Activity of TKI258 against primary cells and cell lines with FGFR1-fusion genes associated with the 8p11 myeloproliferative syndrome

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Abstract

The 8p11 myeloproliferative syndrome (EMS) is an aggressive, atypical stem-cell myeloproliferative disorder associated with chromosome translocations that disrupt and constitutively activate FGFR1 by fusion to diverse partner genes. To explore the possibility of targeted therapy for EMS we have investigated the use of TKI258, a multi-targeted receptor tyrosine kinase inhibitor with activity against FGFR, VEGFR, PDGFR, FLT3 and KIT that is currently being assessed for the treatment of a variety of malignancies in Phase 1 clinical studies. The viability of Ba/F3 cells transformed to IL-3 independence by ZNF198-FGFR1 or BCR-FGFR1 was specifically inhibited by TKI258 with IC_{50} values of 150 nM and 90 nM, respectively. Inhibition was accompanied by dose-dependent inhibition of phosphorylation of each fusion gene, ERK and STAT5. TKI258 also specifically inhibited proliferation and survival of the FGFR1OP2-FGFR1 positive KG1 and KG1A cell lines, resulting in increased levels of apoptosis. Primary cells from EMS patients showed significant, dose-dependent responses in liquid culture and in methylcellulose colony assays compared to controls. This work provides evidence that targeted therapy may be beneficial for patients with EMS.
Introduction

The 8p11 myeloproliferative syndrome (EMS), also known as stem cell leukemia/lymphoma syndrome, is an atypical stem-cell myeloproliferative disorder typically characterized by eosinophilia and in many cases an associated T- or B-cell lymphoma. EMS is caused by constitutive activation of the fibroblast growth factor receptor 1 (FGFR1) by fusion to unrelated partner genes, the most common being ZNF198. Other FGFR1 translocation partner genes are BCR, TRIM24 (TIF1), MYO18A, CEP110, FGFR1OP (FOP), FGFR1OP2, and LOC113386 (HERV-K). The partner protein promotes dimerisation and ligand-independent activation of the FGFR1 kinase in a manner analogous to BCR-ABL. The disease is aggressive and usually progresses to acute myeloid leukemia (AML) usually within one or two years of diagnosis. Currently, stem cell transplantation remains the only curative treatment.

The development of small molecules with inhibitory activity against activated tyrosine kinases has revolutionized therapy for patients with appropriate molecular targets, most notably the BCR-ABL fusion in chronic myeloid leukemia (CML). Treatment of CML patients with the ABL inhibitor imatinib induces durable, complete hematological and cytogenetic remissions in the majority of patients and is likely to substantially improve survival. Imatinib also induces durable responses for myeloproliferative disorders (MPD) with PDGFRα and PDGFRB fusion genes.

These spectacular successes suggest that other conditions characterized by activated tyrosine kinases, such as EMS, may also be treatable by targeted therapy. However imatinib is not active against FGFR1 and therefore alternative compounds are needed. Previous in vitro studies have
demonstrated activity of the tyrosine kinase inhibitors SU5402, PD173074 and PKC412, against Ba/F3 cells transformed with the ZNF198-FGFR1 fusion gene, but none of these compounds is in general clinical use.

TKI258 (formerly CHIR258) (4-amino-5-fluor-3-[5-(4-methylpiperazin-1-yl)-1H-benzimidazol-2-yl]quinolin-2(1H)-one, MW=392.4) is an ATP-competitive inhibitor with activity against class III-IV receptor tyrosine kinases which includes FGFR, VEGFR, PDGFR, FLT3 and KIT. In vitro studies have demonstrated activity of TKI258 against FGFR3 in t(4;14) positive multiple myeloma and this compound is currently being assessed for the treatment of diverse malignancies. Here we report in vitro studies which demonstrate that TKI258 has significant activity against ZNF198-FGFR1 and BCR-FGFR1 transformed Ba/F3 cells, the KG1 and KG1A AML cell lines expressing the FGFR1OP2-FGFR1 fusion gene and primary material from patients with FGFR1 translocations.

Methods

Cell lines

The IL3 independent Ba/F3 clones containing pcDNA3.1/ZNF198-FGFR1 (Ba/F3-ZF) or pcDNA3.1/BCR-FGFR1 (Ba/F3-BF) constructs have been described elsewhere. Ba/F3-ZF, Ba/F3-BF, KG1, KG1A and HEL cell lines were grown in RPMI-1640 supplemented with 10% serum. A Ba/F3 clone containing the pcDNA3.1 vector alone was grown in RPMI-1640 medium supplemented with 10% serum and 10% WEHI3B-conditioned medium as a source of IL3.
Patient material

In vitro response to TKI258 was investigated in cryopreserved cells from five patients with four different FGFR1 fusion genes (Table 1), all of which were confirmed by RT-PCR. As controls we used samples from healthy individuals and from three patients with FGFR1-rearrangement negative MPDs (one case each of JAK2 V617F-positive polycythemia vera, chronic eosinophilic leukemia and unclassified myeloproliferative disorder). The study was approved by the Internal Review Board of South Wiltshire Regional Ethics Committee and informed consent was provided according to the Declaration of Helsinki.

Proliferation and survival assays

Each cell line was seeded at 2x10^4/ml in 96-well plates (100 µl per well) with and without TKI258. TKI258 concentrations ranged from 10 nM to 10 µM with half-log concentration increments. Proliferation was measured at day 0 without inhibitor and after 48 hours by CellTitre 96 One Solution Cell Proliferation MTS assay (Promega, Madison, WI); absorbance was measured on a MRX Microplate Reader (Thermo Labsystems, Waltham, MA). Each experiment was repeated twice. To calculate IC_{50} values, the MTS readings were plotted as a percentage of the control (without inhibitor) and analyzed using Graphpad Prism 4 software (GraphPad Software Inc., San Diego, CA).

Western blotting

Cells were serum-starved and exposed to TKI258 for 2-4 hours or, for Ba/F3-BCR-FGFR1, exposed to inhibitor overnight with serum. Cells were lysed in Triton-X lysis buffer (20 mM Tris-Cl pH 7.8, 1% Triton-X, 150 mM NaCl, 10% glycerol) containing Complete Mini Protease Inhibitor Cocktail (Roche, Basel Switzerland) and 1 mM sodium vanadate (Sigma Aldrich, St.
Louis, MO). Equal volumes of lysate were electrophoresed on an SDS-PAGE gel. Detection was performed with antibodies against FGFR1 (antibody sc-121; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-FGFR (Tyr 653/654, Cell Signalling Technology; Beverly, MA), STAT5 (Cell Signalling Technology; Beverly, MA), phospho-STAT5 (Tyr694) (Cell Signalling Technology; Beverly, MA), ERK (antibody sc-94; Santa Cruz Biotechnology) and phospho-ERK (antibody sc-7383; Santa Cruz Biotechnology). Signals were detected with ECL Plus Western Blotting Detection Reagents (Amersham Biosciences, Little Chalfont, UK).

**Determination of response of primary cells to TKI258.**

Responses were determined using liquid culture and colony assays as described. Briefly, for the liquid culture response assay, cells were seeded at 2x10^6 - 5x10^6 in 1 ml volumes in 24-well plates in duplicate at 0, 20 and 100 nM TKI258. Cell number was counted manually twice weekly and medium was changed weekly.

For colony assays, peripheral blood or bone marrow mononuclear cells were seeded at 2x10^6 cells/ml in 1 ml volumes in 3 cm dishes in Methocult, with cytokines but without erythropoietin (Stem Cell Technologies, Vancouver, BC, Canada) in triplicate at 0, 20 and 100 nM TKI258 or in Human Methylcellulose Base Media without cytokines (R&D Systems, Minneapolis, MN). Colonies of greater than 50 cells were counted on day 7, and of greater than 100 cells on day 14. In order to compare inhibition between samples response was calculated as the mean proportional reduction in colony numbers at days 7 and 14 relative to the number of colonies grown without TKI258. For example, a response of 0.66 indicates that the number of colonies in a treated culture was 0.66 times the number seen in untreated cultures from the same patient.
Fluorescence in situ hybridization (FISH)

Colonies for FISH were either plucked into phosphate buffered saline (PBS) and cytopun onto slides, dried briefly and fixed and stored in 3:1 methanol/acetic acid at -20°C, or were plucked into 3:1 methanol/acetic acid fixative, stored at -20°C until required then washed in fixative and dropped onto slides. FISH was performed using standard methods as previously described20 except that cells were refixed with 1% paraformaldehyde before hybridization. Clones flanking FGFR1 (5´ FGFR1 PAC RP1-224C10; 3´ FGFR1 PAC RP1-162N14) were obtained from the Sanger Institute (Hinxton, UK).

Apoptosis assays

Caspase 3 activity was measured using the Ac-DEVD-pNA substrate in a colorimetric assay as previously described.21 Cells were exposed to inhibitor for 48 hours. Lysates were prepared in 50 mM HEPES, pH 7.4, 0.1% CHAPS, 1 mM DTT, 0.1 mM EDTA, 0.1% Triton X-100 and protein concentration was measured by the Bradford method (BioRad Laboratories, Hercules, CA). To each reaction 100 µg of protein in 73 µl lysis buffer, 25 µl 4 x reaction buffer (200 mM HEPES, pH 7.4, 0.4% CHAPS, 40 mM DTT, 4 mM EDTA, 400 mM NaCl, 40% glycerol) and 2 µl of 10 mM Ac-DEVD-pNA (BioMol, Plymouth Meeting, PA) were added. Reactions were incubated at 37°C and the increase in absorbance over a three hour period measured on an MRX Microplate Reader (Thermo Labsystems, Waltham, MA). In addition, TdT-mediated dUTP nick end-labeling (TUNEL) assay was performed with the DeadEnd Colorimetric TUNEL System (Promega, Madison WI) according to the manufacturer’s instructions following 48 hours exposure to inhibitor.
Results

TKI258 inhibits proliferation and survival of ZNF198-FGFR1 and BCR-FGFR1 transformed Ba/F3 cells

ZNF198-FGFR1 and other EMS-associated FGFR1 fusion genes have been shown previously to encode chimeric proteins with constitutive tyrosine kinase activity that can transform Ba/F3 cells to IL3 independence and induce a fatal MPD in mice. To determine if TKI258 can inhibit this transforming activity, we compared the growth of Ba/F3-ZF cells, Ba/F3-BF cells and control Ba/F3-pcDNA3.1 cells with increasing concentrations of TKI258. Reduced proliferation and survival was seen for both Ba/F3-ZF and Ba/F3-BF with cellular IC₅₀ values for Ba/F3-ZF, Ba/F3-BF and Ba/F3-pcDNA3.1 after 48 hours exposure to TKI258 being 150 nM, 90 nM and 1000 nM respectively (Figure 1).

TKI258 treatment results in dose-dependent reduction in phosphorylation of ZNF198-FGFR1, BCR-FGFR1 and downstream signaling molecules

Western blotting was used to examine the effect of TKI258 treatment on phosphorylation of ZNF198-FGFR1, BCR-FGFR1 and the downstream signaling molecules STAT5 and ERK. After exposure to TKI258 all molecules showed a dose dependent reduction in phosphorylation with increasing concentrations of TKI258 (Figure 2).

TKI258 inhibits proliferation and survival and induces apoptosis in the KG1 and KG1A cell lines

The KG1 cell line and its derivative KG1A have recently been shown to harbor the FGFR1OP2-FGFR1 fusion gene. We therefore examined the inhibitory effect of TKI258 on KG1 and
KG1A compared to the erythroleukemia cell line HEL, which harbors the JAK2 V617F mutation and would not be expected to be responsive to TKI258. A cell viability assay demonstrated reduced survival of both KG1 and KG1A compared to HEL (Figure 3A). Cellular IC₅₀ values for KG1, KG1A and HEL after 48 hours exposure to TKI258 were 180, 180 and 1500 nM respectively. Analysis of the data as a percentage of the day 0 reading (Figure 3B) demonstrated that cell death (i.e. at inhibitor concentrations where the curve falls below 100%) in the KG1 and KG1A cell lines occurred at 1-1.5 log concentration values below that of HEL. Caspase activation, a marker of apoptosis, was elevated in KG1 and KG1A at 200-500 nM TKI258 compared to HEL which showed a minimal increase in caspase activation even at 1000 nM TKI258 (Figure 3C). Increased apoptosis of KG1 cells compared to HEL cells was also confirmed using the TUNEL assay (Figure 3D).

In addition, a dose-dependant reduction in phosphorylation of FGFR1OP2-FGFR1 and STAT5 were seen in KG1 cells (Figure 3E). Interestingly we were unable to detect constitutive phosphorylation of ERK in serum starved cells (results not shown), a finding also described by others.²⁴ ERK is however phosphorylated in response to serum and is therefore phosphorylated by a pathway independent of FGFR1OP2-FGFR1.

**TKI258 inhibits survival and proliferation of primary cells from EMS patients**

To determine if TKI258 is active against primary EMS cells, we examined the effects of this compound in liquid culture and colony assays. In liquid culture assays using cells from three control MPD patients without FGFR1 translocations (of which only two are shown in Figure 4), no difference was seen in the number of cells surviving in the presence of absence of TKI258. In contrast, all four patients with FGFR1 translocations showed clear reductions in relative cell
numbers in cultures containing both 20 and 100 nM TKI258 compared with cultures without inhibitor (Figure 4). Furthermore, a dose dependent effect was apparent with a greater reduction for all four patients tested with 100nM TKI258 compared to 20nM.

Colony growth from ten normal peripheral blood samples was moderately inhibited by TKI258 with a mean response of 0.75 (range of 0.47 - 0.87). No obvious differences were observed between colonies from normal individuals from treated and untreated cultures. For the 4 patients (cases 1-3, 5) for whom sufficient material was available, the median response was significantly lower at 0.51 (range 0.24-0.58; P=0.02, Mann-Whitney; Figure 5A). The degree of colony reduction was dependent on the dose of TKI258: for the normal controls responses at 20 nM and 100 nM were 0.87 and 0.66, respectively (Figure 3). For the 4 patients the mean responses were 0.65 and 0.27 (P=0.08 and 0.01 for 20 nM and 100 nM compared to controls, respectively, Mann-Whitney; Figure 5B). All colonies had a morphological appearance indicative of colony forming units granulocyte macrophage (CFU-GM), however case 1 also grew erythroid burst forming units (BFU-E). Interestingly, the CFU-GM in this case were unaffected by TKI258 (response = 1.04), but the BFU-E were strongly inhibited (response = 0.13) with no growth at all at 100nm.

Colony FISH analysis was performed to determine if TKI258 selectively reduced the number of \textit{FGFR1}-rearranged cells. Collectively for the four patients there was no difference between the proportion of colonies that showed split \textit{FGFR1} signals before treatment (106 of 119; 89%) compared to those after treatment (41 of 47; 87%). For cases 2 and 5 all colonies before and after treatment had \textit{FGFR1} split signals. Patients 1 and 3 had 18% and 38% unrearranged colonies, respectively, prior to treatment but there was no significant change after incubation with TKI258.
Discussion

Significant advances have been made in the use of small molecule inhibitors for the treatment of some malignancies with aberrant tyrosine kinase activation e.g. the use of imatinib for the treatment of BCR-ABL positive CML and MPDs with translocations involving PDGFR A or PDGFRB. The development of inhibitors of the FGFR tyrosine kinases for the treatment of patients with t(4;14) positive multiple myeloma and MPDs in which there is involvement of FGFR3 and FGFR1, respectively, has however made less progress although there is evidence from in vitro and mouse model data that targeting FGFRs will be of therapeutic value. FGFR3 is upregulated as a result of the t(4;14) translocation in about 15% of myeloma patients and inhibition has been demonstrated in t(4;14) positive cell line models and/or mouse xenograft models with the FGFR inhibitors SU54027, PD173074, PKC412 and TKI258. As a result of this work, TKI258 is currently being assessed in clinical trials for the treatment of myeloma and other malignancies.

For MPDs with translocations involving FGFR1, inhibition has been demonstrated with SU5402, PD173074 and PKC412 using transformed Ba/F3 cell lines and with PKC412 in a mouse xenograft model. Treatment of a single ZNF198-FGFR1 positive EMS patient with progressing disease with PKC412 for six months resulted in a partial hematological response but no cytogenetic response and there is clearly a need to examine other compounds with activity against FGFR1. We have therefore investigated the inhibitory activity of TKI258 in Ba/F3-ZNF198-FGFR1 and BCR-FGFR1 cell line models, in the FGFR1OP2-FGFR1-positive cell lines KG1 and KG1A and in primary cells from EMS patients with FGFR1 translocations.
In the Ba/F3-ZNF198-FGFR1 and BCR-FGFR1 cell lines, treatment with TKI258 resulted in the inhibition of cell proliferation and survival accompanied by an inhibition of phosphorylation of the respective fusion proteins, ERK and STAT5. These results are thus similar to those previously demonstrated for the inhibitors SU5402, PD173074 and PKC412 in similar Ba/F3 cell line experiments.7,8 The AML KG1 cell line, and its derivative KG1A, has provided the first example of a patient-derived cell line with an FGFR1 fusion gene. We have demonstrated activity of TKI258 on proliferation, survival and apoptosis in both cell lines with cellular IC$_{50}$ values similar to those obtained in the Ba/F3-ZNF198-FGFR1 cell line. This suggests that transformed EMS still depends on the constitutive activity of FGFR1 fusions similarly to transformed CML which still depends on BCR-ABL.

Importantly, we have also demonstrated for the first time that targeting FGFR1 selectively inhibits the growth and proliferation of primary cells from EMS patients. In a liquid culture assay, cells from all four EMS patients tested showed substantially reduced, dose-dependent survival over two weeks compared to three MPD patients without FGFR1 translocations, who showed no differential cell survival with and without TKI258. Using methylcellulose assays, we showed a significant, dose dependent decrease in colony numbers grown in the presence of TKI258 compared to controls. However more detailed examination of the colony results failed to show clear evidence of selection for normal cells. The reasons for this are unclear and may relate to the fact that EMS is an aggressive disease, however for cases 2 and 5 only FGFR1-rearrangement positive colonies were seen in the pretreatment cultures and it is possible that the proportion of normal clonogenic cells was simply so low that no differential effect could be observed. Similar findings have been described for CML CFU-GM treated with imatinib.27 Interestingly, case 1 grew spontaneous BFU-E that were strongly inhibited by TKI-258. This is
consistent with reports indicating that occasional patients with \textit{FGFR1} fusions present with polycythemia vera before progressing to EMS.\textsuperscript{28}

EMS is an aggressive disease with a median time to transformation of 6-9 months and with about 10\% of patients presenting with acute leukaemia.\textsuperscript{1} In this study we have demonstrated the effectiveness of \textit{FGFR1} fusion inhibition in both cell lines and primary cells, suggesting that targeted therapy is likely to be beneficial for patients with EMS.

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The authors have no competing financial interests.

Author contributions: AC and FHG performed the laboratory analysis. All authors contributed to the design of the study and the final manuscript.
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Figure Legends

Figure 1. TKI258 inhibits proliferation and survival of Ba/F3 cells transformed to IL3-independence by ZNF198-FGFR1 or BCR-FGFR1. Cells (■ Ba/F3-ZNF198-FGFR1 and ● Ba/F3-BCR-FGFR1 grown in the absence of IL3; ▲ Ba/F3-pcDNA3.1 grown in the presence of IL3) were exposed to TKI258 for 48 hours. Values are means +/- SE of three independent experiments.

Figure 2. TKI258 treatment induces dose-dependent inhibition of phosphorylation of ZNF198-FGFR1, BCR-FGFR1, ERK and STAT5. Phosphorylation of ERK, STAT5 and the BCR-FGFR1 and ZNF198-FGFR1 fusion proteins after exposure to TKI258 was assessed by western blotting.

Figure 3: TKI258 inhibits proliferation and survival of KG1 and KG1A and induces apoptosis. TKI258 treatment results in (A) reduced viability and (B) increased cell death (where the curves fall below 100% of day 0 reading) in KG1 and KG1A cell lines assessed at 48 hours, in contrast to the non-responsive HEL cell line. Values are means +/- SE of three independent experiments. (C) TKI258 treatment resulted in a dose-dependent increase in apoptosis, assessed by a colorimetric caspase assay, in KG1 and KG1A compared with HEL. Values are means +/- SD of three separate experiments and are normalized to 1.0 at 0 nM TKI258). (D) Increased apoptosis in KG1 cells was confirmed by the TUNEL assay. Values are means +/- SD of two separate experiments. (E) Reduced phosphorylation of the FGFR1OP2-FGFR1 fusion protein and STAT5 after TKI258 treatment.
Figure 4: TKI258 treatment results in a dose-dependent reduction in cell survival in four EMS patients but not in two MPD patients without FGFR1 rearrangements. Cell counts (mean +/- SE) are plotted relative to untreated control (dotted line at 1.0) at each time point for 20 nM (▲) and 100 nM (▼) TKI258-treated cell cultures.

Figure 5: Hemopoietic colonies from EMS patients show a greater, dose-dependent response to TKI258 than normal peripheral blood cells. A. Overall median responses in methylcellulose colony assays of ten normal peripheral blood samples and EMS cases 1,2,3 and 5 (P=0.02). B. Dose response in colony reduction (+/- SD). A greater differential response was seen at 100 nM than 20 nM compared to normal controls (p = 0.01 and 0.08 respectively).
References


Figure 1
Figure 2
Figure 3
Figure 3 (continued)
Figure 4
Figure 5
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