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Paediatric Update

FLT3 Inhibitors: a paradigm for the development of targeted therapeutics for paediatric cancer

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Abstract

The area of molecularly-targeted cancer therapeutics is generating tremendous interest and excitement. While clinical investigation of these agents has been largely limited to adults, clinical trials for paediatric cancer patients with many of these agents are now underway. This paper reviews the current status of molecularly-targeted therapies for paediatric malignancies, with special attention given to one class of agents, inhibitors of the FLT3 receptor tyrosine kinase. FLT3 is expressed and activated in many human leukemias, including a significant percentage of pediatric AML and infant and childhood ALL, especially in the setting of MLL gene rearrangement. Activating mutations of FLT3 portend a poor prognosis in pediatric AML. Activated FLT3 can be effectively and selectively targeted by small molecule inhibitors, and these agents have shown promise in early phase clinical trials in adults with AML. Limited preclinical data with FLT3 inhibitors in MLL-rearranged ALL have also been reported. Challenges and future directions for the use of FLT3 inhibitors and other targeted agents in paediatric cancer are discussed.

1. Introduction

The promise of molecularly-targeted cancer therapy has generated a tremendous amount of excitement in recent years. Imatinib mesylate, an inhibitor of the BCR-ABL fusion protein tyrosine kinase that has shown remarkable activity in chronic myelogenous leukaemia (CML), is perhaps the most celebrated example of a therapy developed to target a specific molecular mechanism of cancer [1]. While most of the clinical research undertaken with molecularly-targeted therapies to date has involved adult patients, there are both theoretical and practical reasons to believe that paediatric cancer patients will also benefit from the development and use of these agents. The purpose of this article is to review molecularly-targeted therapies with promise for the treatment of paediatric malignancies, with special attention given to one class of agents, inhibitors of the FLT3 receptor tyrosine kinase.

Targeted cancer therapy can be defined as therapy designed to interfere with a specific molecular pathway important in the genesis and/or maintenance of the malignant phenotype. This contrasts with traditional cancer chemotherapy agents, which interfere with some aspect of the global cellular machinery that is shared by malignant and non-malignant cells. The promise of targeted therapy is that it will more efficiently eradicate malignant cells while leaving normal host cells largely unaffected. Translating that promise into reality for cancer patients is proving a challenging task, reflecting the enormous complexity of the molecular basis of human cancer.

2. Molecularly-targeted therapies in paediatric malignancies

2.1. Current Children's Oncology Group (COG)/ National Cancer Institute (NCI) clinical trials

The number of potential targets for cancer therapy is prohibitively large to discuss here, so a few examples of

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Table 1
Targeted cancer therapies being studied in current paediatric clinical trials

Type of Target	Specific Target	Agent name	Type of agent	Proposed Mechanism	Tumour type
Tyrosine kinases	BCR-ABL	Imatinib mesylate (Gleevec)	Small molecule	Inhibits oncogenic kinase	CML, Ph + ALL
	c-KIT	Imatinib mesylate (Gleevec)	Small molecule	Inhibits oncogenic kinase	Sarcomas
	HER2	Trastuzumab (Herceptin)	mAb	Blocks ligand binding	Osteosarcoma
	EGFR	ZD1839 (Iressa)	Small molecule	Inhibits oncogenic kinase	Solid tumours
Surface markers	CD20	Rituximab (Rituxan)	mAb	Opsonises tumour cells	NHL, HD, PTLD
	CD20	^{90}Y-Ibritumomab (90Y-Zevalin)	mAb-conjugate	Delivers toxin	NHL, HD
	CD33	Gemtuzumab ozogamicin (Myelotarg)	mAb-conjugate	Delivers toxin	AML
	GD2	Anti-GD2 (ch14.18)	mAb	Opsonises tumour cells	Neuroblastoma
Anti-apoptotic proteins	Bcl-2	G3139 (Genasense)	mRNA antisense	Facilitates apoptosis	Solid tumours
Fusion proteins	PML-RARα	All *trans*-retinoic acid (tretinoin)	Retinoid	Promotes differentiation	APML
	EWS-FLI1	Peptide-pulsed APC's	Cellular	Immunotherapy	Ewing's sarcoma
	PAX-FKHR	Peptide-pulsed APC's	Cellular	Immunotherapy	Rhabdomyosarcoma
Farnesyl transferase inhibitors	RAS	R115777 (tipifarnib)	Small molecule	Prevents RAS activation	Leukaemia, solid tumours
Histone deacetylase inhibitors	HDAC	Depsipeptide	Peptide competitive inhibitor	Prevents deactivation of tumour suppressors	Solid tumours
Drug resistance mechanisms	MDR1	PSC-833 (valspodar)	P-gp 170 inhibitor	Prevents drug efflux	Leukaemia

APC, antigen-presenting cell; mAb, monoclonal antibody; CML, chronic myelogenous leukaemia; Ph+, Philadelphia chromosome-positive; ALL, acute lymphoblastic leukaemia; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's disease; PTLD, post-transplant lymphoproliferative disorder; AML, acute myelogenous leukaemia; EGFR, epidermal growth factor receptor; APML, acute promyelocytic leukaemia.

targets with particular relevance for paediatric malignancies will be reviewed. Table 1 shows a list of targeted therapies currently being studied in the United States in the Children's Oncology Group (COG) or National Cancer Institute (NCI) sponsored clinical trials [2,3]. Of the therapies listed, only all *trans*-retinoic acid (ATRA), a retinoid that induces differentiation of acute promyelocytic leukaemia (APML) blasts with the PML-RARα fusion protein, has a proven track record of efficacy in paediatric patients in randomised controlled clinical trials [4,5]. Another targeted agent that has demonstrated some efficacy is 3F8, a monoclonal antibody against ganglioside G_{D2}. Neuroblastoma cells have high level surface expression of G_{D2} compared with normal tissues, and binding of the antibody leads to complement-mediated tumour cell lysis. The results of a phase II trial of 3F8 in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) for refractory neuroblastoma demonstrated anti-tumour activity, particularly for minimal residual disease in the bone marrow [6]. The remainder of these therapies is as yet unproven in the paediatric population. However, in the adult population there have been several studies demonstrating promising results for targeted therapies, including imatinib mesylate for CML and gastrointestinal stromal tumours, trastuzumab for breast cancer, ZD1839 for non-small cell lung cancer, rituximab for lymphomas, and gemtuzumab ozogamicin for acute myelogenous leukaemia (AML) [7–21].

2.2. Target validation

The degree of validation of the targets of these therapies as appropriate ones for the tumours being treated has been variable. In paediatric patients with CML, for example, the BCR-ABL fusion protein should be no less valid a target than in adult patients. However, in patients with Ewing's sarcoma, it is not as clear that c-KIT, a receptor tyrosine kinase that transduces proliferative and survival signals when activated, is a rational target for inhibition. While c-KIT is expressed in many sarcomas, there is little data to suggest that c-KIT has abnormal function or is essential to the development or maintenance of the malignant phenotype in these cells [22,23]. This is in contrast with gastrointestinal stromal tumours in adults, where there are activating mutations of c-KIT that are more clearly oncogenic [10,24,25]. Targeting the HER2 protein in osteosarcoma is also based on scant validating data. Human epidermal growth factor receptor 2 (HER2) is a receptor tyrosine kinase whose overexpression and autocrine activation in certain forms of breast cancer has been clearly demonstrated to contribute to the malignant phenotype. This has led to the development of therapies targeted to HER2, such as trastuzumab, a monoclonal antibody which blocks the binding of the

epidermal growth factor to HER2. Retrospective analyses have shown HER2 overexpression in approximately 40% of osteosarcomas, with a correlation of overexpression with metastasis, poor tumour necrosis after neoadjuvant chemotherapy, and decreased survival. However, other studies have not shown a negative prognostic impact [26–30]. These associations await confirmation in prospective studies.

3. FLT3 as an example of the development of molecularly-targeted therapy

3.1. FLT3 in normal tissues

3.1.1. Structure of the FLT3 receptor

One promising potential target for inhibition in paediatric cancer is the FLT3 receptor tyrosine kinase. FMS-like receptor tyrosine kinase-3 (FLT3) is a member of the class III receptor tyrosine kinase family that also includes KIT, platelet-derived growth factor receptor (PDGFR) and FMS [31]. It has an N-terminal extracellular domain with five immunoglobulin-like motifs, a transmembrane domain, a juxtamembrane domain and a tyrosine kinase domain at the C-terminus interrupted by the kinase insert region (Fig. 1). The extracellular domain is heavily glycosylated, resulting in migration on Western Blots as two bands of 130 kD and 160 kD, both greater than the 113 kD molecular weight predicted by the amino acid sequence [32,33].

3.1.2. Range of tissue expression of FLT3

The FLT3 receptor is expressed in normal human bone marrow selectively in CD34+ immature haematopoietic stem/progenitor cells and in dendritic cell progenitors [32,34,35]. On the basis of targeted disruption studies of FLT3 or FLT3-ligand in mice, FLT3 signalling appears to be important in the development of haematopoietic stem cells, B-cell progenitors, dendritic cells and natural killer (NK) cells [36,37]. FLT3 is also expressed in placenta, gonads and brain, where its function is unknown [38,39]

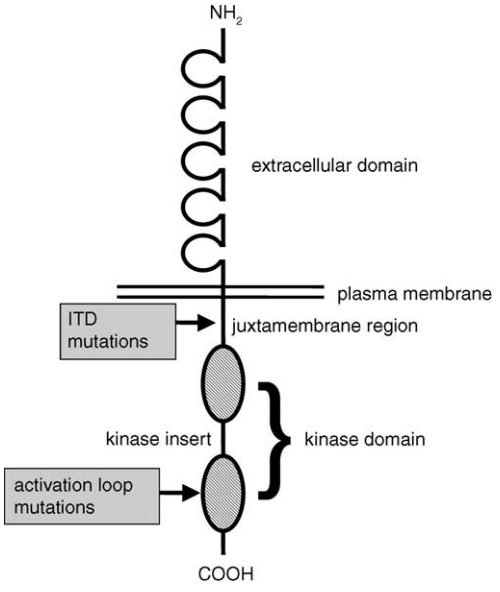

Fig. 1. Schematic of the structure of the FLT3 receptor. ITD, internal tandem duplications.

3.1.3. Normal FLT3 receptor function

The ligand for FLT3 (FLT3-ligand, or FL) is produced by bone marrow stromal cells and many haematopoietic cell types in membrane-bound and soluble forms [40–42]. Binding of ligand to receptor induces dimerisation of the receptor and activation of the kinase domains, which then autophosphorylate and catalyse phosphorylation of substrate proteins [31]. Many important signalling proteins can be activated by FL stimulation of the FLT3 receptor, including signal transducer and activator of transcription 5 (STAT5) phosphatidyl initosol-3-kinase (PI3K), phospholipase C-gamma (PLC-γ), RAS, mitogen activated protein kinase (MAPK), extracellular-signal regulated kinase (ERK1/2), SHC, Src-homology 2 containing protein tyrosine phosphate (SHP2) and Src-homology 2 containing inositol phosphatase (SHIP) [43–48]. These pathways are involved in cellular proliferation, differentiation and survival. A schematic of some of the signalling pathways involving FLT3 is shown in Fig. 2.

3.2. FLT3 in human leukaemia

3.2.1. FLT3 and its ligand are aberrantly expressed in many haematological malignancies

FLT3 is aberrantly expressed in most human leukaemias, including greater than 90% of AML, nearly 100% of B-lineage acute lymphoblastic leukaemia (ALL), and a smaller percentage of T-lineage ALL, CML in blast crisis and chronic lymphoid leukaemia (CLL) [49–51]. FLT3 is also aberrantly expressed in a similar proportion of cell lines derived from human leukaemias [52,53]. A summary of these expression data and the methods used to detect expression is shown in Table 2. Interestingly, nearly all leukaemic cell lines express FLT3-ligand (FL), and thus 40 of 110 cell lines studied expressed both FLT3 receptor and FL [52]. In analogy to the cell line data, we have found that co-expression of FL (detected by flow cytometric analysis) and FLT3 (detected by Western Blotting) by primary AML blasts is also a common occurrence [54]. In a number of cases of cell lines and primary AML samples, this leads to constitutive receptor phosphorylation/activation. These data suggest that FLT3 signalling may be important in certain subtypes of human leukaemia, and that in some cases, autocrine, paracrine or intracrine signalling may be involved.

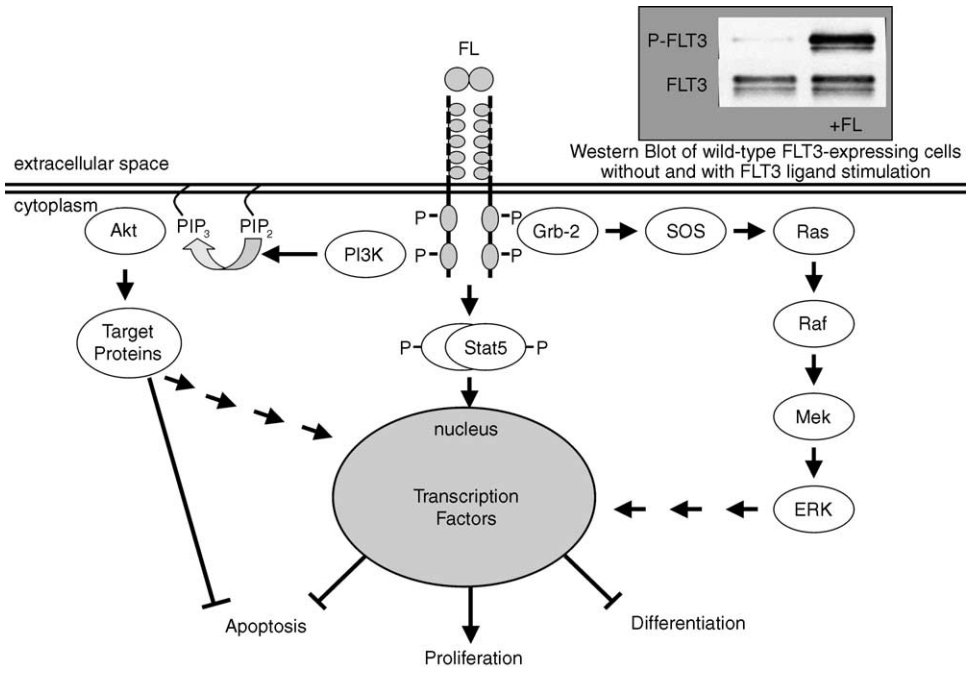

Fig. 2. Downstream signalling pathways for FLT3.

Table 2
Compiled data on FLT3 expression in human leukaemia cell lines and patient samples

	Cell lines	Patient samples
Cell line/patient sample phenotype	No. (%) FLT3 +	No. (%) FLT3 +
Pre-B ALL	16/17 (94%)	57/58 (98%)
B-ALL/Burkitt	4/9 (44%)	1/4 (25%)
T-ALL	1/14 (7%)	8/25 (32%)
AML	25/52 (48%)	95/107 (89%)
M0-M3	11/16 (69%)	50/55 (91%)
M4/M5	12/17 (71%)	36/42 (86%)
M6	0/9 (0%)	2/2 (100%)
M7	2/10 (20%)	7/8 (88%)
CML in blast crisis	6/16 (38%)	15/24 (63%)

Cell lines: From Meierhoff and colleagues 1995 (Ref. 52; method = Northern Blot for RNA), Small and colleagues data not shown (method = Western Blot for protein), Rosnet and colleagues 1996 (Ref. 50; method = flow cytometry for surface protein), Quentmeier and colleagues 2003 (Ref. 100; method = Western Blot for protein). **Patient samples**: From Birg and colleagues 1992 (Ref. 51; method = Northern Blot for RNA), Carow and colleagues 1996 (Ref. 49; method = Western Blot for protein and RNase protection for RNA), Rosnet and colleagues 1996 (Ref. 50; method = flow cytometry for surface protein).

FLT3 has also been found to be overexpressed at some of the highest levels in cases of ALL with rearrangements of the *MLL* (mixed lineage leukemia) gene at 11q23. Microarray gene expression profiles of leukaemic blasts from children (mostly infants) with B-precursor ALL with MLL rearrangements revealed a pattern of expression distinguishing these leukaemias from B-precursor ALL without MLL rearrangements and from AML [55]. The gene whose overexpression best distinguished the MLL leukaemias from the others was FLT3. A cell line (SEMK2-M1) derived from a child with an MLL leukaemia has been recently shown to overexpress FLT3 on the basis of intrachromosomal amplification of the entire FLT3 locus. [56] It remains to be seen whether this mechanism of overexpression occurs in primary patient samples.

3.2.2. *FLT3 activating mutations are present in many leukaemias*

While overexpression of wild-type FLT3 with its ligand is one potential mechanism of FLT3 involvement in leukaemia, the existence of FLT3 activating mutations makes a much stronger case for the importance of this gene. Internal tandem duplications (ITD) were first reported in patients with AML [57]. ITDs are duplications of variable numbers of base pairs, always in multiples of three such that the reading frame remains intact, in the region coding for the juxtamembrane domain of the receptor. This results in the insertion of repeated amino acid sequences in this domain. In addition, often three or six additional base pairs are also inserted, resulting in the insertion of one or two novel amino acids prior to the tandem repeat. The functional result of an ITD is ligand-independent dimerisation with activation of the kinase domain and constitutive phosphorylation of the receptor [58–60].

Another class of activating mutations occur within the so-called "activation loop" of the kinase domain.

Most of these mutations are point mutations that cause substitutions of other amino acids (most commonly tyrosine) for the aspartate at position 835 or the isoleucine at position 836 [61,62]. More recently, other novel activation loop mutations have been reported, including deletion of the aspartate residue at position 835, deletion of the isoleucine residue at position 836, and insertion of a glycine and a serine residue between positions 840 and 841, among others [56,62–64]. A compilation of the FLT3 kinase domain mutations which have been reported is given in Table 3.

Table 3
Activation loop mutations of the FLT3 kinase domain

Designation	Description	Reference
D835Y	Asp- > Tyr	[61]
D835V	Asp- > Val	[61]
D835E	Asp- > Glu	[61]
D835H	Asp- > His	[61]
D835N	Asp- > Asn	[61]
D835G	Asp- > Gly	[63]
D835A	Asp- > Ala	[76]
D835L + K	Asp- > Leu + Lys	[63]
Δ835	Deletion of Asp	[62]
I836T	Ile- > Thr	[63]
I836S	Ile- > Ser	[63]
I836L + D	Ile- > Leu + Asp	[61]
I836M + R	Ile- > Met + Arg	[63]
Δ836	Deletion of Ile	[63]
840 + GS	Insertion of Gly and Ser between Ser (840) and Asn (841)	[64]

3.2.3. Mechanism of FLT3 activation by mutations

The juxtamembrane domain and the activation loop of the tyrosine kinase domain are both thought to be important to the autoinhibitory regulation of the receptor, based largely on data collected from related receptor and cytoplasmic tyrosine kinases [65–68]. Ligand binding normally releases the receptor from this autoinhibition, leading to activation. It is hypothesised that ITDs and point mutations both abrogate the autoinhibitory function, resulting in constitutive activation of the tyrosine kinase activity that is independent of ligand binding [59,60].

3.2.4. Incidence of activating mutations in adult and paediatric leukaemias

These activating mutations are found in many adult and paediatric leukaemias, most notably in AML. Frequencies for ITDs and point mutations are summarised by leukaemia type in Table 4 [56–58,61–63,69–89]. ITDs are the most common, with a prevalence of 17–34% in adult AML and 10–17% in paediatric AML. They

Table 4
Compilation of FLT3 mutation analyses in adults and children by leukaemia type

Diagnosis	Adults		Children	
	ITD no. + /no. pts (%) • no. + /no. pts [Ref.]	Point mutation no. + /no. pts (%) • no. + /no. pts [Ref.]	ITD no. + /no. pts (%) • no. + /no. pts [Ref.]	Point mutation no. + /no. pts (%) • no. + /no. pts [Ref.]
AML	**1039/4552 (23%)** •5/30 [57] • 15/74 [58] • 22/112 [69] • 47/140 [70] • 46/201 [71] • 18/81 [72] • 14/106 [73] • 227/854 [74] • 23/82 [75] • 119/523 [76] • 40/159 [77] • 200/979 [63] • 231/1003 [78] • 32/208 [79]	**160/1937 (8%)** •30/429 [61] •7/97 [62] •75/979 [63] •32/224 [76] •16/208 [79]	**68/490 (14%)** •20/74 [81] •9/80 [82] •15/91 [83] •12/87 [84] •7/64 [85] •5/94 [86]	**7/91 (8%)** [89]
ALL	**0/105 (0%)** •0/50 [57] •0/55 [69]	**1/36 (3%)** [61]	**3/234 (1%)** •2/60 [84] •1/174 [87]	**5/30 (17%)** of infant ALL with MLL rearrangements [56]
CML	**0/40 (0%)** •0/20 [69] •0/20 [88]	**0/20 (0%)** [88]	ND	ND
MDS	**8/129 (6%)** •7/92 [80] •1/37 [69]	**1/29 (3%)** [61]	**0/32 (0%)** [84]	ND

ND, No data reported; ITD, internal tandem duplications; pts, patients; MDS, myelodysplastic syndrome.

occur in all French-American-British (FAB) classification subtypes, are most common in M3 (APL), and can occur together with any cytogenetic abnormality. Point mutations occur in approximately 8% of AML. Thus, a significant proportion of patients with AML (25–42% of adults, 18–26% of children) have activating mutations of the FLT3 tyrosine kinase, making it the most common somatic genetic alteration in this disease. FLT3 mutations occur much less frequently in other types of leukaemia. However, point mutations have been found to be particularly common in infant and childhood MLL leukaemias, with 5 point mutations being found in 30 patients (17%) in a recently published series [56].

3.2.5. Evidence for pathogenic role of FLT3 activating mutations in leukaemia

3.2.5.1. Patients with FLT3 activating mutations have a worse prognosis. The mere presence of an activating mutation or overexpression of FLT3 in a leukaemia would not, by itself, prove that the mutation is important in the biology of the disease. However, several lines of evidence do support this notion. A number of studies have demonstrated that both adult and paediatric AML patients with activating mutations, particularly ITDs, have a worse prognosis than those without mutations. Table 5 summarises the published literature on the prognostic significance of ITDs in childhood AML [81–86]. For example, in a retrospective study utilising samples from a United States (U.S.) cooperative group trial, the 8-year overall and event-free survival were 13% and 7%, respectively, for patients with ITDs and 50% and 44% for patients with wild-type FLT3 ($P = 0.02$ and $P = 0.002$) [83]. The ITD patients were also noted to have a significantly higher leucocyte counts at diagnosis (73 800/μl versus 28 400/μl, $P = 0.05$). Failures of remission induction were significantly more common in patients with ITDs, with remission induction rates of 74% in the ITD-negative group and 40% in the ITD-positive group ($P = 0.005$). Three of the six studies noted that ITDs were more frequently associated with an older age at diagnosis [81,82,85]. None of these paediatric studies looked at patients with point mutations, although several adult studies have been unable to demonstrate a significant prognostic impact of point mutations [63,76,79].

3.2.5.2. Effects of FLT3 activation in model systems. Another line of evidence demonstrating the importance of FLT3 constitutively activating mutations is their ability to transform haematopoietic cell lines. The murine cell lines Ba/F3 and 32D are, respectively, lymphoid and myeloid non-leukaemic progenitor cell lines that normally require interleukin-3 (IL-3) for proliferation and survival. Transfection of constitutively activated FLT3 sequences into these cells results in growth which is independent of IL-3. Transfection of FLT3 wild-type sequences does not have this effect. [58,90–92] In addition, when Ba/F3 or 32D cells which have been transformed by transfection with a FLT3 ITD are injected into syngeneic mice (Balb/c and C3H, respectively), the mice quickly die of a leukaemia-like illness. [90–92] The presence of a functioning kinase domain is necessary for transformation and leukaemogenesis [92].

While able to transform cell lines, two lines of experiments demonstrate that constitutive FLT3 activation alone is not sufficient to fully transform primary haematopoietic cells. Transgenic mice in which FLT3 was constitutively activated through fusion with *TEL* developed myeloproliferative disease after a lag of at least six months. These mice showed increased colony-forming unit (CFU) activity from their bone marrow and spleen and over time developed splenomegaly and extramedullary haematopoiesis with increasing penetrance. However, mice followed for greater than two years failed to develop leukaemia [93]. In another experimental system, bone marrow from Balb/c mice that was transduced with a FLT3 ITD-containing retrovirus also caused a myeloproliferative disorder when transplanted back into lethally irradiated healthy mice [94]. In contrast to the transgenic model, the myeloproliferative disease which develops in these mice is fatal and occurs with little lag. The difference between the phenotypes of these two models might be explained by the higher level

Table 5
Prognostic and clinical data on FLT3 ITD mutations in childhood AML

Number of patients	Number (%) ITD	Parameter	ITD +	ITD-	p-value	Clinical data	Reference
45	10 (22%)	5 year EFS (non-APML patients)	20%	49%	$P = 0.033$	ITD + older, higher WBC	[81]
91	15 (16%)	8 year OS	13%	50%	$P = 0.02$	ITD + higher WBC, more induction failures	[83]
		8 year EFS	7%	44%	$P = 0.002$		
87	12 (14%)	4 year OS	0%	?	?	ITD + >50% induction failure rate	[84]
64	7 (11%)	5 year EFS	14%	69%	$P = 0.003$	ITD + older, higher WBC, more induction failures	[85]
94	5 (5%)	5 year EFS	20%	60%	$P = 0.004$	No difference in age or WBC	[86]

EFS, Event-free survival; OS, overall survival; WBC, white blood cell.

expression and/or additional gene activation at integration sites of the retrovirus model.

Another murine model demonstrates the potential cooperative effects of FLT3 activating mutations and other genetic alterations found in leukaemia. PML/RARα transgenic Balb/c mice develop an APML-like disease, but it occurs with long latency and incomplete penetrance. When bone marrow from these mice is transduced with FLT3 ITD and transplanted back into lethally irradiated healthy mice, it causes murine APL with shorter latency and complete penetrance [95]. Taken together, these data provide strong evidence that activating mutations of FLT3 can contribute to the process of leukaemogenesis, but is not sufficient by itself to fully transform primary haematopoietic cells.

3.2.6. Mechanisms of FLT3-induced leukaemia

3.2.6.1. Internal tandem duplications. The exact pathway by which activating mutations of FLT3 lead to leukaemia is not completely clear. However, experimental data suggests that signalling through FLT3 may contribute through enhancing proliferation, inhibiting apoptosis and blocking differentiation.

There is evidence that the anti-apoptotic pathway involving PI3-kinase and Akt activation is involved in signalling through both FLT3 wild-type and ITD receptors. The p85 subunit of PI3-kinase is phosphorylated in response to activation of the wild-type receptor by ligand. This process involves a complex of several proteins, including CBL, GAB2, SHP-2, and SHIP [43,44]. Akt, a serine/threonine protein kinase downstream of PI3-kinase, has been shown to be constitutively activated in ITD-transfected 32D cells [91,96]. In one study, additional phosphorylation of Akt was seen upon addition of ligand, suggesting that FLT3 ITD receptors may retain the ability to respond to FL [91].

The proliferative pathway involving RAS and MAP kinase has also been shown to be activated in FLT3 signalling. Ba/F3 cells transfected with human wild-type FLT3 and exposed to exogenous FL demonstrated transient activation of MAPK. When this activation was inhibited with PD98059, a MEK inhibitor, the cells no longer proliferated in the presence of FL [45]. Ba/F3 cells transfected with FLT3 ITD show constitutive phosphorylation of MAP kinase and their growth is also inhibited by PD98059 [97,98]. 32D cells transfected with FLT3 ITD show activation of MAPK which is augmented by the addition of FL. When these same cells are co-transfected with an inducible dominant-negative RAS construct, colony formation was decreased by over 50% [91]. MAPK is also constitutively phosphorylated in many samples of primary AML blasts [90,97].

STAT5 is a member of a family of signal transducers and activators of transcription that has been shown to participate in multiple processes in haematopoietic cells, including proliferation, prevention of apoptosis and differentiation. It is the one signalling protein whose activation has been convincingly shown to differ between FLT3 wild-type and constitutively activated signalling. Ba/F3 cells transfected with a constitutively activated *TEL-FLT3* fusion product showed constitutive activation of STAT5, as did Ba/F3 and 32D cells transfected with FLT3 ITDs [92,97,98]. FLT3 wild-type transfectants, on the other hand, did not show constitutive activation of STAT5 and, further, showed only weak or transient activation of STAT5 with exposure to exogenous ligand [91,99]. Recent reports have found that MV4-11, a human AML cell line with a naturally occurring FLT3 ITD mutation, demonstrated constitutive STAT5 activation, but that cell lines with wild-type FLT3 did not show activated STAT5 when stimulated with FL [90,100]. STAT5 target genes, such as the serine/threonine kinases PIM-1 and PIM-2, have also been found to be upregulated by FLT3 ITDs [101,102]. The PIM family, originally identified as proviral insertion sites involved in leukaemia, has been shown to be involved in transformation in many models of lymphoid and myeloid malignancies through proliferative and anti-apoptotic mechanisms [103–107]. Finally, constitutive STAT5 activation has also been shown in primary AML blasts with ITD mutations [90,97].

FLT3 ITD signalling has also been implicated in blocking differentiation. Transfection of FLT3 ITD into 32D cells results in a block of G-CSF-induced myeloid differentiation [108]. Analyses of 32D cells transfected with FLT3 ITD showed that the expression of two transcription factors known to be important in myeloid differentiation, C/EBPα and PU.1, are both significantly repressed in the ITD cells, whereas expression in wild-type FLT3 transfected cells was unchanged [101,109,136].

Another potential mechanism by which FLT3 ITD may contribute to the leukaemic phenotype is inhibition of phosphatase activity. The effects of the FLT3 ITD on activation of tyrosine kinase signalling may be enhanced by the inhibition of cellular phosphatases such as SHP-1, which could lead to amplification of the proliferative, anti-apoptotic and differentiation-blocking effects. Data from our laboratory has shown that Ba/F3 cells transfected with FLT3 ITD demonstrate suppression of SHP-1 expression, while those transfected with wild-type FLT3 do not [110].

3.2.6.2. Activation loop mutations. While the cooperative role of FLT3 ITDs in leukaemia is well supported, the biological significance of activation loop mutations is less clear. 32D and Ba/F3 cells transfected with the Asp835Tyr mutation demonstrate constitutive receptor phosphorylation and IL-3-independent proliferation

[56,61,111]. Ba/F3 cells transduced with either of two other activating loop mutations (840GS and Δ836) similarly result in FLT3 activation and transformation [56,112]. On the other hand, point mutations do not appear to affect prognosis to the same degree as ITDs, at least in adult AML [63,76,79]. It may be that there is a qualitative or quantitative difference in FLT3 signalling induced by the ITD and the point mutation, even though both clearly result in constitutive kinase domain activation and autophosphorylation.

3.2.6.3. Overexpression/activation of wild-type FLT3. The significance of overexpression and activation of wild-type FLT3 in leukaemias is also not clear. Ba/F3 cells overexpressing wild-type FLT3 were not factor-independent *in vitro*, but caused leukaemia when injected in Balb/c mice, perhaps as a result of ligand expression by the mice [92]. Balb/c mice transplanted with bone marrow transduced with a constitutively expressed *FL* gene develop leukaemia, albeit with a long latency [113]. These experiments suggest that autocrine and paracrine signalling through wild-type receptors may also play a role in leukaemogenesis.

There are several human leukaemia-derived cell lines that demonstrate constitutive activation of FLT3 without identified FLT3 activating mutations. Examples include EOL-1, an AML-derived line, and REH, a pre-B ALL-derived line [90] [Small and colleagues, unpublished data]. The SEMK2-M1 cell line, as mentioned earlier, overexpresses wild-type FLT3 on the basis of intrachromosomal amplification of the entire locus and demonstrates constitutive phosphorylation of the FLT3 receptor, presumably due to coexpression of FLT3 ligand [56]. We have also identified constitutive activation of wild-type FLT3 in primary leukaemic blasts [54,90]. However, the presence of constitutive activation of FLT3 does not necessarily implicate FLT3 signalling as the primary cause of the leukaemic phenotype. As discussed in the next section, experiments with FLT3 inhibitors can help clarify the biological significance of FLT3 activation in these model systems.

3.3. FLT3 inhibitors

3.3.1. Discovery of FLT3 kinase inhibitors

The identification of activated FLT3 as a contributor to the cause of many leukaemias has led to its consideration as a potential target for therapy. The success of imatinib mesylate, a small molecule inhibitor of the constitutively activated ABL tyrosine kinase that acts by competing with adenosine triphosphate (ATP) for binding to its active site, has certainly contributed to the excitement about the development of FLT3 inhibitors. Several small molecule tyrosine kinase inhibitors with activity against FLT3, all of which work like imatinib to compete with ATP binding, have now been reported. A summary of the clinically relevant FLT3 inhibitors and their comparative characteristics, including stage of clinical trial development, are summarised in Table 6.

The first FLT3 kinase inhibitors to be reported were AG1295 and AG1296, drugs of the tyrphostin class that inhibit autophosphorylation of FLT3 and FLT3 ITDs with an IC_{50} (concentration causing 50% growth inhibition) of 0.3–0.5 μM. These drugs were shown to be selectively cytotoxic to FLT3 ITD-transfected cell lines and primary AML samples with FLT3 ITDs [98,114,115]. Activation of downstream signalling molecules, such as STAT5 and CBL, was prevented by FLT3 inhibition with a similar IC_{50} [114]. These compounds are not very soluble and are not amenable to use

Table 6
Summary of characteristics of FLT3 inhibitors in clinical development

Compound	Chemical class	FLT3 IC_{50} (nM)	Other RTKs potently inhibited	Stage of clinical development
CEP-701	Indolocarbazole	3	TrkA	Phase II
PKC412	Staurosporine	10*	PKC VEGFR PDGFR KIT	Phase II
MLN518 (CT53518)	Quinazoline	10–220	PDGFR KIT	Phase I
SU5416	Indolinone	100	KIT VEGFR	Phase II
SU5614	Indolinone	10	KIT VEGFR	Phase I
SU11248	Indolinone	50–300	VEGFR PDGFR KIT	Phase I

* = IC_{50} in *in vitro* kinase assay was reported as 528 nM (see text). RTK, receptor tyrosine kinases; IC_{50}, concentration causing 50% inhibition of FLT3 phosphorylation; PKC, protein kinase C; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

in patients, but demonstrated for the first time the proof of concept that FLT3 inhibitors could kill leukaemia cells in which FLT3 signalling plays an important role. This data drove the search for FLT3 inhibitors which could be used in clinical trials and a number of companies now have drugs with these characteristics.

CEP-701 is a indolocarbazole derivative whose ability to selectively inhibit FLT3 was discovered in our laboratory by screening a library of small molecule tyrosine kinase inhibitors for their ability to selectively inhibit the proliferation of Ba/F3 cells transfected with FLT3 ITD, while at the same time allowing rescue by the addition of IL-3 [90]. The IC_{50} for inhibition of proliferation was 5 nM, for inhibition of FLT3 autophosphorylation was 2 nM, and for inhibition of FLT3 *in vitro* kinase activity was 3 nM. In contrast, no inhibition of proliferation of parental Ba/F3 cells occurred at concentrations as high as 200 nM. Inhibition of the most closely related receptor tyrosine kinases, platelet-derived growth factor receptor-β (PDGFR-β), FMS and KIT, occurred only at concentrations of 500–1000 nM or greater. Another kinase known to be inhibited potently by CEP-701 is TrkA with an IC_{50} of approximately 3 nM [116]. CEP-701 is cytotoxic to human AML cell lines with activated FLT3 and, like AG1295 and AG1296, is selectively cytotoxic to primary patient samples with FLT3 ITDs. These cytotoxic effects closely parallel the inhibition of FLT3 phosphorylation and downstream target phosphorylation of STAT5 and MAPK. CEP-701 also prolongs survival in Balb/c mice injected with FLT3 ITD-transfected Ba/F3 cells. Based on these preclinical data, CEP-701 is currently being tested in phase II trials in adults with relapsed or refractory AML and FLT3 activating mutations. The preliminary results have been promising, with a number of patients achieving reductions in peripheral blast counts, and one patient demonstrating reduction of bone marrow blasts from 25% to less than 5% at day 28 of therapy [117]. Clinical responses have correlated with demonstration of inhibition of FLT3 in the patients' leukaemic blasts.

PKC412 is a staurosporine derivative that was originally described as an inhibitor of protein kinase C (PKC), and later discovered to inhibit many tyrosine kinases, including vascular endothelial growth factor receptor (VEGFR), PDGFR, KIT and FLT3 [111]. In experiments similar to those described for CEP-701 above, PKC412 was identified as a FLT3 inhibitor based on its selective inhibition of Ba/F3-FLT3 ITD proliferation. The IC_{50} for inhibition of proliferation and for inhibition of phosphorylation was on the order of 10 nM, but for inhibition of *in vitro* kinase activity was 528 nM, suggesting that the conformation of the naked kinase domain is somehow altered such that the drug does not bind as well. PKC412 prolonged the survival of mice transplanted with FLT3 ITD-transduced bone marrow, who, as mentioned above, develop a fatal myeloproliferative disorder. PKC412 is also being tested in a phase II trial in adults with relapsed or refractory AML with FLT3 activating mutations. Preliminary trial results showed that of eight patients treated, six had transient reductions in peripheral blast counts, one of which had a reduction in bone marrow blasts from 80% to 30% [118].

SU5416 and SU5614 are indolinone compounds that were initially identified as VEGFR and KIT inhibitors, and were subsequently found to inhibit FLT3 as well. Both compounds have been shown to be potent inhibitors of FLT3 with IC_{50} in the 10–100 nM range, and are selectively cytotoxic to Ba/F3 cells transfected with FLT3 activating mutations and human leukaemia cells lines with activated FLT3 [112,119]. SU5416 was studied as a VEGFR, KIT and FLT3 inhibitor in refractory AML/myelodysplastic syndrome (MDS) patients in a phase II study and showed modest clinical activity, with 5% partial responses and 2% haematological improvement [120]. SU5614 is in phase I testing.

SU11248 is an indolinone compound originally identified as an inhibitor of VEGFR and PDGFR, then noted to inhibit FLT3 autophosphorylation with an IC_{50} of 50–250 nM. It has been shown to induce cytotoxicity in cell lines with activated FLT3, and to inhibit growth of MV4-11 cells, a human AML cell line with a FLT3 ITD mutation, in a subcutaneous (s.c.) tumour xenograft model and a murine bone marrow engraftment model [121]. A single dose phase I study showed that $>50\%$ inhibition of FLT3 phosphorylation could be achieved in AML patients at tolerable doses. A repeat dosing phase I study is ongoing [122,123].

MLN518 (formerly CT53518) was discovered initially as an inhibitor of PDGFR and KIT, and has subsequently been reported to inhibit FLT3 autophosphorylation with an IC_{50} of 220 nM [124]. It selectively induces apoptosis in cell lines expressing FLT3 ITD, and prolongs survival in nude mice injected with Ba/F3-FLT3 ITD cells and in mice transplanted with marrow transduced with FLT3 ITD. It is being tested in a phase I clinical trial [125].

3.3.2. Hsp90 Inhibitors and FLT3

Another class of compounds that have shown inhibitory activity in model systems of FLT3 ITD is Heat shock protein 90 (Hsp90) inhibitors. Herbimycin A (HA) was found to be selectively cytotoxic to 32D cells transfected with FLT3 ITD, to inhibit autophosphorylation of FLT3 in ITD-transfected Cos7 cells, and to reduce tumour weight in a 32D/FLT3 ITD murine s.c. tumour model [126]. Interestingly, HA did not inhibit the phosphorylation of FL-stimulated wild-type FLT3 in Cos7 cells. On the basis of these results, HA was postulated to be a FLT3 tyrosine kinase inhibitor. However, further study revealed that HA did not affect the FLT3 kinase activity directly, but rather dissociated

the complex formed by FLT3 ITD and Hsp90 [96]. Wild-type FLT3 was not found to associate with Hsp90, explaining the lack of effect of HA on ligand-stimulated phosphorylation. Another Hsp90 inhibitor, radicicol, has similar effects in these systems. These results suggest that the FLT3-ITD protein, with its elongated juxtamembrane region, may require Hsp90 for proper folding and stabilisation. Hsp90 has been postulated to play this role for other mutated proteins, including the BCR-ABL tyrosine kinase [127–129]. The feasibility of including these compounds in the treatment of patients with FLT3 ITD-positive leukaemia remains to be seen.

3.3.3. Potential paediatric applications of FLT3 inhibitors

While clinical trials of FLT3 inhibitors have to date included only adult patients, the promise of FLT3 inhibitors is certainly not limited to adults. As discussed previously, children with AML also harbour FLT3 activating mutations in approximately 18–25% of cases, and these patients have a very poor prognosis with current therapies. Demonstration of efficacy in the adult studies will hopefully lead to extension of the study population to include paediatric patients. Inhibition of FLT3 in these patients with activating mutations should, at a minimum, render them as curable as patients without mutations, presuming that FLT3 inhibition can be effectively incorporated into standard chemotherapy regimens. This would signify a substantial improvement in the cure rates for these children (for example, from 7% to 44% 8 year event-free survival) [83].

Other paediatric patients for whom FLT3 inhibitors may prove efficacious are infants and children with B-precursor ALL and rearrangements involving the *MLL* gene at 11q23. As discussed previously, these leukaemias have been shown to overexpress FLT3 at even higher levels when compared with standard B-precursor ALL and AML. These patients also have a dismal prognosis. FLT3-activating point mutations have been found in 16% of these cases. A cell line derived from a patient with this form of leukaemia demonstrated amplification of the FLT3 locus and constitutive phosphorylation of the receptor. A FLT3 inhibitor (PKC412) has been shown to be cytotoxic to this cell line *in vitro* and *in vivo* and to primary patient samples with MLL leukaemia and FLT3 overexpression [56]. Inclusion of these patients in clinical trials of FLT3 inhibitors seems warranted.

Finally, AML and ALL patients whose leukaemic blasts are shown to overexpress wild-type FLT3 that is constitutively activated by FL through autocrine, paracrine or intracrine mechanisms comprise an additional population that should be considered for future trials. In order to show that the clinical effects of these agents are, in fact, due to inhibition of the intended target, it will be important in the design of these trials to correlate clinical responses with the level of FLT3 expression, presence of constitutive phosphorylation, activation of downstream signalling proteins (STAT5, MAPK, AKT, etc.) and the response of each of these alterations to FLT3 inhibition.

4. Challenges and future directions for targeted therapy

There remain several challenges in the use of targeted therapies such as FLT3 inhibitors. Optimal treatment strategies are likely to require the combination of targeted and standard therapies, or of multiple targeted therapies, since most cancers are the result of more than one oncogenic "hit." These combinations will need to be studied in an attempt to identify synergism or at least additivity.

Another challenge is that many targeted therapies are not highly specific for their purported target. This is particularly true for small molecule inhibitors. In the case of some FLT3 inhibitors, other tyrosine kinases in the same class (e.g. KIT and PDGFR) are inhibited as potently as FLT3. This raises the possibility of undesirable toxicities mediated through non-targeted kinases,

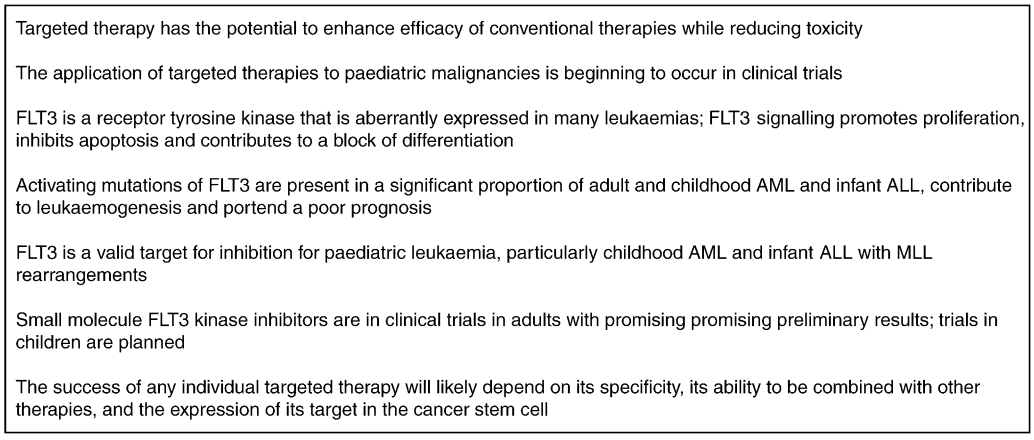

Fig. 3. 'Take home' messages.

many of which are unknown. Optimisation of drug design to improve specificity may be required as these therapies move forward.

The emerging concept of cancer stem cells presents a challenge as well. Normal regenerating human tissues contain pluripotent stem cells with unlimited self-renewing capacity that comprise only a small fraction of the total number of cells. Similarly, a tumour contains cancer stem cells with the same properties. This finding has been well documented in the case of leukaemias, but more recently is proving to be true for solid tumours as well [130–132]. For a targeted therapy to be successful in eradicating a tumour, the target must exist and be active in the cancer stem cell. This concept is being explored in the case of FLT3 activating mutations in AML. FLT3 mutations appear to occur both as early and late "hits." The fact that primary AML samples with FLT3 ITDs have comparatively greater engraftment potential in non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice than non-mutated samples suggests that the ITD does, in fact, exist in the leukaemic stem cell [72,133]. On the other hand, patients have been identified who are ITD-positive at diagnosis, but negative at relapse [134,135]. In these patients, the ITD mutation was likely not present in the leukaemic stem cell, but only in a subclone of leukaemic progenitors which had a growth advantage due to the proliferative and survival effect of the ITD. Other patients have been identified who are negative for an ITD at diagnosis, but positive at relapse, suggesting that the acquistion of new FLT3 mutations may contribute to relapse in some cases [134,135]. Enthusiasm generated by early clinical activity of targeted therapies must be tempered until the durability of these responses can be demonstrated, and this durability will likely depend on the presence and activity of the target in the cancer stem cell.

Despite these challenges, targeted therapies hold tremendous promise for improving the treatment of many forms of cancer, both in adults and children. FLT3 inhibitors provide an excellent example of how the development of targeted therapies can follow in a logical manner from discoveries in the laboratory. Optimisation of these therapies will require well-designed clinical trials and effective translation of the lessons learned from the bedside to the bench and back to the bedside (Fig. 3).

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Note added in Proof

Two articles have been published since this review was submitted which have added significantly to our understanding of the role of FLT3 in pediatric leukemia. In one study, it was noted that in a microarray gene expression analysis of childhood ALL, cases with hyperdiploidy (modal number of chromosomes greater than 50) had relatively high expression of FLT3 [137]. FLT3 genotype analysis of these cases revealed FLT3 mutations in 24% (6 of 25). Three were D835 or I386 substitutions in the kinase domain (point mutations), and three were novel in-frame deletions in the juxtamembrane domain. Another study found D835/I386 mutations in 4 of 19 (21%) cases of childhood hyperdiploid ALL [138]. Thus, it appears that another group of patients who may benefit from therapy with FLT3 inhibitor is children with hyperdiploid ALL.

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