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Ponatinib suppresses the development of myeloid and lymphoid malignancies associated with FGFR1 abnormalities

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Abstract

Myeloid and lymphoid malignancies associated with FGFR1 abnormalities are characterized by constitutive activated FGFR1 kinase and rapid transformation to acute myeloid leukemia and lymphoblastic lymphoma. Molecular targeted therapies have not been widely used for SCLL. Ponatinib (AP24534), that potently inhibits native and mutant BCR-ABL, also targets the fibroblast growth factor receptor (FGFR) family. Using murine BaF3 cells stably transformed with six different FGFR1 fusion genes, as well as human KG1 cells expressing activated chimeric FGFR1 and five newly established murine SCLL cell lines, we show that Ponatinib (< 50 nM) can effectively inhibit phosphoactivation of the fusion kinases and their downstream effectors, such as PLCγ, Stat5 and Src. Ponatinib also significantly extended survival of mice transplanted with different SCLL cell lines. Ponatinib administered at 30 mg/kg daily also significantly delayed, or even prevented, tumorigenesis of KG1 cells in xenotransplanted mice. Furthermore, we demonstrate that Ponatinib specifically inhibits cell growth and clonogenicity of normal human CD34+ progenitor cells transformed by chimeric FGFR1 fusion kinases. Overall, our data provide convincing evidence to suggest that pharmacologic inhibition of FGFR1 fusion kinases with Ponatinib is likely to be beneficial for patients with SCLL and perhaps for other human disorders associated with dysregulated FGFR1 activity.

Keywords

myeloproliferative disease; ponatinib; FGFR1; preclinical trial

Introduction

Stem cell leukemia/lymphoma (SCLL), also known as the 8p11 myeloproliferative syndrome (EMS)[1], has now been designated ‘myeloid and lymphoid neoplasms associated

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Conflict of interest

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with FGFR1 abnormalities (MLNAF)' in the recent WHO classification[2]. MLNAF is characterized by reciprocal chromosome translocations that fuse the FGFR1 kinase domain to a variety of fusion partner genes. These partners provide dimerization domains that facilitate constitutive and ligand-independent activation of the FGFR1 kinase, which leads to a variety of downstream signaling effects involving classical FGFR1 targets such as PLCγ, members of the Stat family of transcription factors[3–6] and Src[7]. Currently eleven FGFR1-associated fusion partners have been described, and the most common rearrangement in MLNAF involves a t(8;13)(p11;q11) translocation which fuses ZMYM2 (previously known as ZNF198) with FGFR1 (see review by[8]). We have developed a transduction/transplantation animal model for ZMYM2-FGFR1 which develops myeloproliferative disorder and T-cell lymphoma[9]. The T-cell lymphomas are CD4+/CD8+, have lost T-cell receptor alpha (Tcra) function and show upregulation of anti-apoptotic genes such as Bcl2 and Il2r[9]. In addition, activated mutation of Notch1 was observed in all T-cell lymphomas induced by ZMYM2-FGFR[10]. These observations could explain why the majority of ZMYM2-FGFR1 MLNAF patients develop T-lymphomas[1, 11].

MLNAF is an aggressive disease, usually presenting with lymphoblastic lymphoma in association with a myeloproliferative disorder, accompanied with/without peripheral eosinophilia. The disorder generally progresses to overt acute myelogenous leukemia within a year of diagnosis[12–14]. The overall mortality rate for MLNAF is >80%[15], which is significantly higher than in other myeloproliferative disorders, due largely to its aggressive nature and resistance to the current chemotherapy regimens used, which are mostly modified from regimens used for other leukemias/lymphomas[15]. Even allogenic bone marrow transplantation has only a limited associated survival. The molecular characterization of the various aspects of MLNAF, however, offers new targets and we have shown in our *in vivo* animal studies that targeting Notch with gamma secretase inhibitors, and Src with Dasatinib, has significant efficacy[7, 10]. The consistent feature of all of the variant fusion kinases, however, is the activation of the FGFR1 kinase, which provides an opportunity to use inhibitors of this function to treat MLNAF.

FGFR1 belongs to a large group of protein tyrosine kinases that play crucial roles in controlling cell growth, differentiation and survival, among other functions[16]. There have been two reports describing targeting FGFR1 in MLNAF using either PKC412[4] or TKI258 [17]. PKC412 (Midostaurin), a multiple serine/threonine and tyrosine kinases inhibitor, was shown to have efficacy in the treatment of one MLNAF patient carrying the ZMYM2-FGFR1 fusion gene[4]. However, it appears that this compound lacks specificity for FGFR activity at the 500 nM (IC_{50} dose) used[18]. TKI258 (Dovitinib) was shown to specifically inhibit proliferation and survival of the KG1 and KG1A cell lines carrying the FGFR1OP2-FGFR1 chimeric kinase, as well as primary cells from 4 MLNAF patients associated with different FGFR1 rearrangements[17]. Recently, Ponatinib (AP24534), a potent, orally active inhibitor of Bcr-Abl kinase and its mutants, was also shown to be effective against FGFR tyrosine kinase activity at nanomolar concentrations[19], although not specifically in the context of MLNAF rearrangements. Ponatinib is currently being investigated in a phase II clinical trial for patients with CML (http://clinicaltrials.gov NCT01207440). Here we show that Ponatinib effectively inhibited the activation of several

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different FGFR1 fusion kinases and their downstream effectors, resulting in cell growth inhibition and apoptotic death. In these studies, Ponatinib was more effective than TKI258 in inhibiting in vitro growth of the human MLNAF KG-1 cells. Importantly, Ponatinib treatment resulted in statistically significant prolonged survival in ZMYM2-FGFR1 and CEP110-FGFR1 models of MLNAF in syngeneic transplantation mouse models. Ponatinib was also effective against human KG1 cells in an immunocompromized murine xenotransplantation model. These data indicate that Ponatinib may be effective in the treatment of neoplasms associated with chimeric FGFR1 kinases, and perhaps for other human disorders associated with deregulated FGFR1 activity.

Materials and methods

Inhibitors

Ponatinib was obtained from Ariad Pharmaceuticals, Inc.; PD173074 was obtained from Cayman Chemical; TKI258 (dovitinib) and PKC412 (midostaurin)) were purchased from LC laboratories. All inhibitors were dissolved in DMSO and stored at −80°C before use.

Stable transformation of BaF3 cells

Cells from the BaF3 murine pro-B cell line were stably infected with ZMYM2-FGFR1, BCR-FGFR1, CEP110-FGFR1 or the control MIEG3 vector as described previously[7]. Using the same protocol we also established BaF3 cells stably expressing CUX1-FGFR1 (a kind gift from Dr. Els Lierman, Department of human genetics, KU Leuven, Leuven, Belgium) and FGFROP2-FGFR1 which was cloned from human KG1 cells. The FOP1-FGFR1 fusion gene was synthesized from its individual component parts and fused using a 6 bp linker following PCR amplification. All transformed BaF3 cells co-express GFP and show IL3 growth independence.

Cell culture and proliferation assays

All cell lines were cultured in RPMI (Invitrogen) with 5% FBS (Hyclone), at 37°C in 10% CO_2. For drug treatments, 40,000 cells/well were seeded in 96-well plates and incubated overnight, then treated with the either DMSO (control) or the drugs indicated in the results section at concentrations defined by the experiments. Cell viability was determined using Cell Titer-Glo luminescence cell viability kits (Promega) and a SpectraMaxR M5e (Molecular Probe) luminescence plate reader.

Cell apoptosis assay and cell cycle analysis

For analysis of apoptosis, cells were stained with Annexin V and 7-AAD (BD Biosciences) following the manufacturer's protocol. Cell cycle analysis was performed using standard Flow cytometry procedures following either propidium iodide staining alone, or together with BrdU incorporation. These cells were then stained with anti-BrdU-APC (eBioscience), as described previously [20].

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Flow cytometry analysis

Spleen tissues were passed through a cell strainer (BD, Bedford, MA) to generate single cell suspensions. Bone marrow cells were isolated by flushing mouse femurs with PBS and stained using specific conjugated monoclonal antibodies (Table S1). For intracellular staining of phospho-Stat5, cells were fixed and permeabilized before reacting with a phospho-Stat5 (Y694) antibody (BD Biosciences) according to standard procedures. Following staining, cells were washed once in staining medium and then analyzed using an LSR II cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR).

Immunoprecipitation and western blot analyses

Proteins were isolated as described previously[10]. Whole-cell lysates (50 μg) were separated using SDS-PAGE and immunoblotted with specific antibodies (details available on request). To detect phospho-FGFR1, 500 μg of each whole-cell lysate was first immunoprecipitated with anti-FGFR1 antibody (Santa Cruze Biotechnology) and followed by Western blot with anti-phosphotyrosine antibody (PY20, Sigma).

Molecular analyses

Total RNA was isolated using Trizol (Invitrogen) and retro-transcribed with a QuantiTect reverse transcription kit (Qiagen). Amplification was performed using standard PCR (New England Bio.) in combination with specific primers and conditions which are available on request.

Animals and treatment schedule

In the first experiment, female Balb/c mice (Harlan 6–8 weeks old) were injected with ZNF112 or CEP2A cells at 2×10^6 per mouse through the tail vein. One week after injection, mice were randomized to treatment with vehicle or Ponatinib (20 mg/kg/day). Ponatinib was dissolved in 25 mM citrate buffer (pH 2.75) according to the manufacturer's suggestion, and given orally using a gavage needle twice daily. The control group of mice was given an equal volume of the citrate buffer by gavage. All treatments were performed 6 days per week for 4 weeks. In a second experiment, 5×10^6 of KG1 cells/mouse were injected intravenously into female NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, Jackson Laboratory). One-week post transplantation these mice were randomly divided into treatment (Ponatinib, n = 9) and control (n = 10) groups. The mice in the Ponatinib group were first treated with 40 mg/kg for 6 days, and following one-week recovery without drug treatment the mice were then switched to 30 mg/kg treatment with a regimen of 4-days-on and 3-days-off for an additional 4 weeks. The control mice were treated as described above. All animal experiments were carried out under protocols approved by the Institutional Animal Care and Use Committee of the Georgia Health Sciences University.

Histopathology

Tissues were fixed in 10% neutral buffered formalin for at least 48 hours. The sternum bones were decalcified in 6% formic acid for 2–3 days, and then embedded in paraffin. Standard H&E staining was performed using 3 μm sections.

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Human CD34+ cord blood cell infection and methylcellulose culture

Human cord blood cells were obtained from the Cord Blood Bank of Georgia Health Sciences University under approved IRB protocols. CD34+ cells were isolated using the EasySep Cord Blood CD34 Positive Selection Kit (StemCell Technologies) following the manufacturer's protocol, and expanded in StemSpan SFEM medium (StemCell) supplemented with recombinant human cytokines: LDL 10ug/ml, Flt-3 100ng/ml, SCF 100ng/ml, TPO 50ng/ml, Il-3 20ng/ml and IL6 20ng/ml (R & D Systems). After 24h pre-stimulation, CD34+ cells were infected with either BCR-FGFR1, CEP110-FGFR1 or the control MIEG3 vector as described previously[9]. After four rounds of infection, the infected cells were treated with either DMSO or 50 nM Ponatinib (BCR-FGFR1 infected CD34+ cells were treated with 100 nM) for 48 h in the growth medium, and then plated into methylcellulose media (MethoCult H4435, StemCell Technologies) at 1500 cell/well in 12-well plates. Each treatment was triplicated and colonies were counted and classified under a microscope at 50x magnification.

Results

To investigate whether Ponatinib inhibits proliferation in cells expressing various FGFR1-chimeric kinases *in vitro*, we infected BaF3 cells with retroviral constructs and selected pools of IL3 independent cells. This assay has been used extensively to establish whether exogenous genes have oncogenic abilities. Cells transformed with human ZMYM2-FGFR1, BCR-FGFR1, CEP110-FGFR1, FGFR1OP2-FGFR1, FOP-FGFR1 and CUX1-FGFR1, grew rapidly and demonstrated auto activation of the various fusion kinases[7].

Ponatinib inhibits chimeric FGFR1 transformed cell growth at nanomolar concentrations

Using the Cell Titre-Glo assay, Ponatinib was shown to proportionally reduce cell growth at various IC_{50} values, ranging from 20 to 50 nM in BaF3 cells expressing the various fusion kinases. Cells infected with the empty MIEG3 vector and maintained on IL3, were not affected by Ponatinib up to 100 nM (Figure 1). Thus, Ponatinib clearly has a profound effect on the ability of the FGFR1 fusion kinases to promote cell growth in this model system.

Previously we developed a series of cell lines from primary MLNAF for three of the variant fusion kinases. ZNF112 expresses the ZMYM-FGFR1[10], BBC1 and BBC2 express BCR-FGFR1[21] and CEP2A and CEP5A carry CEP110-FGFR1[7]. ZNF112, CEP2A and CEP5A were derived from T-cell lymphomas, while BBC1 and BBC2 were derived from B-cell lymphomas. Ponatinib also inhibits proliferation of these murine MLNAF cell lines (Figure 1), where IC_{50} values ranged between 10–20 nM for ZNF112, CEP2A and CEP5A and 20–50 nM for BBC1/2. These data demonstrate that Ponatinib is also effective against cells derived from the primary MLNAF disease.

Ponatinib induces cell cycle arrest, apoptosis and death associated with a decrease of chimeric FGFR1 phosphorylation

When Ponatinib was used to treat BaF3 cell pools stably expressing ZMYM2-FGFR1 and CEP110-FGFR1 (50 nM) or BCR-FGFR1 (100 nM) for 48 h, reduced phosphorylation FGFR1 levels was seen (Figure 2A). The percentage of cells in S-phase was also

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dramatically decreased, while the percentage of apoptotic cells was increased in the three different chimeric kinase-transformed BaF3 cells (Figure 2B) which suggests that their survival depends on activated FGFR1. When four murine MLNAF cell lines were treated with 50 nM Ponatinib for 48 h, similar results were seen, except for BBC2 cells, which required 100 nM Ponatinib. The effects on S-phase and apoptosis correlated with decreased levels of phosphoactivation of the chimeric kinases in all MLNAF cell lines (Figures 2C and 2D).

Ponatinib inhibited downstream molecular components of chimeric FGFR1

We next analyzed known consequences of FGFR1 activation following Ponatinib treatment of the derivative BaF3 and MLNAF cell lines. PLCγ is a well-known downstream target of FGFR1[22] and we have previously demonstrated that constitutive expression of FGFR1 chimeric kinases increases Src activation[7]. In this analysis we also compared the effects of Ponatinib inhibition of FGFR1 with those of Dasatinib inhibition of Src. In transformed BaF3 cells, Ponatinib showed significant effects on PLCγ activation, whereas Dasatinib generally did not (Figure 3). Both Dasatinib and Ponatinib treatment, however, led to an almost complete loss of Src activation, which is consistent with a direct effect of Dasatinib on Src activation, as well as Src being a downstream consequence of FGFR1 activation[7]. Phosphoactivated Stat5 is increased in FGFR1 chimeric kinase expressing cells[3] and when the murine MLNAF cell lines were treated Ponatinib pStat5 levels were also decreased. In the MLNAF cell lines, Ponatinib remarkably reduced pPLCγ and pSrc levels in all cases. Similarly, pStat5 levels were beyond the limits of detection in all but BBC2 (Figure 3).

Ponatinib significantly reduces leukemogenesis in vivo

The *in vitro* studies described above suggested that Ponatinib is potentially an effective single agent against chimeric FGFR1 expressing cells. To determine whether the same was true *in vivo*, we grafted ZNF112 and CEP2A cells into syngeneic mice which we have previously shown results in MLNAF[7] (and unpublished observations). Control animals were treated with vehicle, and the experimental mice were treated with 20 mg/kg Ponatinib for 4 weeks following the regimen described in the Methods. The mean survival time in the control groups of ZNF112 and CEP2A mice was 28 and 35 days, respectively (Figures 4A and C). In Ponatinib treated ZNF112 cohorts survival averaged 76 days ($p = 0.0001$) and mice engrafted with CEP2A survived on average 62 days ($p = 0.006$). Three mice in both groups were still alive after 160 days when the experiment was terminated. Consistently, the mean spleen weight in the Ponatinib treated group was significantly ($p < 0.05$) less than in the vehicle treated group in both ZNF112 and CEP2A transplanted mouse models (Figure 4B and D).

Ponatinib induces growth inhibition and apoptosis in human KG1 cells

To date, there have been no reported human cell lines carrying the common FGFR1 chimeric kinases, although the KG1 cell line, originally described as being derived from an AML patient[23], expresses the rare FGFR1OP2-FGFR1 variant. KG1 cells show constitutive activation of the chimeric FGFR1 protein[10], suggesting that the original patient probably suffered from MLNAF. Cell proliferation assays demonstrated an IC_{50} of ~20 nM for KG1

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cells (Figure 5A), which is similar to that seen for all of the murine MLNAF cell lines analyzed, with the exception of those expressing BCR-FGFR1 (Figure 1). Consistent with this observation, Ponatinib also decreased the phosphorylation levels of FGFR1OP2-FGFR1, and its downstream effectors, at a low 25 nM dose (Figure 5B). By comparison, although another FGFR1 inhibitor, TKI258, was active against the chimeric FGFR1 kinase in KG1 cells, a ~10-fold higher concentration was required to achieve the same effect (Figure 5B). To further compare the relative efficacy between TKI258 and Ponatinib, we used different doses of the two drugs to treat KG1 cells *in vitro* for 24 h and observed that both drugs reduced FGFR1OP2-FGFR1 phosphorylation levels in a dose-dependent manner, as well as the phosphoactivation of its downstream effectors, such as pPLCγ, and pSrc (Figure 5B). Using quantitative Phosphoflow analysis (Figure 5C), 25 nM Ponatinib treatment resulted in a marked reduction in pStat5 activation, whereas the same response required 250 nM of TKI258. This 10-fold difference in active concentration was also reflected in the proportional decrease in activation of the downstream targets pPLCγ and pSrc (Figure 5B).

To determine the consequences of inactivating FGFR1 function using both kinase inhibitors, we demonstrated that 14.1% of cells exhibited apoptosis after treatment with 25 nM Ponatinib over a 72 hour period, whereas 500 nM TKI258 was required to achieve the same response (Figure 5D). While this level of apoptosis is relatively low, it is three times that seen in cells treated with the vehicle alone. Cell cycle analysis demonstrated that after 72 h treatment with 25 nM Ponatinib, 88% of KG1 cells were in G_0/G_1. Treatment with 250 nM TKI258 resulted in 72% of cells arrested in G_0/G_1, even though TKI258 at this concentration does not induce significant apoptosis in KG1 cells (Figure 5E). As shown in Figure 5B, however, 250 nM TKI258 did not completely prevent activation of the chimeric FGFR1, PLCγ or pSrc. It seems, therefore, that residual, low levels of FGFR1 kinase activity can protect the cells from undergoing apoptosis.

Ponatinib significantly prolongs survival of mice xenotransplanted with KG1 cells

Ponatinib treatment can significantly prolong survival in the murine MLNAF model described above, although it is important to determine whether human cells are similarly affected *in vivo*. We, therefore, investigated the anti-tumorigenic activity of Ponatinib using KG1 cells in a xenotransplant mouse model. Previous studies of KG1 cells have predominantly used subcutaneous xenografts[24–26], so we first investigated whether we could establish an orthotopic xenograft model that is more representative of leukemia. We therefore injected 3 female NSG mice intravenously with 5×10^6 KG1 cells. After 40–60 days all these animals succumbed to the disease, demonstrating hypercellularity in the bone marrow, infiltrates into the lungs and kidneys (Figure 6C) as well as intra-abdominal tumors (where > 90% of cells were positive for human CD45, data not shown). The spleens, however, were only slightly enlarged. To enrich for cells that were more prone to homing to the bone marrow microenvironment, we recovered the KG1 cells from the BM of these mice and expanded them *in vitro*. The immunophenotype of these recovered KG1 cells are identical to the parental cells, and protein expression level of FGFR1OP2-FGFR1 was also the same (data not shown). To determine the effect of Ponatinib on the disease produced by these reselected cells, we injected 5×10^6/mouse intravenously into NSG mice. One-week

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post transplantation the mice were randomly divided into treatment and control groups. The treatment group (n = 9) were originally orally dosed with 40 mg/kg per day, but after only one week of treatment they exhibited an obvious decrease in body weight. We therefore suspended the treatment for one week and then on resumption of the treatment, reduced the dose to 30 mg/kg following an interrupted regimen of 4-days-on and 3-days-off for an additional 4 weeks. In this study, the mice treated with 30 mg/kg Ponatinib showed a significantly longer survival time than mice from the control group. Significantly, 44% of the treated mice are still alive after 4-months post-transplantation (Figure 6A). Consistent with this observation, flow cytometry analysis, using anti-human CD45 antibodies, demonstrated that there were remarkably fewer KG1 cells in the bone marrows and spleens from the Ponatinib treated mice compared to the control mice (Figure 6B). Furthermore, histopathological analysis showed that mice in the control group showed hypercellularity in bone marrow and extensive infiltration of KG1 cells into the lungs and livers. In contrast, in the Ponatinib treated mice, a normal cytoarchitecture of these organs was observed (Figure 6C). These experiments, therefore, demonstrate that treatment with Ponatinib alone can significantly reduce leukemogenesis both in the murine MLNAF model as well as in human KG1 xenotransplanted mice, further indicating a potential chemotherapeutic role for Ponatinib in MLNAF patients.

Ponatinib specifically inhibits cell growth and clonogenicity of normal human CD34+ progenitor cells transformed by chimeric FGFR1 fusion kinases

The studies presented above demonstrate that Ponatinib is effective in treating cells expressing FGFR1 fusion kinases in MLNAF models. Testing its efficacy in the human disease, however, is difficult because of the rarity of MLNAF. To overcome this challenge, we sorted normal human cord blood CD34+ progenitor cells and infected them with retroviral supernatant carrying either CEP110-FGFR1, BCR-FGFR1 or control MIEG3 vector. After four rounds of infection, the average infection efficiency was shown to be >60% as determined by flow cytometry analysis of GFP+ cells (Figure 7A). To determine whether Ponatinib was effective against these human cells, we compared transformed CD34+ cells with those carrying the empty vector following treatment for 48h. The concentration of Ponatinib used in these experiments were based on the BaF3 cell model system described above. Flow cytometry analyses demonstrated that 50 nM Ponatinib did not change the percentage of GFP+ cells in empty vector infected cells (Figure 7A), but significantly ($p < 0.01$) decreased the GFP+ population cells in the CD34+ cells transformed with either CEP110-FGFR1 or BCR-FGFR1 (Figure 7A). In addition, Ponatinib treatment induced increased apoptosis in the FGFR1 fusion gene transformed CD34+ cells compared with the MIEG3 infected cells (Figure 7B). Together these experiments demonstrated that human FGFR1 fusion kinase-transformed cells are sensitive to Ponatinib. To determine whether Ponatinib could specifically block differentiation of CD34+ progenitor cells, we seeded transformed or MIEG3 infected cells into semi-solid methycellulose medium. The cells were pre-treated with Ponatinib or DMSO for 48 h, and cultured with the full complement of growth factors (see Methods) for 9 days. The specific types of colonies (erythroid, granular cell, myeloid cell or granulocyte/macrophage) and numbers of colonies were classified and counted under a microscope. Since in this analysis, Ponatinib did not have any effect on the types of colonies formed, all lineage-specific colonies were pooled as

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shown in Figure 7C. Ponatinib significantly reduced the colony formation efficacy of both CEP110-FGFR1 and BCR-FGFR1 transformed CD34+ cells, but not in MIEG3 infected CD34+ cells at 50 nM concentrations. In summary, Ponatinib, by targeting FGFR1 kinase activity, specifically inhibits cell proliferation in chimeric FGFR1 transformed cells and further induced these cells to undergo apoptosis. Compared to other FGFR1 inhibitors, therefore, it appears that Ponatinib may be a more potent FGFR1 inhibitor that merits further evaluation in MLNAF patients, as well as in other FGFR1 dependent solid tumors.

Discussion

All patients reported to date with MLNAF carry a chimeric FGFR1 kinase, suggesting that constitutive activation of this kinase is the primary initiating event, although it appears that other genetic events are required for disease development[6, 9, 10]. Sustained function of the chimeric kinase, however, appears to be required to maintain cell viability, since inactivating its function with various drugs reduces cell survival. Previous studies, for example, have shown that PKC412 (midostaurin) and TKI258 (dovitinib) increases growth inhibition of FGFR1-transformed BaF3 cells and human leukemic KG1 cells[4, 17]. Proliferation of primary MLNAF cells was also inhibited by TKI258[17]. In one ZMYM2-FGFR1 patient with advanced disease, treatment with PKC412 induced a partial response, but recent data suggests that this compound lacks specificity for FGFR activity at the 500 nM (IC_{50} dose) used[18]. Despite these indications of efficacy against MLNAF, there is clearly a need to examine other compounds that can more efficiently target FGFR1 activity. Although Ponatinib, which is an orally active, multitargeted kinase inhibitor was originally developed for treatment of both refractory and mutant BCR-ABL-positive chronic myeloid leukemia (CML)[19], analysis of the spectrum of kinases that were also down regulated showed that FGFR kinases were inhibited at nanomolar concentrations[19]. Furthermore, Ponatinib effectively inhibited KG1 cell growth *in vitro*[27]. These observations prompted us to investigate whether it could be also used as a therapeutic agent to treat MLNAF. In both the human disease, and murine models of MLNAF, there is evidence that, although MPD is the common component of the syndrome, the specific leukemia/lymphoma (T-cell or B-cell) that develops depends to some extent on the specific individual variant chimeric kinase that is expressed. Some patients presented coincidently with bilineage (myeloid and lymphoid) neoplasms[15]. These observations, therefore, suggest that targeting the genetic abnormality that is common to all of the variant diseases in the syndrome would be the most successful. Indeed, we found that Ponatinib can efficiently inhibit both MLNAF lymphoid cells and human myeloid KG1 cells *in vitro* as well as *in vivo*. The effective dose of Ponatinib required in these experiments was 5–10 times lower than TKI258, which is consistent with results from Gozgit et al.[28] in BaF3 cells engineered to express constitutively activated versions of each of the 4 FGFRs. Similarly, in mouse models of MLNAF, Ponatinib (20 mg/kg) was also shown more effective than the 100 mg/kg described for PKC412[4], which is also consistent with our in vitro experiment treating ZNF112 cells with PKC412, which was 10-fold less effective in inducing the same level of cell growth inhibition seen for Ponatinib. Overall, these observations provide preclinical evidence that Ponatinib may prove more effective than other FGFR1 inhibitors as part of a treatment strategy for MLNAF.

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Despite the significant effect of Ponatinib against FGFR1 kinase function *in vitro*, *in vivo* treatment showed a limited success. At the 30 mg/Kg dose used in our experiments, it was calculated[19] that Ponatinib can reach a mean plasma level of 561 nM, 6-hours after oral administration, which is 10–20 times greater than the IC_{50} values noted in MLNAF cells *in vitro*. Despite this, only 20% of mice with xenografts of murine MLNAF survived, and 40% of mice with xenografted KG1 cells showed long term survival after Ponatinib treatment. It should be noted that this survival rate might have been prolonged if the treatment had been extended beyond 4 weeks. These studies clearly suggest that molecularly targeting a single oncogene/pathway may not be sufficient to prevent disease progression, especially if resistant clones frequently arise after long-term treatment with a single agent[29]. Previous studies[6, 9, 10] showed that leukemia/lymphoma induced by ZMYM2-FGFR1 was oligoclonal, suggesting that additional events, genetic or epigenetic, are required for development and progression of FGFR1-driven leukemogenesis. We previously demonstrated that the consistent deletion of the T-cell receptor alpha (Tcra) gene[9], as well as mutational activation of Notch1[10] are required for ZMYM2–FGFR1 driven murine T-ALL. Treatment of these T-ALL with GSIs showed some efficacy[10]. We have also shown that upregulation of Bcl2[9] and activation of PI3K are common events in MLNAF, and consequently targeting these proteins has a significant effect on cell viability *in vitro* (data not shown). It is possible, therefore, that combining drugs against all of these targets may have greater success in treatment of this disease.

During our *in vivo* experiments in the NSG mice, we observed that daily oral dosing with 40 mg/kg Ponatinib caused remarkable loss of body weight and ruffled fur in all treated mice after only one-week. After interrupting the treatment for one-week the mice recovered, and then we continued to administer the drug at 30 mg/kg using a 4-days-on/3-days-off regimen where the drug appeared to be better tolerated. O'Hare and colleagues[19] reported that Ponatinib showed a wide therapeutic range (5–50mg/kg) which was well tolerated in both SCID and Nude mouse models, although the NSG mice seemed to be more sensitive. In contrast, we did not observe side-effects of Ponatinib treatment (20 mg/kg) in Balb/c mice xenotransplanted with ZNF112 or CEP2A. This increased sensitivity in the NSG mice may be due to their extreme immunodeficiency (absence of mature T, B, and natural killer cells) compared with SCID (lack of T and B cells) and Nude mice (greatly reduced number of T cells).

In conclusion, we provide convincing evidence that Ponatinib can effectively inhibit phosphorylation levels of the various FGFR1 fusion kinases, and their downstream effectors, which subsequently led to cell growth inhibition and death. Consistent with these *in vitro* studies, short term exposure to Ponatinib can significantly extend survival of mice xenografted with different chimeric FGFR1-driven leukemias *in vivo*, as well as cell growth and colony formation efficacy of human CD34+ progenitor cells transformed with chimeric FGFR1 kinases. These results provide a proof-of-principle that molecularly targeting cells expressing chimeric FGFR1 kinases with the novel FGFR1 inhibitor Ponatinib could be a potentially successful approach to the treatment of MLNAF patients, either alone or in combination with other drugs, such as those targeting Src or the Notch1 signaling pathway[10].

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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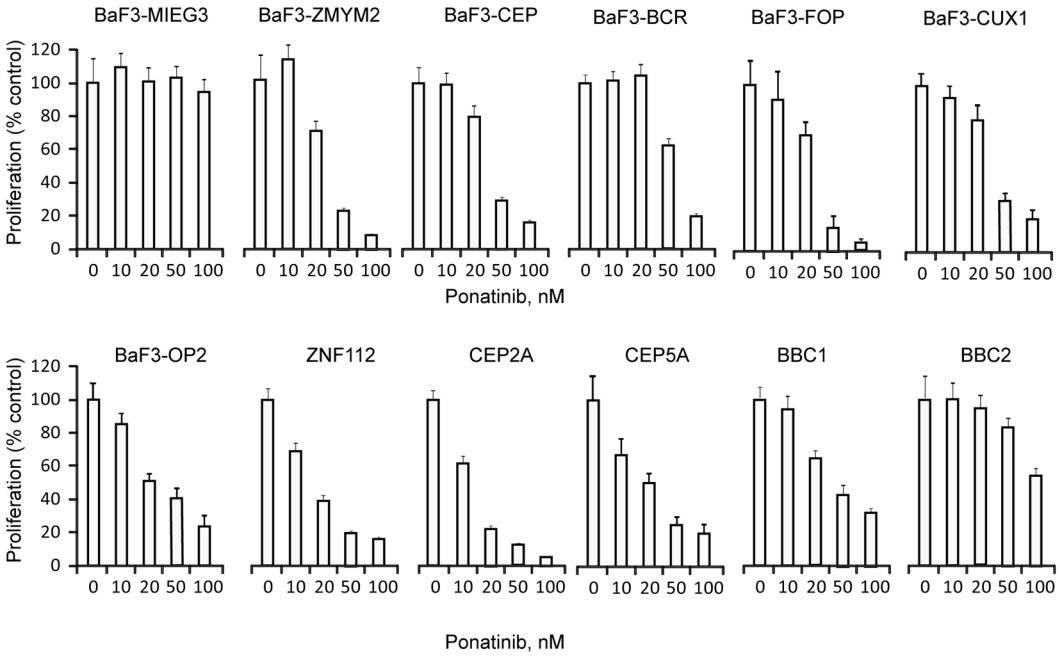

Figure 1. Ponatinib inhibits cell growth in chimeric FGFR1 transformed murine cell lines

Summary of cell growth arrest in BaF3 cells expressing the different fusion kinases as indicated, demonstrates the IC50 for Ponatinib in each case (nM). Similarly, MLNAF murine cell lines (see text) also show IC50s in the nM range. Error bars refer to SD of octuplicate analyses.

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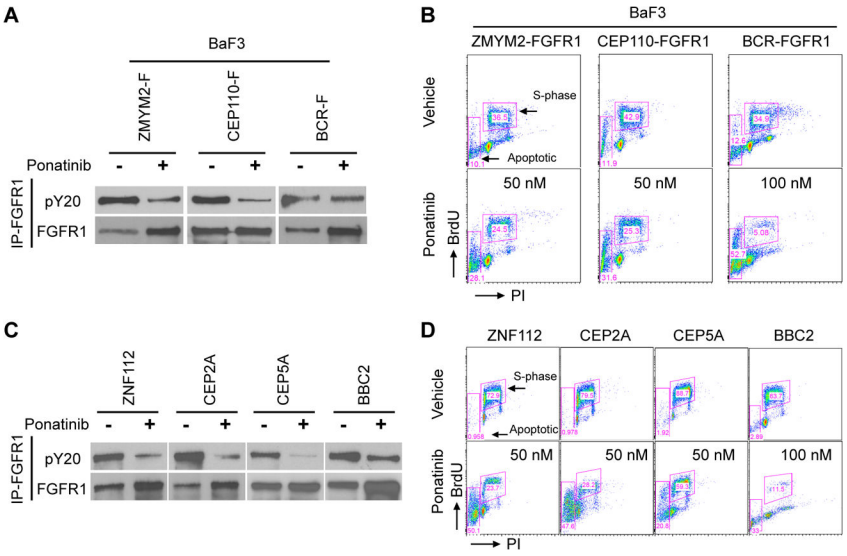

Figure 2. Downregulation of FGFR1 activation results in apoptotic cell death

(**A** and **C**) Western blot analysis shows reduced phospho-FGFR1 levels following Ponatinib treatment of BaF3 cells stably expressing three different chimeric FGFR1 kinases, as well as several MLNAF cell lines. ZNF112 expresses ZMYM2–FGFR1, CEP2A and CEP5A cells express CEP110–FGFR1 and BBC2 expresses BCR-FGFR1. (**B** and **D**) BrdU cell cycle analysis shows a remarkable decrease in the percentage of cells in S-phase and demonstrates increased rates of apoptosis (including apoptotic cell debris) following 48 hour treatment with 50 nM Ponatinib for BaF3-ZMYM2, BAF3-CEP, ZNF112, CEP2A, and CEP5A and 100 nM for BaF3-BCR and BBC2 cells. All experiments were performed at least in duplicate.

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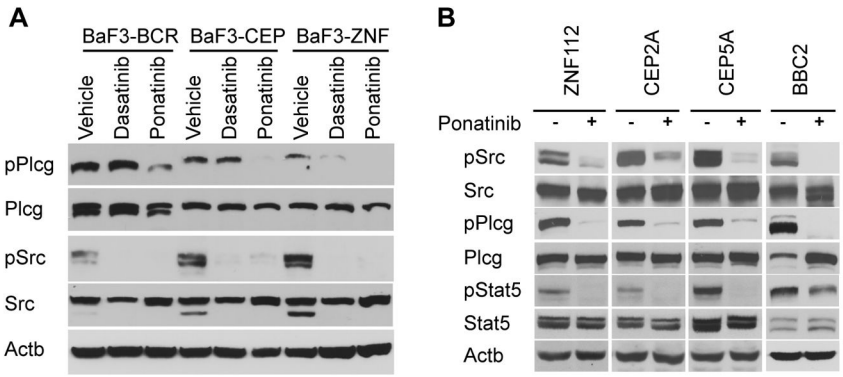

Figure 3. Ponatinib inhibits molecular signaling downstream of chimeric FGFR1

Western blot analyses show that Ponatinib remarkably inhibits phosphorylation levels of chimeric FGFR1 downstream effectors Plcg and Src in (**A**) BaF3 cells transformed by BCR-FGFR1 (BCR), CEP110-FGFR1 (CEP), or ZMYM2-FGFR1 (ZNF). The dose of Ponatinib is the same as described in Figure 2 for the respective cell lines. Dasatinib (1000 nM) was used as a positive control to demonstrate inhibition of Src activation. In (**B**) inhibition of Src, Plcg and Stat5 was seen in MLNAF cell lines treated with Ponatinib; ZNF112 expresses ZMYM2–FGFR1, CEP2A and CEP5A cells express CEP110–FGFR1 and BBC2 expresses BCR–FGFR1.

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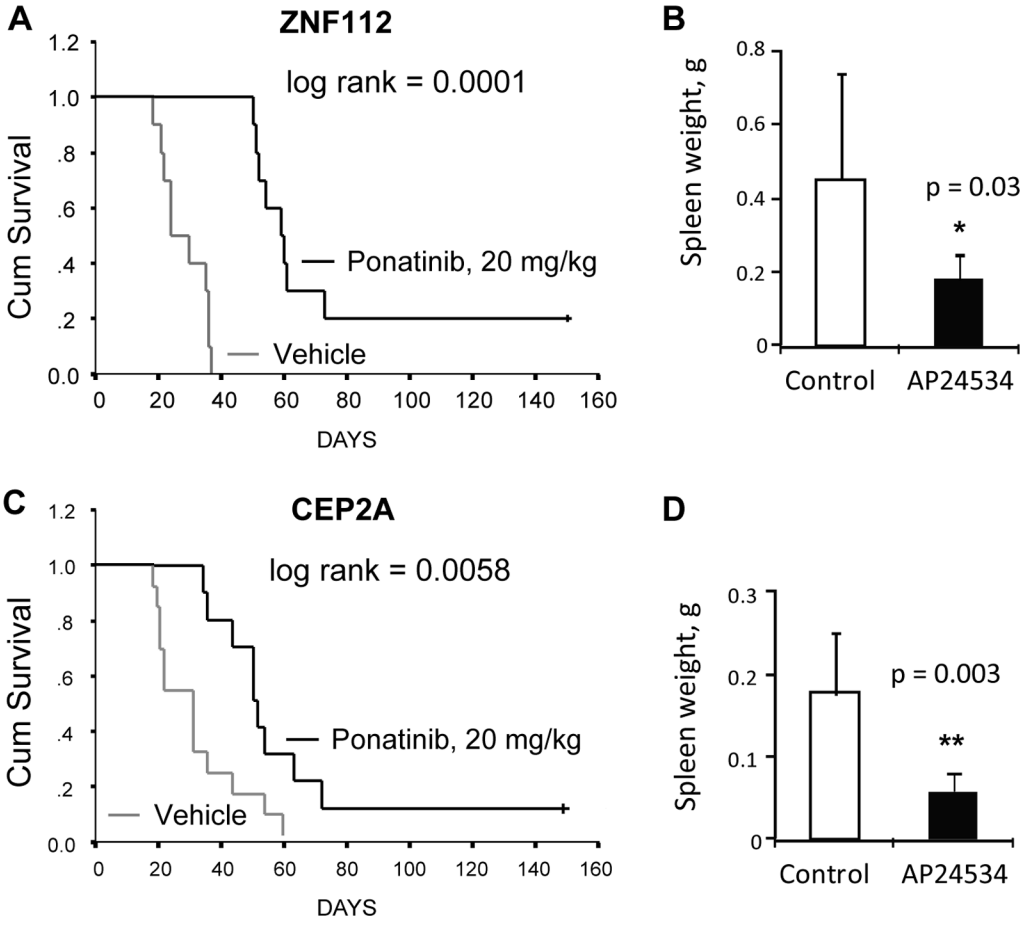

Figure 4. Ponatinib significantly suppresses leukemogenesis in vivo

Ponatinib reduces lymphomagenesis of ZNF112 and CEP2A cells. (**A** and **C**) ZNF112 or CEP2A cells when engrafted into Balb/c mice (n=10 for ZNF112 and n =13 for CEP2A) rapidly succumb when treated with the vehicle alone. When the experimental groups were treated with 20 mg/kg Ponatinib (n = 10 for each cell line), survival was significantly prolonged. (**B** and **D**) Spleen weight in the treated groups engrafted with either ZNF112 or CEP2A cells are significantly reduced compared with the vehicle treated control (* $P < 0.05$, ** $P < 0.01$).

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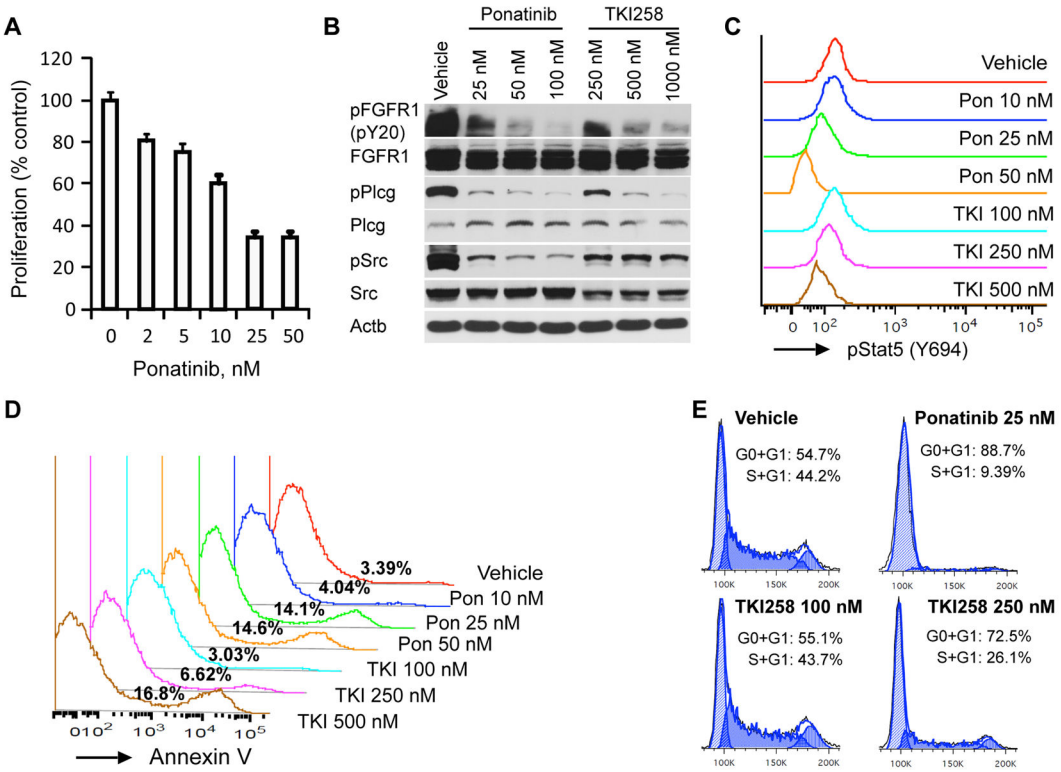

Figure 5. Ponatinib induces growth inhibition and apoptosis in human KG1 cells

A) Cell proliferation assays show that Ponatinib inhibits growth of KG1 cells over 72h at different concentrations. (**B**) Western blot analysis, using various antibodies specific for downstream targets of FGFR1, demonstrates that Ponatinib inhibits the activation of Plcg and Src as a result of inhibiting the FGFR1 fusion kinase. (**C**) Phosphoflow analysis using a phospho-Stat5 (Y694) antibody, demonstrates the relative effects of Ponatinib and TKI258 on KG1 cells. (**D**) Analysis of Annexin V positive cells (apoptosis) in KG-1 cells treated with Ponatinib (Pon) at various concentrations for 72 h, demonstrates that a 10-fold greater concentration of TKI258 (TKI) is required to produce the same effect. (**E**) Cell cycle analysis using propidium iodide staining of KG1 cells after 72 hours drug treatment shows the same enhanced effect of Ponatinib compared with TKI258 treatment for 72 h.

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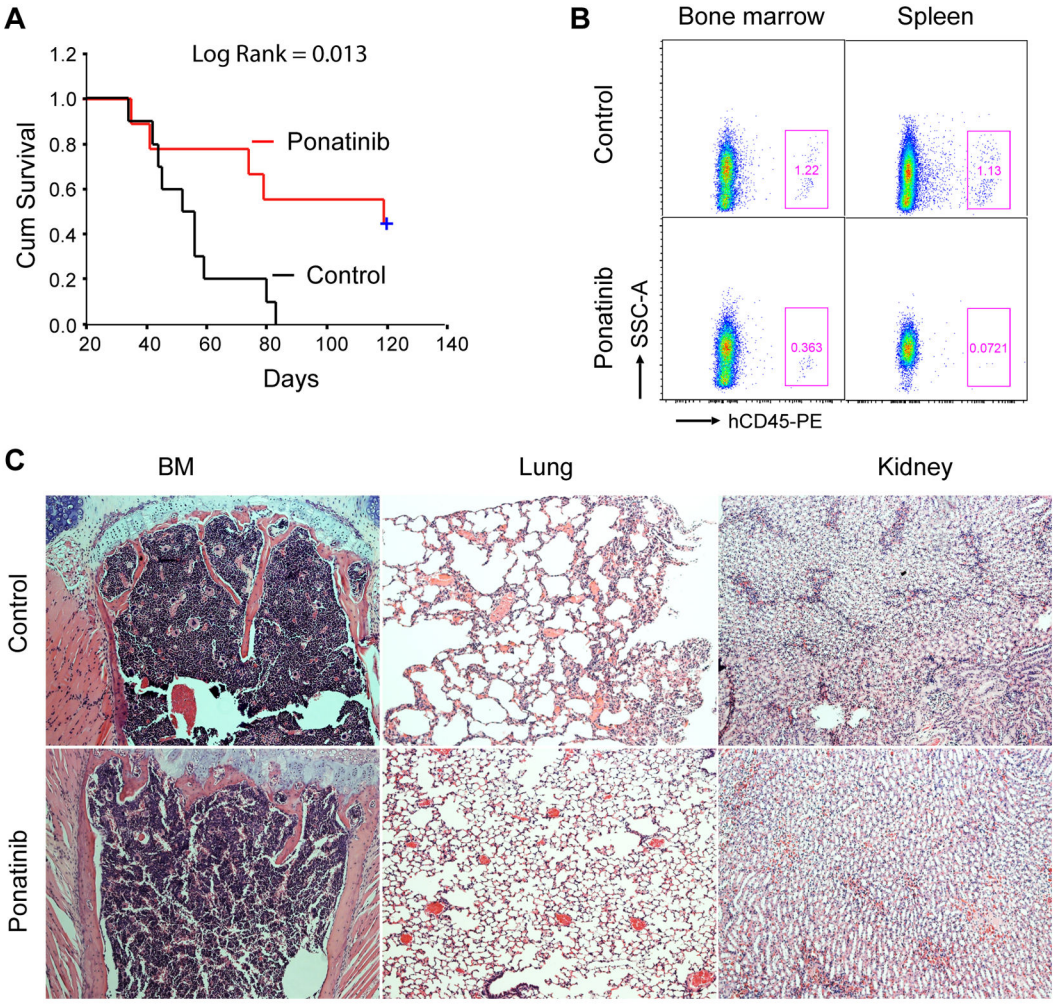

Figure 6. Ponatinib significantly prolongs survival of mice carrying human KG1 xenografts

(**A**) Kaplan-Meier survival analysis of KG1 xenotransplanted mice treated either with Ponatinib (30 mg/kg body weight) or vehicle (control). (**B**) Representative flow cytometry analysis using anti- human CD45 shows decreased CD45+ cells in the bone marrow and spleen in Ponatinib-treated mice compared with the vehicle treated cohort. (**C**) Representative H & E analysis of bone marrow (BM), lung and kidney from Ponatinib treated mice, compared with controls, shows reduced hypercellularity and infiltration respectively.

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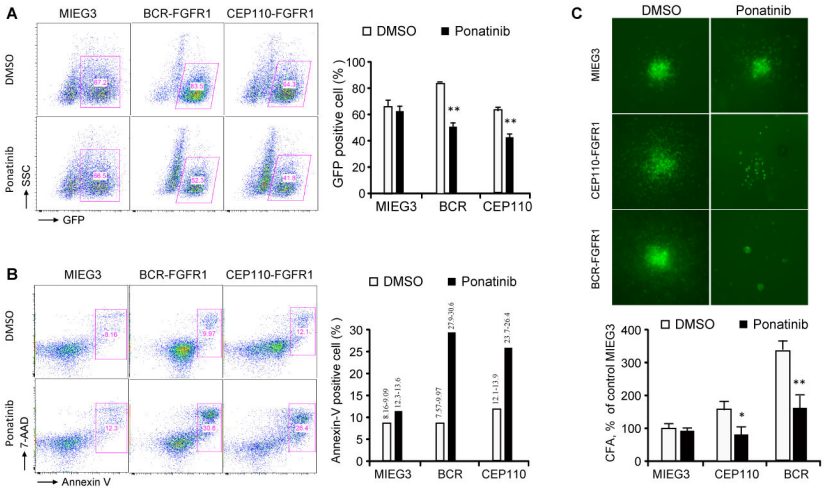

Figure 7. Ponatinib specifically inhibits cell growth and clonogenicity in normal CD34+ cord blood progenitors transformed with chimeric FGFR1 kinases

CEP110-FGFR1, BCR-FGFR1, or control MIEG3 vector transduced normal human CD34+ cord blood cells (>64% cells are GFP positive) were treated with either DMSO or Ponatinib for 48 h, followed by FACS analysis to detect the percentage of GFP positive cells (A) and to measure apoptotic cells using annexin V staining (B). Two independent experiments were performed and the ranges of percentage of annexin V positive cells in both GFP positive and negative cells are shown. (C) Representative images of colonies after 9 days in methylcellulose culture treated with vehicle or Ponatinib (see Materials and methods) are shown in the upper panel. The relative colony formation assay (CFA) is calculated by normalization to DMSO treated MIEG3 transduced CD34+ cells (lower panel). Error bars refer to SD of triplicate analyses. * $p < 0.05$ and ** $p < 0.01$.