attributed to FLT3 inhibitors must be assessed in context of the degree and duration of target inhibition achieved. Inter-patient variations in drug metabolism and protein binding mean that FLT3-inhibitory activity cannot simply be determined by the measurement of plasma drug levels which frequently do not reflect the true level of unbound, active drug present. Direct assays of FLT3 inhibition are not possible in samples from patients with low circulating blasts numbers or when blasts are cleared in response to either the FLT3 inhibitor or accompanying cytotoxic chemotherapy. To circumvent these obstacles the Baltimore group have developed a ‘surrogate’ assay in which a FLT3-ITD-transfected leukaemia cell line (TF/ITD) is incubated with plasma

taken at appropriate time-points from the treated patient (Levis *et al*, 2006). The FLT3 inhibitory activity in the patient plasma is then determined by lysis, immunoprecipitation and phospho-FLT3 immunoblotting of the treated TF/ITD cells. This ‘plasma inhibitory activity’ assay provides a means of assessing FLT3 inhibition over time which has been validated in patients receiving CEP701 and PKC412 (Smith *et al*, 2004;Levis *et al*, 2006;Knapper *et al*, 2006a). By judicious monitoring of plasma inhibitory levels in future studies it may be possible to tailor dosing in individual patients to ensure sustained FLT3-inhibitory levels in all cases.

Resistance

The clinical responses seen so far to FLT3 inhibitors have been almost exclusively short-lived, suggesting the presence of mechanisms by which leukaemic blasts may evade the effects of FLT3 inhibition. The acquisition of secondary tyrosine kinase domain point mutations that interfere with drug binding is a well-documented phenomenon in CML patients receiving therapy with imatinib (Wadleigh *et al*, 2005). Pre-clinical studies using AML cell lines have shown that small variations in the molecular structure of the FLT3 activation loop can greatly influence response to FLT3 inhibitors: cells that express different FLT3-TKD point mutations show distinctly different profiles of *in vitro* drug response (Grundler *et al*, 2003). Cools and colleagues described the results of an *in vitro* screen designed to discover mutations in the ATP-binding pocket of FLT3 that cause drug resistance: point mutations at 4 different positions were identified that conferred varying degrees of resistance to PKC412, with variable cross-reactivity being seen with other inhibitors. One of these, the G697R mutation conferred high-level resistance to PKC412, SU5614 and K-252a as well as to six experimental molecules (Cools *et al*, 2004). In clinical trials of FLT3 inhibitors to date there has been only one report of the acquisition of a secondary FLT3-TKD mutation: a patient that responded to PKC412 but became resistant to the drug after 280 days treatment was found to have developed a point mutation at one of the positions identified by Cools (N676K) that had not been present at diagnosis (Heidel *et al*, 2006).

Increases in FLT3 expression during treatment represent another potential mechanism by which the efficacy of FLT3-inhibitors may be reduced. Laboratory assays

performed alongside the UK phase 2 study of CEP701 demonstrated increases in blast surface FLT3 expression during FLT3 inhibitor treatment in 13 out of 14 patients (Knapper *et al*, 2006a).

Recent laboratory studies using primary AML blasts treated with CEP701 and PKC412 have suggested that FLT3 may not always provide the primary survival signal even in cases with high levels of activated FLT3 expression (Knapper *et al*, 2006b). Furthermore, research using FLT3 inhibitor-resistant leukaemia cell lines, generated through prolonged co-culture with FLT3 inhibitors, has revealed that leukaemia cells may become FLT3 independent by activating compensatory signalling pathways (Piloto *et al*, 2007): in resistant cells, FLT3 itself could still be inhibited but several signalling pathways normally switched off by FLT3 inhibition, including PI-3 kinase/AKT and Ras/MEK/MAPK, remained activated. Newly-acquired activating Ras mutations were found in 2 of the resistant cell lines, suggesting another means by which resistance may be acquired. This Ras-mediated resistance could be overcome by combining a FLT3 inhibitor with an inhibitor of PI3 kinase or MEK, suggesting a future potential treatment strategy.

Stem cell effects

Pertinent to the design of clinical strategies to implement inhibition of FLT3, is the time sequence in which mutations are acquired in the process of leukaemogenesis. There are now data to support that FLT3 mutations frequently occur in leukaemia stem cells, although it appears that they can also represent late events arising in subclonal blast populations. Consistent with an 'early hit' hypothesis, FLT3-ITD mutations have recently been identified in purified CD34⁺/CD38⁻ leukaemia-initiating stem cells, with these cells subsequently shown to successfully engraft FLT3-ITD AML in a NOD/SCID mouse model (Levis *et al*, 2005b). Analysis of paired patient samples has revealed that in 84% of patients with a FLT3-ITD at diagnosis, the mutation is also detectable at relapse (Shih *et al*, 2002). ITDs may sometimes, however, be acquired as late events: patients with myelodysplastic syndrome that lack a FLT3 ITD mutation at first diagnosis frequently develop one at the time of progression to AML, and AML patients who do not have FLT3 mutations at diagnosis may develop them at the time of relapse. In contrast, FLT3-TKD mutations seem to

more usually represent 'late hits', being lost at relapse in more than half of cases (Shih *et al*, 2004).

It has been hypothesised that stem-cell directed therapeutic effects could underlie delayed clinical responses reported in 2 CEP701-treated patients that peaked several weeks after the drug was withdrawn (Knapper *et al*, 2006a). A future strategy in which maintenance FLT3 inhibitor therapy is continued for a prolonged period after initial remission induction may have the potential to inhibit the regrowth of residual leukaemia stem cells. Such a strategy may be of lesser relevance in patients with FLT3-TKD mutations.

Which patients should receive FLT3 inhibitors?

A large body of pre-clinical evidence, assembled using both FLT3-expressing leukaemia cell lines and primary blast samples has shown that cells harbouring FLT3-ITD mutations display a consistently greater sensitivity to the cytotoxic effects of FLT3 inhibitors than those that express only WT FLT3 (Levis *et al*, 2001; Brown *et al*, 2004; Weisberg *et al*, 2002). Subsequent clinical studies have shown that the majority of patients with FLT3 **ITD or TKD** mutations who achieve a satisfactory plasma FLT3-inhibitory level will achieve some degree of clinical response (Smith *et al*, 2004; Stone *et al*, 2005b). **Patient numbers have so far being insufficient to observe any effect of ITD size on response rate, although there is some pre-clinical evidence that blasts from cases with high mutant-to-wild-type FLT3 ratio are more sensitive to the *in vitro* effects of FLT3 inhibition (Brown *et al*, 2004).** (~~Although sometimes seen,~~) Clinical responses to FLT3 inhibitors appear to be less frequent among FLT3 WT patients and appear to reflect the somewhat unpredictable degree of dependence that these leukaemias have on FLT3 signalling. At the time of writing, entry to the majority of ongoing FLT3 inhibitor trials is restricted to cases with mutant FLT3: other evolving molecular targets may ultimately have more relevance for the treatment of WT patients.

FLT3 INHIBITORS IN COMBINATION WITH CYTOTOXIC CHEMOTHERAPY

Clinical responses to FLT3 inhibitor monotherapy have been relatively modest and in light of the accompanying rapid emergence of resistance seen in responding cases it appears likely that these drugs will only have a relatively limited future role as sole therapeutic agents. There is now a move towards employing FLT3 inhibitors in combination with conventional cytotoxic chemotherapy, this approach being supported by evidence from pre-clinical studies. In an *in vitro* model, CEP701 demonstrated synergistic killing of FLT3-ITD-expressing leukaemia cell lines when combined in a time-sequence-specific manner with 4 different chemotherapeutic agents (Levis *et al*, 2004). Pre-treatment of cells with CEP701 before chemotherapy resulted in G₁-phase cell cycle arrest and thus led to antagonism with the S-phase-specific drug cytarabine. Synergistic cytotoxicity was seen, however, when the FLT3 inhibitor was administered simultaneously-with or following chemotherapy. The authors concluded that CEP701 should be administered only after chemotherapy in future combination studies because the simultaneous administration of daunorubicin and CEP701 led to displacement of protein-bound daunorubicin into the serum resulting in potentially toxic anthracycline levels. SU11248 also showed additive or synergistic cytotoxic effects on leukaemia cell lines or primary blast samples when combined with cytarabine or daunorubicin (Yee *et al*, 2004).

Clinical trials of both CEP701 and PKC412 used in combination with chemotherapy are now underway. In the first such study, PKC412 was combined simultaneously with standard cytarabine and daunorubicin-based induction and cytarabine-based consolidation chemotherapy in newly-diagnosed young adult AML patients irrespective of their FLT3 mutation status (Stone *et al*, 2005a). Initial 100mg twice daily dosing of PKC412 was associated with unacceptably high levels of emesis and biochemical liver derangement, perhaps consistent with the high anthracycline level-related toxicity predicted by Levis's pre-clinical CEP701 study (Levis *et al*, 2004). Better tolerability has since been seen at a modified dose of 50mg BD. Two separate phase 3 trials of CEP701 combined with chemotherapy are currently open. In a US-based study which has now expanded internationally, AML patients with FLT3-activating mutations in first relapse are randomised to receive salvage chemotherapy

alone or in combination with oral CEP701, which is introduced upon completion of each course of chemotherapy. Preliminary reports show this combination to be well-tolerated with encouraging rates of clinical response that again correlate well with laboratory assays of FLT3 inhibition (Levis *et al*, 2005a). The UK-based MRC AML15 study for newly diagnosed adult patients below the age of 60 years has recently opened a randomisation that allocates half of patients identified on diagnostic screening to harbour a FLT3 mutation to receive CEP701 following each cycle of induction and consolidation chemotherapy.

In the long term, simultaneous inhibition of multiple pathogenetic targets may be achievable by combining molecularly targeted therapeutic agents. A glimpse of the potential of such an approach was provided by a mouse model of FLT3-enhanced PML-RAR α -positive APL in which a dramatic disease response was seen in mice treated with all-*trans* retinoic acid (ATRA) used in combination with the novel indolinone FLT3 inhibitor SU11657 (Sohal *et al*, 2003).

NEW DIRECTIONS OF FLT3-DIRECTED THERAPY

FLT3 inhibitors in acute lymphoblastic leukaemia

FLT3 is expressed in nearly all cases of B-lineage ALL, with particularly high expression levels seen in infant ALL in association with rearrangements of the MLL gene at chromosome 11q23 (Armstrong *et al*, 2002). Activating mutations in the tyrosine kinase domain of FLT3 have been reported in up to 15% of these MLL-rearranged ALL cases (Armstrong *et al*, 2004). In view of the extremely poor prognosis of infant ALL with a failure of standard therapy to cure the vast majority of patients, FLT3 inhibitors represent a potentially valuable new treatment strategy in this setting. Pre-clinical studies using CEP701 demonstrated selective *in vitro* cytotoxicity in ALL cell lines and in primary paediatric ALL blasts with high levels of FLT3 expression (Brown *et al*, 2005b), with evidence of sequence-dependent synergistic cytotoxic effects when combined with standard chemotherapeutic agents (Brown *et al*, 2005a). PKC412 is also differentially cytotoxic *in vitro* to ALL cells with MLL translocations and overexpression of WT or mutant FLT3 (Armstrong *et al*, 2003; Stam *et al*, 2005). In the US, Children's Oncology Group trials of CEP701 in

combination with chemotherapy are planned in both relapsed FLT3-mutant AML and infants with MLL-rearranged ALL.

Anti-FLT3 antibody therapy

Antibodies targeted against FLT3 have recently been proposed as a potential means of avoiding some of the potential drawbacks of FLT3 inhibitors such as non-specific off-target activity and acquired resistance. IMC-EB10 and IMC-NC7 are fully human monoclonal antibodies that are capable of blocking the binding of FLT3 ligand to FLT3. An inhibitory effect has been demonstrated on both ITD mutant and WT FLT3 in AML cell lines and primary samples (Li *et al*, 2004;Piloto *et al*, 2005) while IMC-EB10 in particular was able to initiate antibody-dependent cell mediated cytotoxicity in FLT3-expressing cells and increase survival in a murine model of FLT3-ITD leukaemia (Piloto *et al*, 2005). Although less *in vitro* cytotoxicity was seen against FLT3-overexpressing ALL cell lines, IMC-EB10 also significantly prolonged survival in a murine ALL model with most of this effect being mediated through natural killer cell activity (Piloto *et al*, 2006). Clinical trials of these antibodies are now being contemplated and there may be future potential for conjugation with cytotoxic agents. It remains to be seen whether their absolute specificity for FLT3 will ultimately be to their advantage or detriment.

CONCLUSIONS

The therapeutic inhibition of FLT3 retains the potential to profoundly improve AML treatment. Although relatively modest in depth and duration, the clinical responses seen in early phase clinical trials of small molecule FLT3 inhibitors, when considered alongside the results of correlative laboratory studies, confirm the clinical anti-leukaemic efficacy of a strategy of sustained FLT3 inhibition in FLT3-dependent AML. There is now a strong body of pre-clinical evidence to support a synergistic relationship between FLT3 inhibitors and conventional cytotoxic agents if administered in the correct time sequence. Several trials of FLT3 inhibitors in combination with chemotherapy are now well underway, while trial protocols to assess the value of FLT3 inhibitors in leukaemia stem cell-directed post-chemotherapy maintenance treatment are also anticipated. Ultimately, it is hoped that,

with increased knowledge of the complex molecular pathogenetic mechanisms underlying AML and improved diagnostic screening techniques, FLT3 inhibitors will be successfully utilised in combination with a range of other molecularly targeted agents tailored to effectively target the signalling pathways underlying the disease in individual patients while avoiding some of the toxicities associated with traditional chemotherapy.

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Table 1: Direct inhibitors of FLT3

Compound	Chemical class	FLT3 IC₅₀	Other targets	Clinical stage
AG1295	Quinoxaline	1000nM	PDGFR, KIT	Lab only
AG1296	Quinoxaline	1000nM	PDGFR, KIT	Lab only
AGL2033	Quinoxaline	700nM	PDGFR, KIT	Lab only
SU5416 (Sexamanib)	3-substituted indolinone	100nM	KIT, VEGFR	Phase 2
SU5614	3-substituted indolinone	10nM	KIT, FMS	Lab only
SU11248 (Sunitinib)	3-substituted indolinone	50nM	KIT, PDGFR, VEGFR	Phase 1
CEP701 (Lestaurtinib)	Indolocarbazole	3nM	TRKA	Phase 3
PKC412	Indolocarbazole	10nM	KIT	Phase 3
MLN-518 (Tandutinib)	Piperazinyl quinazoline	30nM	KIT, PDGFR	Phase 2
CHIR-258	Benzimidazole quinoline	1nM	KIT, FMS, VEGFR, FGFR	Phase 1
BAY 43-9006 (Sorafenib)	Bi-aryl urea	<50nM	B-RAF, PDGFR, VEGFR	Lab only
ABT-869	Urea derivative	4nM	KIT, KDR, PDGFR	Phase 1
Ki23819	Quinoline-urea	10nM	n/a	Lab only
KW-2449	n/a	6nM	KIT, Aurora kinase	Phase 1

FLT3 IC₅₀ refers to the concentration required for 50% *in vitro* inhibition of FLT3 phosphorylation
‘Other targets’ refers to other receptors known to be inhibited by the compound at similar concentrations to FLT3

Table 2: FLT3 inhibitors in published clinical studies in AML

Drug	Trial stage	Route and dose	Patient eligibility	n (AML)	FLT3 mutation	Clinical response	Adverse effects	Reference
SU5416	Phase 2	Intravenous 145mg/m ² twice-weekly	Relapsed / refractory AML >60 yrs and unfit for intensive treatment	42 (42)	7 ITD*	BM blasts <5% without count recovery in 1 case >50% reduction in BM blasts in 7 cases (no responses in ITD cases)	Nausea, headache, bone pain	(Fiedler <i>et al</i> , 2003)
SU5416	Phase 2	Intravenous 145mg/m ² twice-weekly	Relapsed / refractory AML High risk myelodysplastic syndrome	55 (33)	Unknown	BM blasts <5% without count recovery in 1 case >50% reduction in BM blasts in 3 cases	Headache, dyspnoea, fatigue, infusion reactions, thrombosis	(Giles <i>et al</i> , 2003)
SU11248	Phase 1	Oral 50-350mg (Single dose only)	AML (unrestricted)	29 (29)	3 ITD 2 TKD	Not applicable	Nausea, diarrhoea	(O'Farrell <i>et al</i> , 2003b)
CEP701	Phase 1/2	Oral 40-80mg BD	Relapsed / refractory AML with FLT3-activating mutation	17 (17)	16 ITD 1 TKD	BM blasts <5% without count recovery in 1 case Peripheral blood blast clearance in 4 cases	Nausea, diarrhoea, fatigue	(Smith <i>et al</i> , 2004)
PKC412	Phase 2	Oral 75mg TDS	Relapsed / refractory AML or high risk myelodysplastic syndrome with FLT3-activating mutation	20 (19)	18 ITD 2 TKD	BM blasts <5% without count recovery in 3 cases >50% reduction in PB blasts in 14 cases, >50% reduction in BM blasts in 6 cases	Nausea, diarrhoea, fatigue, headache, oedema ? pulmonary infiltrates	(Stone <i>et al</i> , 2005b)
SU11248	Phase 1	Oral 50-75mg daily	Relapsed / refractory AML Unfit for intensive treatment	15 (15)	2 ITD 2 TKD	Peripheral blast clearance or BM blast reduction in 4/4 FLT3 mutant, 2/7 FLT3 WT cases	Dose-limiting fatigue, hypertension at 75mg Oedema, fatigue	(Fiedler <i>et al</i> , 2005)
CEP701	Phase 2	Oral 60-80mg BD	Newly-diagnosed AML >70yrs (or 60-70yrs with comorbidity)	29 (29)	2 ITD 3 TKD	Peripheral blood blast clearance or >50% BM blast reduction in 3/5 FLT mutant, 5/22 FLT3 WT cases	Nausea, diarrhoea, constipation, liver enzyme changes	(Knapper <i>et al</i> , 2006a)
MLN-518	Phase 1	Oral 50-700mg BD	Relapsed / refractory AML Unfit for intensive treatment High risk MDS	40 (39)	8 ITD 1 TKD	Peripheral blast clearance and reduction in BM blasts in 2 ITD cases (at doses above 525mg)	Weakness, fatigue, nausea, diarrhoea, oedema	(DeAngelo <i>et al</i> , 2006)

ITD indicates FLT3 internal tandem duplication, TKD indicates FLT3 tyrosine kinase domain point mutation

*Retrospective FLT3 mutation screening in 35 patients only. No FLT3-TKD mutation analysis performed.

