# Ponatinib as targeted therapy for *FGFR1* fusions associated with the 8p11 myeloproliferative syndrome

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**ABSTRACT**

The 8p11 myeloproliferative syndrome is a rare, aggressive myeloproliferative neoplasm characterized by constitutively active FGFR1 fusion proteins that arise from specific chromosomal translocations and which drive aberrant proliferation. Although FGFR1 inhibitors have shown *in vitro* activity against FGFR1 fusions, none are in use clinically and there is a need to assess additional compounds as potential therapy. Here we use cell lines and primary cells to investigate ponatinib (AP24534). Ponatinib-treated Ba/F3 cells transformed by *ZMYM2-FGFR1* and *BCR-FGFR1* and the *FGFR1OP2-FGFR1* positive KG1A cell line showed reduced proliferation and decreased survival when compared to control cells. Inhibition induced apoptosis and reduced phosphorylation of the FGFR1 fusion proteins and substrates. Ponatinib-treated cells from 8p11 myeloproliferative syndrome patients (n=5) showed reduced colony growth compared to controls. In one evaluable patient, ponatinib specifically reduced numbers of *FGFR1*-fusion gene positive colonies. Ponatinib, therefore, shows considerable promise for the treatment of patients with 8p11 myeloproliferative syndrome.

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## Introduction

Myeloproliferative neoplasms (MPN) carrying fibroblast growth factor receptor-1 (*FGFR1*) fusion genes are rare stem cell disorders that are clinically characterized by eosinophilia, a poor prognosis and frequent association with T- or B-cell lymphoma. *FGFR1* is most commonly fused to *ZMYM2* (*ZNF198*) as a result of the t(8;13)(p11;q12) but rearrangements involving *BCR, FGFR1OP, FGFR1OP2, CEP110, HERV-K, TRIM24, MYO18A, CFS1, LRRFIP1* and *CUX1* have also been described, some as single cases.[1-3] These fusions are all associated with cytogenetically visible rearrangements of 8p11-12 and thus the disorder is commonly referred to as the 8p11 myeloproliferative syndrome (EMS).

Stem cell transplantation is currently the only curative treatment for EMS and although a number of compounds have been investigated for activity against FGFR1 fusion proteins,[2,4-7] none are routinely available for clinical use.

Ponatinib (AP24534) has previously been shown to have inhibitory activity towards FGFRs.[8-10] Here we confirm its activity in cell-line models and have further investigated its potential for the treatment of EMS patients using primary patient material.

## Design and Methods

### Cell line assays

The Ba/F3 cell lines transformed to interleukin-3 (IL3) independence by pcDNA3.1-*BCR-FGFR1* (Ba/F3-BF) or pcDNA3-1-*ZMYM2-FGFR1* (Ba/F3-ZF) are described elsewhere.[11,12] We focused on these two fusions as they are the ones most frequently seen in EMS patients. Cell lines were grown in RPMI-1640 plus either 10% serum (HL60, HEL and transformed Ba/F3 cell lines), 20% serum (KG1A) or 10% serum plus 10% WEHI-conditioned medium as a source of IL3 (Ba/F3-A10, a non-transformed Ba/F3 cell line containing the pcDNA3.1 vector). Ponatinib (AP24534) (supplied by Ariad Pharmaceuticals Inc., Cambridge, MA, USA) was dissolved in dimethyl sulfoxide (10 mM) and stored at -20°C in single-use aliquots.

To assess proliferative response to ponatinib, cell lines were set up at $1x10^5$/mL (Ba/F3 cell lines) or $2\text{-}3x10^5$/mL (KG1A) in 96-well plates in triplicate with ponatinib concentrations ranging from 1 nM to 1 μM with half-log increments in 100 μL volumes in RPMI-1640 plus 10% serum. CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI, USA) was used to measure the number of live cells at Day 0 and at 48 h. Absorbance was measured using an MRX Microplate Reader (Thermo Labsystems, Waltham, MA, USA). Each experiment was performed three times. IC50 values were calculated using Graphpad Prism 4 (GraphPad Software, San Diego, CA, USA).

Western blotting was performed as previously described.[4] Cells were washed to remove serum then incubated with inhibitor for 2-4 h. Antibodies were: anti-STAT5 (#9363, Cell Signaling Technology, Beverley, MA, USA), anti-phospho-STAT5 (#9359, Cell Signaling Technology, Beverley, MA, USA), anti-ERK (sc-94, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospho-ERK (sc-7383, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-FGFR1 (ab31324, Abcam, Cambridge, MA, USA) and anti-phospho-FGFR1 (#3476, Cell Signaling Technology, Beverley, MA, USA). To assess apoptosis, cells were exposed to inhibitor for 48 h. After Western blotting, the following antibodies were used: anti-cleaved caspase-3 (#9664), anti-cleaved lamin A (#2031), anti-cleaved PARP (#9544) and

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anti-GAPDH (#2118) (all from Cell Signaling Technology, Beverley, MA, USA).

### Primary patient material assays

Cryopreserved peripheral blood mononuclear cells from 5 EMS patients taken at or close to the time of diagnosis were used in *in vitro* response assays. *BCR-FGFR1* was identified in 2 cases, *ZMYM2-FGFR1* in 2 cases and *FGFR1OP-FGFR1* in one case (*Online Supplementary Table S1*). Fresh, peripheral blood samples from healthy individuals were used as controls. The study was approved by the relevant regional ethics committee and informed consent was obtained in accordance with the Declaration of Helsinki. Mononuclear cells were set up at $2x10^5$/mL in methylcellulose with cytokines but without erythropoietin (Stem Cell Technologies, Vancouver, BC, Canada) in triplicate for each inhibitor dose. Colonies more than 50 cells were counted on Day 7 and more than 100 cells were counted on Day 14. For each inhibitor concentration the relative colony number was calculated as the proportion of the cell number in the untreated cultures; an index of response was calculated as the mean of the relative colony numbers for all inhibitor concentrations at Days 7 and 14.[4]

Fluorescence *in situ* hybridization (FISH) was used to assess any differential effect of ponatinib on colony growth. After counting on Day 14, colonies were plucked into 3:1 methanol/acetic acid, stored at -20°C until required, and then pipetted onto slides. FISH was performed according to established techniques[13] except that slides were washed briefly in 70% acetic acid immediately after being made and refixed with 1% paraformaldehyde in PBS for 10 min before hybridization. A minimum of 5 cells was examined to determine the FGFR1-fusion status of each colony. Clones flanking FGFR1 (5′-*FGFR1* PAC RP1–224C10; 3′-*FGFR1* PAC RP1–162N14) were obtained from the Sanger Institute (Hinxton, UK).[14]

## Results and Discussion

The treatment of a number of malignancies in which there is aberrant activation of tyrosine kinases has been revolutionized by targeted therapy with selective kinase inhibitors, the paradigm for which is imatinib for *BCR-ABL* positive chronic myeloid leukemia. Imatinib is not active against FGFR1, and therefore a number of other compounds have been assessed experimentally for the treatment of EMS, including PKC412 (midostaurin),[2,5] SU5402,[7,12] PD1730747 and TKI258[2,4] but none of these is currently in general clinical use. Two reports describe the treatment of single patients with PKC412[5] and sorafenib[6] but in neither case was there convincing evidence of a differential effect upon levels of the *FGFR1* fusion gene. There remains, therefore, a need to investigate additional compounds for the treatment of EMS. Here we assess the

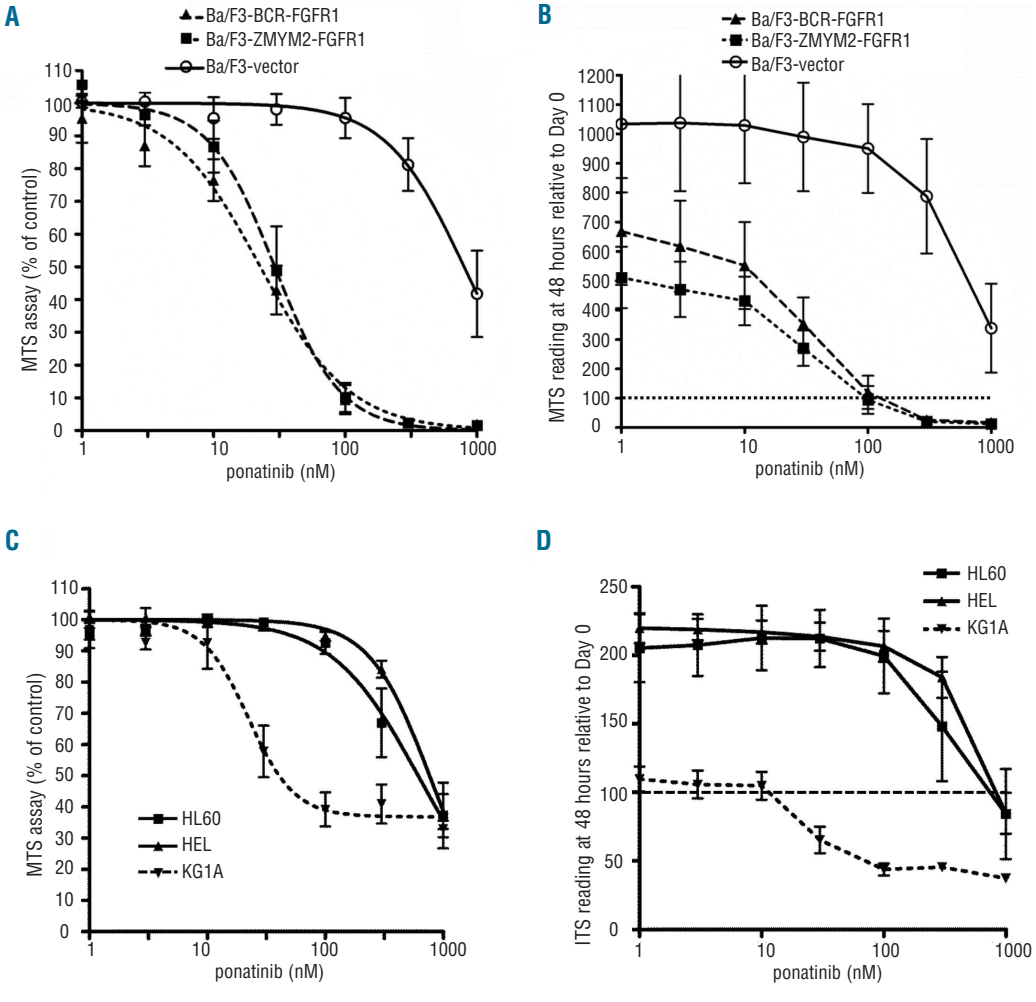


**Figure 1.** Effect of ponatinib on *FGFR1*-fusion gene transformed Ba/F3 cells and the KG1A cell line. (A) Proliferation of Ba/F3 transformed by *ZMYM2-FGFR1* or *BCR-FGFR1* at 48 h was inhibited compared to vector-control cells ($IC_{50}$= 22 nM, 29 nM and 792 nM, respectively). (B) Results expressed relative to Day 0 readings indicate a net loss of FGFR1-transformed cells (where dashed line crosses y-axis at 100%). Proliferation (C) and survival (D) of KG1A is similarly inhibited compared to HL60 and HEL ($IC_{50}$= 41 nM, 616 nM and 764 nM, respectively). All data points are means ± standard error of the mean.

potential of ponatinib for the treatment of EMS patients. Ponatinib is an orally available multi-targeted inhibitor with activity against a number of kinases including ABL, FGFR1, FLT3, KIT and PDGFRA which is currently being evaluated in clinical trials for CML and *BCR-ABL* positive acute lymphoblastic leukemia.[15]

### *Ponatinib inhibits proliferation and survival of Ba/F3 cells transformed by* FGFR1 *fusion genes and the KG1A cell line*

The Ba/F3 cell line is dependent upon IL3 for survival and proliferation but can be transformed to IL3 independence by expression of activated oncogenes such as tyrosine kinase fusion genes. Ba/F3 cells transformed by *BCR-FGFR1* (Ba/F3-BF) and *ZMYM2-FGFR1* (Ba/F3-ZF) are thus rendered IL3 independent providing a model system for assessment of FGFR1 inhibition. With ponatinib treatment, a greater dose-dependent decrease in proliferation and survival was seen in Ba/F3-BF and Ba/F3-ZF than seen with vector alone ($IC_{50}$ values = 22 nM, 29 nM and 792 nM, respectively) (Figure 1A). A greater net reduction in cell number was seen for both fusion genes compared to that of the vector control (Figure 1B) (i.e. where the MTS reading at 48 h falls below the Day 0 MTS reading). Western blotting demonstrated that increasing ponatinib concentration was accompanied by reduced phosphorylation of the FGFR1 fusion protein and downstream signaling proteins ERK and STAT5 (Figure 2A). Increased cleavage of lamin-A and PARP in the Ba/F3-BF cell line with increasing ponatinib dose (caspase-3 cleavage is not a good indicator of apoptosis in Ba/F3 cells)[16] indicated cell death by apoptosis (Figure 2B).

The KG1A cell line harbors an *FGFR1OP2-FGFR1* fusion gene[17] and thus provides another model for assessing FGFR1 inhibition. Ponatinib reduced both proliferation and survival of KG1A compared with the *FGFR1*-fusion

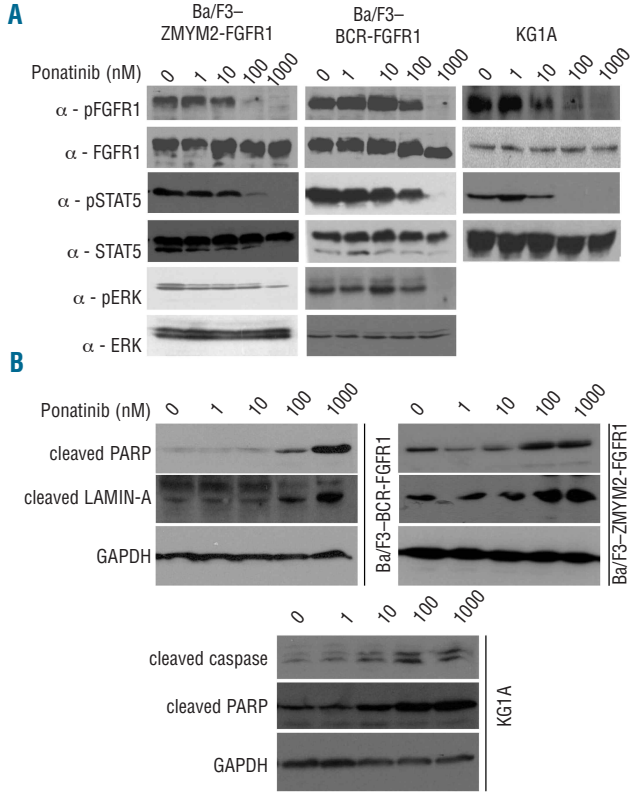


**Figure 2.** Ponatinib treatment results in a dose-dependent inhibition of phosphorylation and apoptosis. Ba/F3-ZF, Ba/F3-BF and KG1A were exposed to ponatinib for 48 h followed by Western blotting using the indicated antibodies (A). *BCR-FGFR1* and *ZMYM2-FGFR1* transformed Ba/F3 cells and KG1A cells were exposed to ponatinib for 48 h followed by Western blot using antibodies for cleaved caspase 3, cleaved PARP or cleaved lamin-A. GAPDH was used as a loading control.

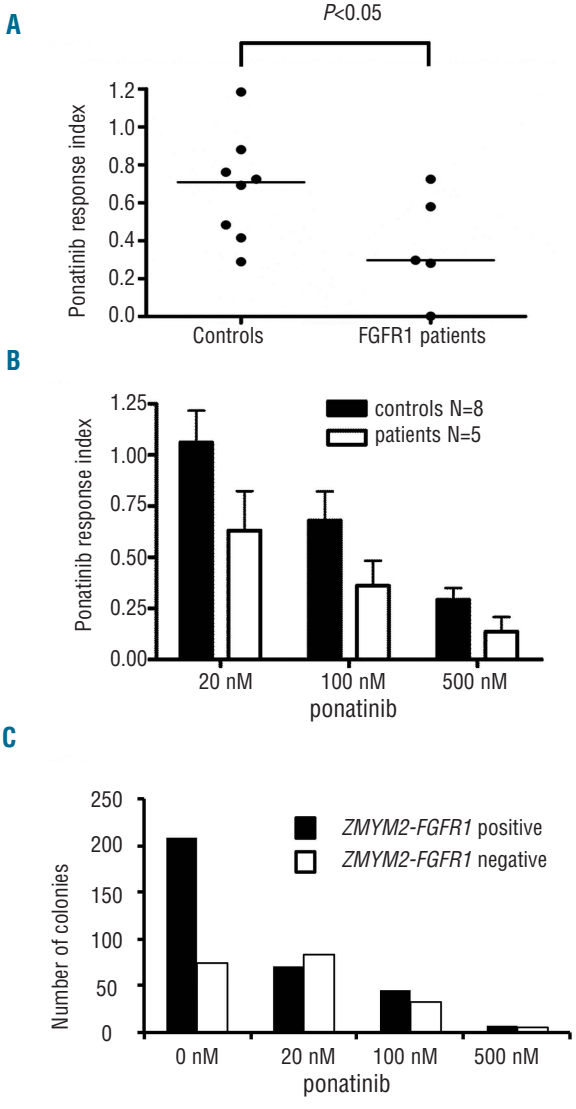


**Figure 3.** Effect of ponatinib upon colony growth of primary cells from patients with *FGFR1* fusion genes. (A) Colony growth of cells from 5 patients was reduced compared to that seen in 8 controls (t-test, $P<0.05$). The y-axis shows the response index i.e. relative reduction in treated *versus* untreated cultures averaged over all concentrations at Days 7 and 14 as described. (B) Colony growth inhibition, i.e. a reduced response index (mean of Days 7 and 14 counts relative to untreated cultures for all 5 patients and 8 controls),[4] was seen at all concentrations of ponatinib. (C) A subset of colonies (n=201) from case F5 with a *ZMYM2-FGFR1* (*Z-F*) fusion were plucked at Day 14 and *FGFR1*-fusion status was determined by FISH, thereby allowing the size of the *Z-F* positive and negative fractions of the Day 14 colony counts to be estimated. *Z-F* positive and negative cells were differentially affected; *Z-F* positive cell number fell most rapidly between 0 nM and 20 nM ponatinib whilst *Z-F* negative cell number only fell at concentrations greater than 20 nM.

gene-negative HL60 and HEL cell lines (Figure 1C, D) ($IC_{50}$=41 nM, 616 nM and 764 nM, respectively). Western blotting demonstrated reduced phosphorylation of the FGFR1 fusion protein and STAT5 (Figure 2A) (ERK is not constitutively phosphorylated in KG1A and is not, therefore, not examined here).[18] Cleaved caspase 3 and cleaved PARP increased with increasing inhibitor concentration indicating increased levels of apoptosis (Figure 2B). These results are consistent with a recent study demonstrating activity of ponatinib against *CUX1-FGFR1* transformed Ba/F3 cells.[19]

### Ponatinib inhibit proliferation and survival of primary cells from EMS patients

Primary cryopreserved cells from 5 patients with *FGFR1* translocations were exposed to ponatinib in methylcellulose colony assays and growth was compared to that seen in normal control samples. Overall, ponatinib treatment resulted in significantly reduced colony growth in patients compared with normal controls (t-test, $P<0.05$) (Figure 3A). Reductions were seen at all ponatinib concentrations (Figure 3B), but due to lower numbers the differences at each concentration were not statistically significant.

For cases 1, 4 and 5, colonies from Day 14 plates were plucked for FISH analysis with *FGFR1*-split apart probes (the total number of colonies analyzed by FISH and the number *FGFR1*-fusion positive at each ponatinib concentration is given in the *Online Supplementary Table S2*). Cells from 2 patients grew only FGFR1-fusion gene positive colonies in cultures treated with 500 nM ponatinib suggesting a very low incidence of *FGFR1*-fusion negative cells. A third patient (case 5, *ZMYM2-FGFR1* positive) showed *ZMYM2-FGFR1* negative colonies in treated cultures allowing us to assess differential growth of fusion gene positive and negative colonies in treated *versus* untreated cultures. The numbers of *ZMYM2-FGFR1* positive colonies showed a greater reduction at lower concentrations of ponatinib than *ZMYM2-FGFR1* negative colonies (Figure 3C), mirroring the effect seen in the Ba/F3 ponatinib response assay (Figure 1A). At 20 nM ponatinib, the proportion of *ZMYM2-FGFR1*-positive colonies fell by 38%, i.e. from 0.74 in untreated cultures to 0.46 ($\chi^2$, $P>0.001$) (*Online Supplementary Table S3*)

In summary, we have shown that ponatinib has clear activity against FGFR1 fusion proteins in EMS and thus has potential for the treatment of this rare subset of MPN patients for whom current therapy is inadequate.

### *Authorship and Disclosures*

*Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.*

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