Flt3 Does Not Play a Critical Role in Murine Myeloid Leukemias Induced by MLL Fusion Genes

Stéphanie Albouhair#, Ester Morgado, Catherine Lavau*#b
Centre National de la Recherche Scientifique, UMR7151, Paris, France

Abstract

Leukemias harboring MLL translocations are frequent in children and adults, and respond poorly to therapies. The receptor tyrosine kinase Flt3 is highly expressed in these leukemias. In vitro studies have shown that pediatric MLL-rearranged ALL cells are sensitive to Flt3 inhibitors and clinical trials are ongoing to measure their therapeutic efficacy. We sought to determine the contribution of Flt3 in the pathogenesis of MLL-rearranged leukemias using a myeloid leukemia mouse model. Bone marrow from Flt3 null mice transduced with MLL-ENL or MLL-CBP was transplanted into host mice and Flt3−/− leukemias were compared to their Flt3 wild type counterparts. Flt3 deficiency did not delay disease onset and had minimal impact on leukemia characteristics. To determine the anti-leukemic effect of Flt3 inhibition we studied the sensitivity of MLL-ENL leukemia cells to the Flt3 inhibitor PKC412 ex vivo. As previously reported for human MLL-rearranged leukemias, murine MLL-ENL leukemia cells with higher Flt3 levels were more sensitive to the cytotoxicity of PKC412. Interestingly, Flt3 deficient leukemia samples also displayed some sensitivity to PKC412. Our findings demonstrate that myeloid leukemias induced by MLL-rearranged genes are not dependent upon Flt3 signaling. They also highlight the discrepancy between the sensitivity of cells to Flt3 inhibition in vitro and the lack of contribution of Flt3 to the pathogenesis of MLL-rearranged leukemias in vivo.

Introduction

Chromosomal translocations involving the Mixed Lineage Leukemia (MLL) gene on chromosome 11q23 are present in approximately 10% of acute leukemias (myeloid- AML or lymphoblastic - ALL). These MLL-rearranged leukemias generally have a poor prognosis and a high rate of relapse following therapy. MLL translocations are particularly prevalent in infant leukemias and in secondary chemotherapy-related leukemias. In these two situations the disease latency is strikingly brief; the average age for diagnosis of infant leukemias is approximately 6 months and secondary leukemias can be detected 26 months after beginning of chemotherapy. This rapid disease onset suggests that MLL fusion genes are powerful oncogenes that, given the role of MLL in chromatin structure, could have pleiotropic effects and alter both signaling and differentiation pathways by having a global impact on gene expression [1]. This hypothesis finds support from gene expression profiling analyses that identified a gene signature distinguishing MLL-rearranged acute leukemias from other ALLs or AMLs [2–5]. This MLL-rearranged leukemia signature includes the HOX1 cluster genes and the HOX cofactor MEIS1 gene which are direct transcriptional targets of MLL [6]. Another gene specifically upregulated in MLL-rearranged leukemias that has been proposed to play a critical role in their pathogenesis is the gene encoding Fms-like tyrosine kinase 3 (Flt3) [3,7–9]. Excessive expression of Flt3 in MLL-rearranged leukemias results from direct transcriptional activation by HOX9A and MEIS1 [10] and post-transcriptional regulation by miR-150, a microRNA whose level is repressed by MLL fusion genes [11]. Flt3 is normally expressed in immature hematopoietic progenitors and, upon activation by its ligand, triggers signal transduction pathways that stimulate cell survival and proliferation. The upregulation of Flt3 and autocrine or paracrine activation by its ligand can lead to excessive Flt3 signaling in leukemia [12].

Mutations that constitutively activate Flt3, either internal tandem duplication in the juxtamembrane region or point mutations in the kinase domain, are detected in approximately one third of AMLs and confer a poor prognosis. Several small-molecule inhibitors have been developed to target Flt3 signaling and are being evaluated in clinical trials to treat AML patients [13]. The selective upregulation of Flt3 in MLL-rearranged ALLs raised the possibility that Flt3 inhibitors could also be beneficial in the treatment of these aggressive leukemias [2]. Two studies have shown that cultured leukemia cells from pediatric MLL-rearranged ALLs are more sensitive to the cytotoxicity of Flt3 inhibitors, PKC412 or CEP-701, than ALL cells with germline MLL [14,15] expressing lower levels of Flt3. These results confirmed previous work showing the sensitivity of MLL-rearranged cell lines to PKC412 [7]. Clinical trials of Flt3
inhibitors, PKC412 and lestaurtinib, to treat pediatric MLL-rearranged ALL are ongoing [16].

Marine models to determine the role of Flt3 in the pathogenesis of MLL-rearranged leukemias have shown contradictory results. Using Flt3 knock out mice, we previously found that leukemias induced by the retroviral transduction of Hoxa9 and Meis1, which recapitulate many features of MLL-rearranged myeloid leukemias, are Flt3 independent [17]. In contrast, a recent study showed that shRNA-mediated knock-down of Flt3 increases the latency of MLL-AF9-induced leukemias in transplanted mice [11], supporting the notion that Flt3 is a worthy drug target. This could, however, not be validated in an earlier study showing that therapeutic inhibition of Flt3 by PKC412 does not reduce tumor burden of MLL-AF9 leukemia in mice [18]. Here, we examine the role of Flt3 in the pathogenesis of MLL-rearranged myeloid leukemias by transducing two MLL fusion genes into Flt3-deficient bone marrow cells. We show that Flt3-null cells can be transformed by MLL-ENL or MLL-CBP in vitro. Moreover, following transplanation into recipient mice, Flt3-null cells induce similar leukemias to their Flt3 wild type counterparts, providing evidence that Flt3 is dispensable to the genesis of leukemias induced by MLL-ENL or MLL-CBP. To explore whether Flt3 signaling in leukemia cells is important ex vivo, we studied the sensitivity of cultured primary MLL-ENL leukemia blasts to the FLT3 inhibitor PKC412. As was reported for human MLL-rearranged blasts, leukemia cells expressing higher levels of Flt3 are more sensitive to the cytotoxicity of PKC412. We also find that PKC412’s cytotoxicity involves off target activity. Altogether, our findings suggest that Flt3 requirements may differ for survival of MLL-rearranged leukemia cells in vitro and leukaemogenesis in vivo.

Materials and Methods

Ethics Statement

Mouse care and experimental procedure were performed in accordance with the animal care and use committee of the Institut Universitaire d’Hématologie and approved protocols from the Comité Régional d’Éthique en Experimenation Animale n°4.

Mice and DNA Constructs

Homozygous Flt3<sup>−/−</sup> mice were kindly provided by Ihor Lemischka (Princeton University) and control wild-type (Flt3<sup>+/+</sup>) 129/Sv mice were purchased from Charles River (Arbresle, France). MSCV-MLL-ENL, MSCV-MLL-CBP, MIE, MIE-MLL-ENL, and MIE-MLL-CBP constructs have been described previously [19,20].

Retroviral Transduction of Bone Marrow Progenitors and Transformation Assay in vitro

Production of retroviral supernatants and transduction of bone marrow (BM) Lin<sup>−</sup> progenitors were performed as previously described [21].

Transplantation of Mice and Characterization of Leukemias

Eight to twelve-week-old lethally irradiated (2 split doses of 500 rad or a single dose of 900 rad) 129/Sv mice were transplanted by tail vein injection of 60–80×10<sup>5</sup> infected progenitors with a radioprotective dose of 10<sup>7</sup> syngenic BM cells. Blood cell counts were performed with a Cell-Dyn 3700 counter (Abbott Diagnostic, Rungis, France). Mice with signs of disease were euthanized and their tissues (spleen, liver, muscle and kidneys) were harvested for histological analyses. BM cytospins were stained with Wright-Giemsa for morphological analyses. BM, peripheral blood and spleen cell suspensions were blocked with rat IgG (10 µg/ml) prior to staining with fluorochrome-conjugated monoclonal antibodies specific for Mac-1, Gr-1, F4/80 or c-Kit (purchased from BD Biosciences or eBioscience) and analyzed by flow cytometry. Flt3 expression was analyzed following preincubation with the blocking anti CD16/CD32 antibody (eBioscience). Dead cells were excluded by propidium iodide staining. Fluorescence was analyzed on a LSR II (BD bioscience, San Jose, CA), using CellQuest software (BD bioscience).

Transplantability of tumors was assessed by tail vein injection of 10<sup>5</sup> nucleated BM cells from a leukemic mouse into 2–4 non-irradiated immunodeficient Rag2<sup>−/−</sup> recipient mice.

Protein Expression Analyses

Whole cell lysates were prepared from 5×10<sup>5</sup> BM cells for Western blot analyses. A 1:5000 dilution of anti-FLT3 SC-340 (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody, followed by rabbit peroxidase-conjugated secondary antibody and visualization using the enhanced chemiluminescence (ECL) reagent (Amersham). Hybridization with the rabbit polyclonal antibody to actin 20–33 (Sigma-Aldrich) was used as loading control.

Cytotoxicity Assays of Primary Cells

PKC412 (Novartis, Basel, Switzerland) was dissolved in DMSO to make initial stock solutions and then diluted in media to obtain desired final concentrations. DMSO frozen BM cells harvested from secondary or tertiary transplanted MLL-ENL leukemic mice were used to study the sensitivity of primary leukemia samples to PKC412. These BM samples consistently contained more than 95% percent of GFP-expressing leukemic blasts. Thawed cells were seeded at high density (8×10<sup>5</sup> cells/mL) and cultured for 48 h in RPMI 1640 medium with 20% FCS and supplements (50 U/mL penicillin G, 50 mg/mL streptomycin, 2 mM L-glutamine and 0.05 mM 2-mercaptoethanol), without recombinant growth factors. Cells were then seeded in 96 well plates at a density of 6–8×10<sup>3</sup> cells/mL with various concentrations of PKC412 in duplicates or triplicates wells. After 3 days, viable cell numbers

Figure 1. MLL fusion genes transform BM progenitors independently of Flt3 in vitro. Myeloid colony forming assays were performed with Flt3<sup>+/+</sup> and Flt3<sup>−/−</sup> progenitors infected by empty MIE vector, MLL-ENL or MLL-CBP-encoding vectors. Numbers of colonies are shown for 10<sup>5</sup> (first passage colonies) or 10<sup>6</sup> (second to fourth passage colonies) seeded cells. Results shown are the mean ± SEM of 3 (MLL-CBP and MIE) or 5 (MLL-ENL) independent experiments.

doi:10.1371/journal.pone.0072261.g001
were determined by scoring trypan blue negative cells with a hemacytometer. Cell viability was reported as percentage of untreated cells. Samples for which the number of viable cells in the untreated wells dropped by more than 70% over the 3-day culture were not analyzed. The cytotoxicity response of primary leukemic cells was also evaluated using a XTT assay according to the manufacturer’s instruction (Roche).

Statistical Analyses

Mice survival curves were compared using the log-rank test. Features of leukemias from the different cohorts were compared using the unpaired t test. Comparisons between untreated and PKC412-treated blasts were done using the paired t test. All statistical tests were performed with Prism 4.0 (GraphPad).

Results

Flt3 is Dispensable for the Transforming Properties of MLL-ENL or MLL-CBP in vitro

To determine whether Flt3 expression is important for the acquisition of transformed properties by primary hematopoietic cells transduced with MLL fusion genes, we used a myeloid progenitor replating assay [23] and compared the number of colonies generated from Flt3+/+ or Flt3−/− BM cells infected with MLL-ENL or MLL-CBP. Flt3 null mice have reduced BM pro-B cells and hematopoietic stem cells but they are healthy and display normal populations of mature hematopoietic cells [22]. In three independent experiments we found that Flt3 genotype did not affect the ability of MLL-ENL or MLL-CBP to sustain the proliferation of colony forming cells (Figure 1). Moreover, MLL-ENL or MLL-CBP colonies derived from Flt3+/+ BM displayed the same morphology and size as their Flt3−/− counterpart (data not shown). Cells pooled from Flt3−/− or Flt3+/+ MLL-ENL tertiary colonies could be transferred into liquid cultures and grown for more than 6 months with cytokines, indicating that Flt3 was not required for cell immortalization.

Flt3 Is Dispensable to the Induction of Leukemias by MLL Fusion Genes

To determine whether Flt3 is required for the onset of leukemias induced by MLL fusion genes, BM progenitors from Flt3−/− or Flt3+/+ mice were transduced with MLL-ENL, or by an empty MIE (MSCV-IRES-eGFP) retroviral vector and injected into lethally irradiated Flt3−/− recipient mice. Mice transplanted with Flt3−/− or Flt3+/+ MIE-transduced BM remained healthy and displayed similar proportions of GFP-expressing peripheral blood leukocytes, indicating that Flt3 deficiency did not impair BM engraftment or hematopoietic repopulating potential (Figure 2A). Mice transplanted with MLL-ENL-transduced Flt3−/− or Flt3+/+ progenitors all died or became moribund and had to be sacrificed within 60 days. The survival curves of both cohorts (n = 9) were very similar (Figure 2B) indicating that Flt3 did not affect leukemia latency. Moreover, considerable heterogeneity in Flt3 protein levels was seen in BM blasts collected at death from Flt3+/+ MLL-ENL mice, and there was no correlation between Flt3 levels and disease latency (Figure 2C and data not shown). The hematological parameters of all the mice were carefully examined and are summarized in Table 1. All mice exhibited features of AMLs as previously described [23,24]. These included a marked increase in number of peripheral white blood cells (WBC), splenomegaly, thrombocytopenia, invasion of BM by leukemia cells and infiltration of peripheral organs such as liver, muscle and kidneys. The only feature that distinguished Flt3−/− and Flt3+/+ MLL-ENL leukemia mice was a reduction in leukocytosis in the absence of Flt3 (Table 1). The myeloid nature of the leukemias was verified by morphology assessment of peripheral blood (PB) smears and BM cytopsins (data not shown). It was confirmed by flow cytometry detection of myeloid markers (Mac-1, Gr-1 and F4/80) on PB and BM blasts (Figure 2D, E and F and data not shown). All these characteristics were consistent with acute monocyctic or myelo-monocytic leukemia and were unaffected by Flt3 genotype. Of note, infiltration of peripheral organs by blasts was very similar in Flt3−/− and Flt3+/+ MLL-ENL leukemias (spine weights in Table 1 and data not shown), indicating that Flt3 signaling is not required for migration of leukemia blasts in vivo.

To examine if Flt3 was important for leukemia transplantability, we studied the ability of Flt3+/+ and Flt3−/− MLL-ENL leukemias to engraft into secondary recipients. BM cells from at least 5 independent primary leukemic mice of each cohort were transplanted into immunodeficient Rag2−/− recipient mice (2 to 4 recipients per donor). All the leukemias were transplantable and induced similar disease regardless of their Flt3 status (Figure 2G, right panel). This provides additional evidence that leukemic blasts are not dependent on Flt3 signaling for tumor expansion and that in addition to being dispensable for MLL-ENL leukemia initiation, Flt3 appears to be dispensable for leukemia maintenance.

Overall these findings demonstrate that the pathogenesis of MLL-ENL acute myeloid leukemias does not require Flt3 signaling.

Table 1. Features of AMLs induced by MLL-ENL or MLL-CBP transduction of Flt3+/+ or Flt3−/− BM cells.

|                | Survival (days) | WBC (x10⁶/mL) | Platelets (x10⁶/mL) | Spleen weight (mg) |
|----------------|-----------------|---------------|---------------------|--------------------|
| Flt3+/+ MLL-ENL| 53±6            | 261±99        | 187±63              | 393±115            |
| Flt3−/− MLL-ENL| 56±5            | 71±52         | 235±211             | 472±129            |
| Flt3+/+ MLL-CBP| 115±36          | 133±104       | 277±87              | 375±184            |
| Flt3−/− MLL-CBP| 116±33          | 89±111        | 212±142             | 376±220            |

Values shown are mean ± SD measured for 9 to 10 mice per cohort, WBC, white blood cells.

*The WBC counts of Flt3+/+ and Flt3−/− MLL-ENL mice were significantly different (p = 0.0004, unpaired t test).

doi:10.1371/journal.pone.0072261.t001

To examine if Flt3 was important for leukemia transplantability, we studied the ability of Flt3+/+ and Flt3−/− MLL-ENL leukemias to engraft into secondary recipients. BM cells from at least 5 independent primary leukemic mice of each cohort were transplanted into immunodeficient Rag2−/− recipient mice (2 to 4 recipients per donor). All the leukemias were transplantable and induced similar disease regardless of their Flt3 status (Figure 2G, right panel). This provides additional evidence that leukemic blasts are not dependent on Flt3 signaling for tumor expansion and that in addition to being dispensable for MLL-ENL leukemia initiation, Flt3 appears to be dispensable for leukemia maintenance.

Overall these findings demonstrate that the pathogenesis of MLL-ENL acute myeloid leukemias does not require Flt3 signaling.
Figure 2. The onset and characteristics of MLL-ENL and MLL-CBP leukemias are not influenced by Flt3 expression. A: Engraftment of empty vector MIE-transplanted mice measured as a percentage of GFP-expressing peripheral blood white blood cells (WBC) detected by flow cytometry. Results of 5 separate mice, transplanted with Flt3+/+ or Flt3−/− MIE-transduced BM, analyzed at either 104 (diamonds) or 111 (triangles) days post-transplantation. The percentage of GFP expression in the MIE-transduced Flt3+/+ or Flt3−/− BM at the time of transplantation was between
conditional Mll-Cbp knockin model showed that Mll-Cbp could induce myeloid hyperplasia but its ability to initiate leukemogenesis required cooperating mutations [25]. This contrasts with Mll-Enl that rapidly induced myeloid leukemias in a chromosomal translocation model [24]. Moreover, we previously found using the retroviral transduction/transplantation model that Mll-Cbp required a longer latency to induce AML leukemias compared to Mll-Enl [19]. We reasoned that under these circumstances, a lack of Flt3 signaling might be more consequential and delay the onset or alter the phenotype of Mll-Cbp leukemias.

In brief, we found that the latency, phenotype, and transplantability of Mll-Cbp leukemias were essentially unaffected by the absence of Flt3 (Figure 2, Table 1 and data not shown). The only characteristic correlating with Flt3 deficiency was an increase in monocyteic markers; Flt3−/− Mll-Cbp Bmi displayed a higher percentage of Mac-1 and F4/80 expressing blasts compared to their Flt3+/− counterparts (n = 7–8 mice per genotype). However, expression of these monocyteic markers was highly variable and a significant difference was only seen for Mac-1 expression.

Of note, Mll-Cbp and Mll-Enl leukemias are distinct clinical entities. The median survival of Mll-Cbp mice (106 or 116 days) is two-fold longer than that of Mll-Enl mice (53 or 56 days). Mll-Cbp and Mll-Enl leukemias have distinguishable immunophenotypes with a higher percentage of Mll-Enl blasts expressing monocyteic (Mac-1 and F4/80) and granulocytic (Gr-1) markers compared to Mll-Cbp blasts (data not shown and Figure 2D–F). Finally, in contrast to Mll-Enl leukemia blasts that proliferate robustly ex vivo in the presence of growth factors, blasts from Mll-Cbp leukemias generally display very limited expansion in culture.

Murine Mll-Enl Myeloid Leukemia Cells are Sensitive to FLT3 Inhibition in vitro

To study the sensitivity of primary Mll-Enl blasts to the small-molecule Flt3 inhibitor PKC412, we determined the number of viable cells following incubation for 3 days with varying drug concentrations. The sensitivity of cells from distinct Mll-Enl leukemic mice was variable but overall PKC412 reduced viability in a dose-dependent manner with IC50 for most samples ranging from 100 to 700 nM (Figure 3A). In agreement with the heterogeneous levels of Flt3 protein seen by immunoblotting (Figure 2), the percentage of Flt3-expressing cells measured by flow cytometry was variable among the leukemia samples. Flt3 levels did not influence the innate ability of the cells to survive/proliferate ex vivo as all samples displayed a similar moderate degree of expansion (approximately 1.5-fold increase in the number of viable cells during the 3-day culture period, Figure 3B).

Incubation with PKC412 (500 nM) induced a significant reduction in cell survival with approximately half of the viable cells recovered (Figure 3B). Interestingly, we observed that samples containing a high percentage of Flt3 expressing cells were more sensitive to PKC412 (compare samples #243 and #12, with respectively 94% and 38% of cells expressing Flt3, to samples #94, #35 and #12 with less than 3% of cells expressing Flt3; Figure 3A). Furthermore, in samples initially containing a high percentage of Flt3-expressing blasts, exposure to PKC412 caused an important reduction of the Flt3-positive subset (Figure 3C) suggesting a differential cytotoxicity of the drug towards Flt3-expressing cells. PKC412 exposure induced myeloid maturation in most samples, which could be observed by flow cytometry and morphological analyses (Figure 3D). Overall, these findings suggest that Flt3 targeting by PKC412 is detrimental to cell survival ex vivo.

Flt3−/− Mll-Enl Leukemic Cells are Sensitive to FLT3-inhibitors in vitro

To explore whether the toxicity of PKC412 towards Mll-Enl leukemia cells was solely mediated by Flt3 inhibition, we studied the sensitivity of Flt3−/− Mll-Enl leukemias. Flt3 deficient cells displayed a dose-response to PKC412 with IC50 ranging from 400 to 5000 nM (Figure 4A); this range was similar to the IC50 measured for Mll-Enl Flt3+/− cells expressing low levels of Flt3 (samples #94, #35, and #842 in Figure 3A). Again we found that exposure of Flt3−/− blasts to PKC412 induced myeloid maturation visible by a shift in Mac-1 and Gr-1 expression (Figure 4C) and changes in morphology (data not shown).

Discussion

Our objective was to determine the role of Flt3 in the pathogenesis of Mll-rearranged leukemias and to use a murine leukemia model to study the sensitivity of blasts to FLT3 inhibition ex vivo. Using genetically modified mice lacking Flt3 as a source of hematopoietic precursors, we found that Flt3 signaling was dispensable for in vitro and in vivo oncogenic transformation by Mll-Enl or Mll-Cbp. With either fusion genes, leukemia onset and progression, infiltration of peripheral organs or leukemia transplantability were unchanged in the absence of Flt3. The presence of Flt3 did have a notable effect on leukocyteosis with a considerable reduction in circulating blasts in Flt3−/− Mll-Enl leukemias compared to their Flt3+/− counterparts. This did not seem to be caused by a lesser proliferation rate of Flt3−/− Mll-Enl blasts as it was not accompanied by reduced leukemia infiltration of organs or longer disease latency, and the proliferation potential of the Flt3−/− and Flt3+/− Mll-Enl blasts was comparable upon ex vivo culture (Figures 3B and 4B).

The lower number of circulating blasts may instead be connected with Flt3’s known role in controlling migration of hematopoietic precursors from the BM to the peripheral blood [26]. This property could
also explain the effectiveness of FLT3 inhibitors in lowering peripheral blood blasts seen in clinical trials [13].

Interestingly a spontaneous reduction of Flt3 expression upon transplantation of leukemia cells to secondary recipient mice was seen with two independent primary *MLL-ENL* leukemias.
This indicates that sustained Flt3 expression does not confer a selective advantage to expanding leukemic clones in vivo. It also indicates that in addition to being dispensable for the initiation of myeloid leukemias, Flt3 is dispensable for leukemia maintenance. This contrasts with a report showing that shRNA-mediated knock down of Flt3 significantly, albeit moderately, delayed the onset of MLL-AF9 AMLs in transplanted mice [11]. The reasons for this discrepancy are unclear but we cannot rule out differences in Flt3 requirement between MLL-AF9 and MLL-ENL or MLL-CBP leukemias.

The sensitivity of primary human MLL-rearranged ALL samples to FLT3 inhibitors was shown to be proportional to the level of FLT3 expression [14,15]. In line with this, our data with murine MLL-ENL AMLs also show that PKC412's cytotoxicity is more pronounced toward samples expressing higher levels of Flt3. This argues that PKC412's cytotoxicity is mediated by Flt3 inhibition and that the Flt3 pathway contributes to cell survival in culture. However, this is at odds with previously published in vivo studies of FLT3 inhibitors performed with either xenotransplants of human cell lines [7] or transplantation of retrovirally transduced mouse BM [18]. In these investigations the therapeutic efficacy of PKC412 was restricted to MLL-rearranged leukemia cells carrying either FLT3 amplifications or FLT3 activating mutations; whereas no therapeutic benefit could be observed in cells in which FLT3 was unaltered [7,18]. This suggests that in contrast to FLT3 activating mutations or amplifications, FLT3 signaling resulting from overexpression of wild type FLT3 may not cause a proliferative advantage to MLL-rearranged leukemias in vivo; this is consistent with our findings that Flt3 deficiency does not delay MLL-rearranged leukemias. These findings highlight a disparity between the absence of Flt3 dependency of MLL-rearranged leukemias in vivo and their sensitivity to FLT3 inhibition ex vivo.

Figure 4. Flt3−/− MLL-ENL leukemias are sensitive to PKC412. A: Cytotoxic dose response of BM blasts from five Flt3−/− MLL-ENL leukemic mice. Viable cells were counted after 3 days of culture in presence of increasing concentrations of PKC412 and shown as percentage of untreated control cells. Data points represent means of duplicate or triplicate wells, ±/− SD. B: Fold expansion of viable cells during 3 days of culture (number of live cells at the end of culture divided by number of cells initially seeded) either in absence or presence of 500 nM PKC412. Mean ±/− SD of results from 6 Flt3−/− MLL-ENL leukemic mice. C: Flow cytometry analysis of Mac-1 and Gr-1 expression after 3 days of culture of samples #54 and #762 either without or with PKC412 (700 nM). The percentages of cells in the quadrants are indicated. Similar induction of maturation was seen in blasts from 5 different Flt3−/− MLL-ENL leukemic mice.

doi:10.1371/journal.pone.0072261.g004

(Figure 2C). This indicates that sustained Flt3 expression does not confer a selective advantage to expanding leukemic clones in vivo. It also indicates that in addition to being dispensable for the initiation of myeloid leukemias, Flt3 is dispensable for leukemia maintenance. This contrasts with a report showing that shRNA-mediated knock down of Flt3 significantly, albeit moderately, delayed the onset of MLL-AF9 AMLs in transplanted mice [11]. The reasons for this discrepancy are unclear but we cannot rule out differences in Flt3 requirement between MLL-AF9 and MLL-ENL or MLL-CBP leukemias.
Two explanations can be proposed to address this paradox. First, primary leukemia cells adopt very different biological properties when they are cultured ex vivo, notably their growth slows down considerably. Hence Flt3 signaling might be important for cell survival ex vivo while being dispensable to the proliferation of leukemia cells in vivo, conceivably because of abundant stimuli in the bone marrow environment. Second, PKC412 while being more active towards cells expressing high levels of Flt3, also exerts its cell toxicity through the combined inhibition of other molecular targets. Like most small molecule kinase inhibitors that target the ATP binding site, PKC412 inhibits multiple kinases other than Flt3-deficient cells is evidence of PKC412’s off-target activity. This ability to inhibit multiple targets may play a considerable role in the anti-leukemic activity of Flt3 inhibitors. This is supported by studies of primary human MLL-rearranged leukemia cells in which PKC412 doses required for repression of cell growth were considerably higher than those sufficient to fully inhibit Flt3 phosphorylation [15]. Another report comparing several Flt3 inhibitors on primary AML samples also concluded that less selective compounds were most effective at inducing cytotoxicity [27]. Conversely, highly specific strategies like siRNA or inhibitory monoclonal antibodies directed against particular kinases might be less efficient. The discrepancy between our in vitro and our in vivo findings highlight the need to incorporate several experimental models in preclinical evaluation of novel drugs. In vitro assays are inherently more artefactual. For instance we observed that increasing fetal bovine serum content or adding cytokines to the culture media greatly influenced the cells’ sensitivity to PKC412 (data not shown). The murine in vivo assay also has caveats in recapitulating the human MLL-rearranged leukemias. Although we showed that Flt3 was dispensable for the generation of leukemias induced by two distinct MLL fusion genes, both diseases were myeloid malignancies and we cannot rule out that Flt3 may be important in the pathogenesis of MLL-rearranged lymphoid malignancies. However, identification of a common gene expression signature of MLL-rearranged leukemias regardless of lineage [4,20] suggests that mechanisms underlying MLL fusion gene-induced ALLs and AMLs are, to some extent, conserved. The ultimate evaluation of the therapeutic benefit of Flt3 inhibitors against MLL-rearranged leukemias awaits results of clinical trials in patients. Their analysis should also determine whether Flt3 inhibition per se or off-target activity mediates the efficiency of these compounds.

Acknowledgments

We are very grateful to Doro Lemischka for the Flt3−/− mouse and to Toshio Kitamura for the Plat-E cells. We also thank Marika Pla for mouse husbandry and for Rag2−/− mice, Novartis for the PKC412 and Marcelle Lavau, Olivier Bernard and Dan Wechsler for critically reading the manuscript.

Author Contributions

Conceived and designed the experiments: SA CL. Performed the experiments: SA EM CL. Analyzed the data: SA EM CL. Wrote the paper: SA CL.

References

1. Eguchi M, Eguchi-Ishimae M, Greaves M (2005) The role of the MLL gene in infant leukemia. Int J Hematol 78: 390–401.
2. Armstrong SA, Staunton JE, Silverman LB, Pieters R, den Boer ML, et al. (2002) MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet 30: 41–47.
3. Kohlmann A, Schoch C, Dugas M, Schnitter S, Hiddemann W, et al. (2005) New insights into MLL gene rearranged acute leukemias using gene expression profiling: shared pathways, lineage commitment, and partner genes. Leukemia 19: 953–964.
4. Ross ME, Mahfouz R, Onciu M, Liu HC, Zhou X, et al. (2004) FLT3 ligand inhibitors on primary AML samples also concluded that less selective compounds were most effective at inducing cytotoxicity [27].
5. Zheng R, Levis M, Piloto O, Brown P, Baldwin BR, et al. (2004) FLT3 ligand activity mediates the efficiency of these compounds.

---

11. Jiang X, Huang H, Li Z, Li Y, Wang X, et al. (2012) Blockade of miR-150 associated genes. Mol Cell Biol 26: 3902–3916.
12. Kindler T, Lipka DB, Fischer T (2010) FLT3 as a therapeutic target in AML: still challenging after all these years. Blood 116: 5089–5102.
13. Brown P, Levis M, Shurtleff S, Campana D, Downie J, et al. (2005) Flt3 inhibition selectively kills childhood acute lymphoblastic leukemia cells with high levels of Flt3 expression. Blood 105: 812–820.
14. Forster A, Pannell R, Drynan LF, McCormack M, Collins EC, et al. (2003) Targeted disruption of the flk2/flt3 gene leads to deficiencies in primitive hematopoiesis. Proc Natl Acad Sci U S A 100: 7853–7858.
15. Mackarehtschian K, Hardin JD, Moore KA, Boast S, Goff SP, et al. (1995) Targeting paediatric acute lymphoblastic leukaemia: novel therapies currently in development. Br J Haematol 131: 295–311.
16. Lee-Sherick AB, Linger RM, Gore L, Keating AK, Graham DK (2010) Conditional MLL-CBP targets GMP and models therapy-related myeloproliferative disease. Embo J 24: 368–381.
17. Morgado E, Allouihair S, Lavau C (2007) Flt3 is dispensable to the Hoxa9/myb leukemia. Cancer Cell 22: 524–535.
18. Lee-Sherrick AB, Linger RM, Gore L, Keating AK, Graham DK (2010) Targeting paediatric acute lymphoblastic leukemia: novel therapies currently in development. Br J Haematol 151: 295–311.
19. Mohri M, Takahashi T, Motegi Y, Amano T, Takahashi T, et al. (2003) Flt3 is dispensable in vivo in mice. Blood 88: 2004–2012.
20. Stubb MC, Kan YM, Kasperaviciute A, Wright RD, Feng Z, et al. (2008) MLL-AF9 and FLT3 interaction in acute myelogenous leukemia: development of a model for rapid therapeutic assessment. Leukemia 22: 66–77.
21. Lee S, Tsukada R, Briskin M, Ganser A, Yee LM, et al. (2005) Transmuting potential of the T-cell acute lymphoblastic leukemia-associated homeobox gene HOXA13, TLX1, and TLX3. Genes Chromosomes Cancer 45: 846–855.