

Irreversible pan-ERBB inhibitor canertinib elicits anti-leukaemic effects and induces the regression of FLT3-ITD transformed cells in mice

Amanda Nordigården, Jenny Zetterblad, Cecilia Trinks, Henrik Green, Pernilla Eliasson, Pia Druid, Kourosh Lotfi, Lars Ronnstrand, Thomas Walz and Jan-Ingvar Jönsson

Linköping University Post Print

N.B.: When citing this work, cite the original article.

This is the authors' version of the following article:

Amanda Nordigården, Jenny Zetterblad, Cecilia Trinks, Henrik Green, Pernilla Eliasson, Pia Druid, Kourosh Lotfi, Lars Ronnstrand, Thomas Walz and Jan-Ingvar Jönsson, Irreversible pan-ERBB inhibitor canertinib elicits anti-leukaemic effects and induces the regression of FLT3-ITD transformed cells in mice, 2011, British Journal of Haematology, (155), 2, 198-208.

which has been published in final form at:

<http://dx.doi.org/10.1111/j.1365-2141.2011.08819.x>

Copyright: Blackwell Publishing

<http://www.blackwellpublishing.com/>

Postprint available at: Linköping University Electronic Press

<http://urn.kb.se/resolve?urn=urn:nbn:se:liu:diva-72031>

Irreversible pan-ERBB inhibitor canertinib elicits anti-leukaemic effects and induces the regression of FLT3-ITD transformed cells in mice

Amanda Nordigården¹, Jenny Zetterblad¹, Cecilia Trinks², Henrik Gréen³, Pernilla Eliasson¹, Pia Druid¹, Kourosh Lotfi^{3,4}, Lars Rönnstrand⁵, Thomas M. Walz², and Jan-Ingvar Jönsson¹

¹ Experimental Haematology unit, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden;

² Division of Oncology, Department of Clinical and Experimental Medicine, Faculty of Health Sciences, Linköping University, Linköping, Sweden;

³ Clinical Pharmacology, Department of Medical and Health Sciences, Faculty of Health Sciences, Linköping University, Linköping, Sweden;

⁴ Division of Haematology, Department of Clinical and Experimental Medicine, Linköping University Hospital, Linköping, Sweden.

⁵ Experimental Clinical Chemistry, Department of Laboratory Medicine, Lund University, Skåne University Hospital, Malmö, Sweden.

Running title: Canertinib has anti-leukaemic effects and inhibits FLT3

Correspondence: Dr. Jan-Ingvar Jönsson, Department of Clinical and Experimental Medicine, Linköping University, SE-581 85 Linköping, Sweden.

e-mail: jan-ingvar.jonsson@liu.se; Phone: +46-101032391; Fax: +46-101031375

Summary

Recent findings have indicated that tyrosine kinase inhibitors (TKIs) targeting the ERBB receptor family display anti-leukaemic effects despite the lack of receptor expression on human leukemic cells. The occurrence of activating mutations in the gene encoding FMS-like tyrosine kinase 3 (FLT3) in patients with acute myeloid leukaemia (AML) has rendered inhibition of this receptor a promising therapeutic target. Due to possibility of cross-reactivity, we have investigated the effect of the irreversible pan-ERBB inhibitor canertinib (CI-1033) on leukemic cells expressing FLT3. The drug had anti-proliferative and apoptotic effects on primary AML cells and human leukaemic cell lines expressing mutated FLT3. In several AML patient samples, a blast cell population expressing FLT3-ITD was eradicated by canertinib. Canertinib inhibited receptor autophosphorylation and kinase activity of both mutated and FLT3 ligand stimulated wildtype FLT3, leading to inhibition of the PI3-kinase and MAP kinase pathways. Apoptotic induction was dependent on pro-apoptotic BH3-only protein BCL2L1/BIM since RNAi silencing attenuated apoptosis. Moreover, the drug induced regression of cells expressing FLT3-ITD in a murine in vivo-transplantation model at previously described tolerated doses. These results indicate that canertinib as an irreversible TKI could constitute a novel treatment regimen in patients with mutated or overexpressed FLT3.

Introduction

Despite a remarkable progress in understanding the pathogenesis of acute myelogenous leukaemia (AML), cytarabine and anthracyclins are still cornerstones of therapy. The effectiveness is limited however due to resistance, and relapse is a major cause of treatment failure. To develop novel strategies much focus has been on defining protein kinases as critical components of signal transduction in leukaemic cells. Activating mutations in the *FMS-like tyrosine kinase 3 (FLT3)* gene are recognized as one the most common mutations found in AML, and are detected in approximately one-third of the patients. The majority are internal tandem duplication (ITD) mutations associated with an increased relapse rate and decreased overall survival in response to standard chemotherapy. Activation loop point mutations in one of the tyrosine kinase domains (TKD) have also been found. Both classes of mutations lead to constitutive activation of signalling molecules including PI3-kinase and RAS signalling, and growth factor-independent growth. FLT3-ITDs induce a lethal myeloproliferative disease in murine bone marrow transplantation models (Kelly, *et al* 2002, Lee, *et al* 2005, Li, *et al* 2008).

Due to the frequent occurrence of *FLT3* mutations, and overexpression of wildtype FLT3 in many patients with AML or acute lymphoblastic leukaemia (ALL), tyrosine kinase inhibitors (TKI) with blocking effects of FLT3 signalling have developed as an attractive therapeutical approach (Kindler, *et al* 2010). Some TKIs display strong *in vitro* efficacy on transformed FLT3-ITD expressing cell lines and primary AML cells, and eradicate FLT3-ITD-expressing cells in animal models. However, the effectiveness is limited and some patients display primary or secondary resistance to the inhibitors. Therefore, development of novel TKIs targeting FLT3 more efficiently and/or inhibiting alternative pathways is valid to enable long-term therapeutic benefits.

Most kinase inhibitors in clinical development target the kinase ATP site and there is a possibility of cross-reactivity between compounds (Fabian *et al*, 2005). One group of anti-cancer targets is the ERBB or epidermal growth factor (EGF) protein family of four structurally related (ERBB1-4) receptor tyrosine kinases, known to play significant roles in human cancer cell proliferation (Rocha-Lima, *et al* 2007). The potential of ERBB inhibitors for AML therapy has been explored in recent studies. Gefitinib (Iressa™, ZD1839), investigated in clinical trials for advanced or metastatic non-small-cell lung cancer (NSCLC), has been shown to impair the viability of AML patient cells *in vitro* and to induce differentiation of human HL-60 and U-937 leukaemic cell lines (Stegmaier, *et al* 2005). Since these cells lack ERBB receptor expression, gefitinib appears to act on off-targets. Recently, the ERBB1/EGFR-inhibitor erlotinib was shown to have activity on AML cell lines and cells from patients with myelodysplastic syndrome (Boehrer, *et al* 2008, Slichenmyer, *et al* 2001, Smaill, *et al* 2000). Although erlotinib was unable to induce cell death of HL-60 and U-937, differentiation and killing of the KG-1 cell line was triggered. While the authors were unable to explain erlotinib-induced cell death, an association to JAK2/STAT5 was suggested.

Canertinib (CI-1033) is a quinazoline-based novel member of the ERBB inhibitors that, in contrast to gefitinib and erlotinib, irreversibly inhibits all members of the family by covalently binding to cysteine residues in the kinase domains. This results in prolonged inhibition of ATP-binding, preventing its catalytic activity and downstream signalling (Slichenmyer *et al*, 2001; Smaill *et al*, 2000). Canertinib has shown promise in blocking tumor growth for breast cancer, ovarian cancer, esophageal cancer, and advanced non-small cell lung cancer, either as single therapy, or when used in combination with DNA-damaging agents (Ako, *et al* 2007, Campos, *et al* 2005, Janne, *et al* 2007, Ocana and Amir 2009), but has not been in clinical trials of human leukaemic patients. Recently, we reported that canertinib has anti-leukaemic effects on some leukaemic cell lines in an ERBB-independent

manner (Trinks, *et al* 2010). We now extend our findings and demonstrate that canertinib has an inhibiting selectivity in cells expressing FLT3-ITD or FLT3-TKD as well as cells with FLT3 ligand activated receptor. Treatment with canertinib of primary AML cells carrying FLT3-ITD led to significant apoptosis induction. More importantly, progressive growth of hematopoietic progenitor cells expressing FLT3-ITD in a transplantation model was prevented in canertinib-treated mice.

Materials and Methods

Reagents, cytokines, and antibodies

AG1295 and camptothecin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Canertinib (CI-1033) was a gift from Pfizer Pharmaceuticals (Ann Arbor, MI, USA).

Inhibitors were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. Recombinant cytokines were purchased from PeproTech (London, UK). The following antibodies were used: antibodies for phospho-Ser⁴⁷³-AKT, phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴) and caspase-3 (Asp¹⁷⁵) (Cell Signaling Technology, Danvers, MA), antibodies for phospho-Thr³²-FOXO3a and phospho-Ser²⁵³-FOXO3a (Upstate Biotechnology, Charlottesville, VA), BCL2L1/BIM antibody (Affinity BioReagents, Golden, CO), and anti-pTyr (PY99) antibodies (Santa Cruz BioTechnology, Santa Cruz, CA), antibody to glyceraldehyde-3-phosphate dehydrogenase/GAPDH (Millipore, Billerica, MA) and antibody to human ERBB1-4 (Santa Cruz). The anti-FLT3 antibody was raised against synthetic peptides corresponding to amino acids 740-757 of human FLT3 and purified as described (Razumovskaya, *et al* 2009).

Secondary antibodies used were horseradish peroxidase (HRP)-conjugated from Jackson Laboratories (Bar Harbor, Maine), GE Healthcare Life Sciences (Milwaukee, WI), or Dako (Glostrup, Denmark). Antibodies for detection of human CD markers (CD33-phycoerythrin/PE, CD34-fluorescein isothiocyanate/FITC, and CD135-PE) on AML patient cells by flow cytometry were purchased from BD Biosciences (San Jose, CA).

Cells

MV4;11 was cultured in Iscove's modified Dulbecco's medium (IMDM; PAA laboratories, Les Mureaux, France) with 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, 1 % penicillin-streptomycin, and 5 ng/ml human GM-CSF at 37°C in 5% CO₂. FDC-P1 cells were cultured in the same medium supplemented with an IL-3 containing

supernatant. All other cell lines were cultured in RPMI-1640 (PAA) with 10% FBS, 2 mM L-glutamine, and 1 % penicillin-streptomycin at 37°C in 5% CO₂.

Patient samples

The ethical committee at the Faculty of Health Sciences, Linköping, Sweden, approved the study and informed consent was obtained from all patients. Bone marrow or peripheral blood samples from AML patients according to “the World Health Organization (WHO) classification of tumors for 2008” (Arber, *et al* 2008) were enriched for mononuclear cells on Ficoll-Hypaque (GE Healthcare) and cryopreserved in liquid nitrogen. Unthawed cells were allowed to rest for 4 hours in RPMI-1640 with 20% FBS before adding TKI, and analysed after 24-72 hours. Mutation analysis of the *FLT3* gene was performed by multiplex PCR including digestion of amplicon with EcoRV for detection of ITD and D835 mutations.

Establishment of cells expressing wildtype FLT3, FLT3-ITD, and FLT3-TKD/D835

Ba/F3 cells stably expressing wildtype, ITD, or D835Y of FLT3, were done by transfecting EcoPack cells with the corresponding pMSCV-FLT3 constructs and virus-containing supernatants were used for infections. FDC-P1 cells expressing wildtype FLT3 or FLT3-ITD have been previously described (Nordigården, *et al* 2009). FLT3 expression was confirmed by flow cytometry.

Detection of apoptosis

Cells were labeled with Annexin V-FITC (BD Biosciences, San Jose, CA) and 5 µg/mL propidium iodide (Sigma), and analyzed using a FACS Canto and Diva software (BD Biosciences).

Assessment of proliferation

Cells were cultured in triplicates on 96-well tissue culture plates for 48 hours. At the end of the experiment, cultures were labelled for 4 hours with 0.5 $\mu\text{Ci}/\text{well}$ [^3H]-thymidine, harvested and counted in a β -scintillation counter. Sensitivity to canertinib was also assessed by the MTT assay as previously described (Green, *et al* 2003). IC_{50} values for cell survival were calculated as percent of survival compared to control cells.

Immunoprecipitations and immunoblotting

For immunoprecipitations, lysates were precleared with protein A sepharose (GE Healthcare) for 1 hour at 4°C followed by incubation overnight at 4°C with the indicated antibody. Immunocomplexes were captured with protein A sepharose. Following eletrophoresis and transfer to polyvinylidene fluoride membranes (Invitrogen), immunoblotting was performed with primary antibody overnight at 4°C and HRP-coupled secondary antibody for 1 hour. Blots were visualized by chemiluminescence (ECL, GE Healthcare).

In vitro-kinase assay

Ba/F3 cells expressing wildtype FLT3 were starved in cytokine-deprived medium overnight. One hour before stimulation with 50 ng/mL FL at 37°C for 15 minutes, canertinib was added (0.1-10 μM). In the case of Ba/F3 cells expressing FLT3-ITD or FLT3-D835Y, cells were treated the same way but not stimulated with FL. Cell lysis and immunoprecipitations were performed using an anti-human FLT3 serum. FLT3 kinase activity was assayed using myelin basic protein (Sigma) as a substrate. The amount of radioactivity incorporated into MBP was assayed using autoradiogram.

Gene silencing with small interfering RNAs (siRNAs)

Silencing of *BCL2L1/BIM* was performed by transfection of *SMART*pool siRNAs (Dharmacon Research Inc., Lafayette, CO) into FDC-P1/FLT3-ITD cells as previously described (Nordigården, *et al* 2009).

Realtime polymerase chain reaction (PCR)

Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) and cDNA was synthesized with SuperScript III and random hexamers (Invitrogen). Real-time quantitative PCR (qPCR) reactions were performed in triplicates with *BCL2L1/BIM*-specific primers as previously described (Nordigården, *et al* 2009). Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays and qPCR were performed as previously described (Nordigården, *et al* 2009) with a rabbit polyclonal anti-human FOXO3a antiserum (provided by Dr. E. Lam, London) and primers to the Forkhead response element (FHRE) region in the murine *BCL2L1/BIM* promoter.

In vivo experiments

All animal experiments were conducted at the Linköping University animal facility in line with Swedish legal regulations and with permission from Linköping animal ethical committee. FDC-P1 cells (2×10^6) transduced with retrovirus expressing FLT3-ITD and enhanced yellow fluorescence protein (EYFP) were injected into the tail vein of syngenic DBA/2 mice in 0.2 mL PBS per mouse. Seven days later, the level of engraftment was analysed by flow cytometry on peripheral blood samples collected by lateral-tail vein bleeding. Treatment was initiated on day 9 by intraperitoneal injections of 0.5 mL of

canertinib (40 mg/kg/day or 80 mg/kg/day) in PBS and continued for four consecutive days (day 10-13). On day 14, the experiment was terminated and a new blood sample was taken to determine the engraftment after treatment. Mice were then sacrificed and spleen weights were recorded.

Statistical analysis

Statistical analysis was performed using the Student *t* test and *p* values of less than 0.05 were considered statistically significant.

Results

Canertinib causes apoptosis of AML blasts primarily from patients harbouring FLT3-ITD

We recently demonstrated the anti-proliferative and apoptotic effects of the pan-ERBB inhibitor canertinib at high doses ($>5 \mu\text{M}$) in human leukaemic cell lines HL-60 and U-937 not expressing ERBB receptors (Trinks, *et al* 2010). These findings promoted us to investigate the *in vitro*-effects of canertinib in a panel of de novo-diagnosed AML patient samples. We first decided to determine the effects of canertinib on mononuclear cells from ten AML patients with FLT3-ITD. Two cases lacking mutations were also included. In the case of FLT3-ITD positive samples, the size of the ITD varied from 21 to 162 base pairs, and the FLT3-ITD expression was estimated to 11% to 57% of the blast cell DNA. Apoptosis was determined at 24, 48, and 72 hours by flow cytometry on Annexin V-stained cells. The cytotoxic effects of canertinib at 72 hours at concentrations between $5 \mu\text{M}$ and $40 \mu\text{M}$ is shown in Figure 1A-B. In five of the eight patients expressing FLT3-ITD cell viability decreased significantly. For instance, at $20 \mu\text{M}$ of canertinib viability decreased with 22%, 10%, 77%, and 28%, respectively, in patients #I-IV. Interestingly, the viability correlated to the expression level of *FLT3-ITD* by PCR analysis, where the amount of *FLT3-ITD* compared to the total DNA content was estimated to 25%, 11%, 57%, and 19% (not shown).

In several AML samples expressing FLT3-ITD, a cell population defined by flow cytometry to an intermediate-high forward scatter and low side scatter profile, indicative for a blast cell phenotype, was completely lost after treatment at $5 \mu\text{M}$ of canertinib for 24 hours. Three examples are given in Figure 2 where the difference is clearly visualised when comparing the lower FACS blot after canertinib treatment to the upper blot without canertinib. This sensitive population was further characterised by cytospin preparations of FACS-sorted cells from cultures not treated with canertinib. In all cases, the cells displayed a typical blast cell appearance (Figure 2, lower panel). Expression of CD34 and CD33, two

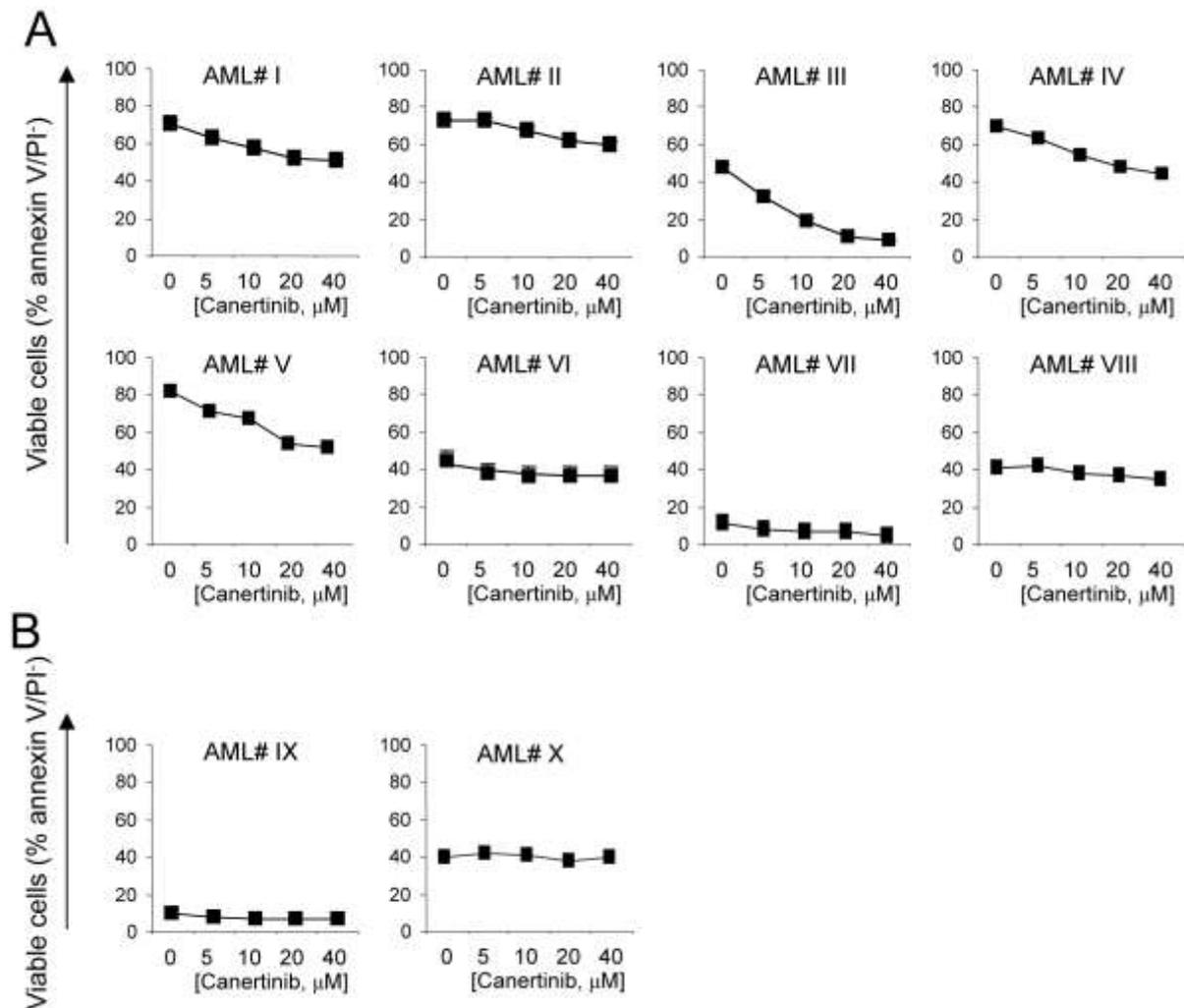


Figure 1. Canertinib displays cytotoxicity on primary AML cells expressing immature surface markers

(A-C) Mononuclear cells from blood or bone marrow of ten patients diagnosed with AML, of which eight had FLT3-ITD (A) and two patients lacked FLT3 mutations (B), were assayed for apoptosis after canertinib treatment. Cells were cultured for 72 hours, stained with Annexin V-APC and propidium iodide, and analysed by flow cytometry. Data are mean \pm SEM from duplicate samples.

markers used to distinguish immature human hematopoietic cells (Taussig, *et al* 2005), was preferentially expressed in a significant fraction of the cells (indicated in the upper right quadrant in dot plots in Figure 2). In three of the samples, we determined the *FLT3-ITD* RNA expression in the cell population ablated by canertinib compared to the whole cell sample by qPCR. The analysis revealed higher *FLT3-ITD* expression levels, ranging from 1.5- to 5-fold,

in canertinib-sensitive cells (not shown), indicating that canertinib induces apoptosis of AML cells expressing FLT3-ITD.

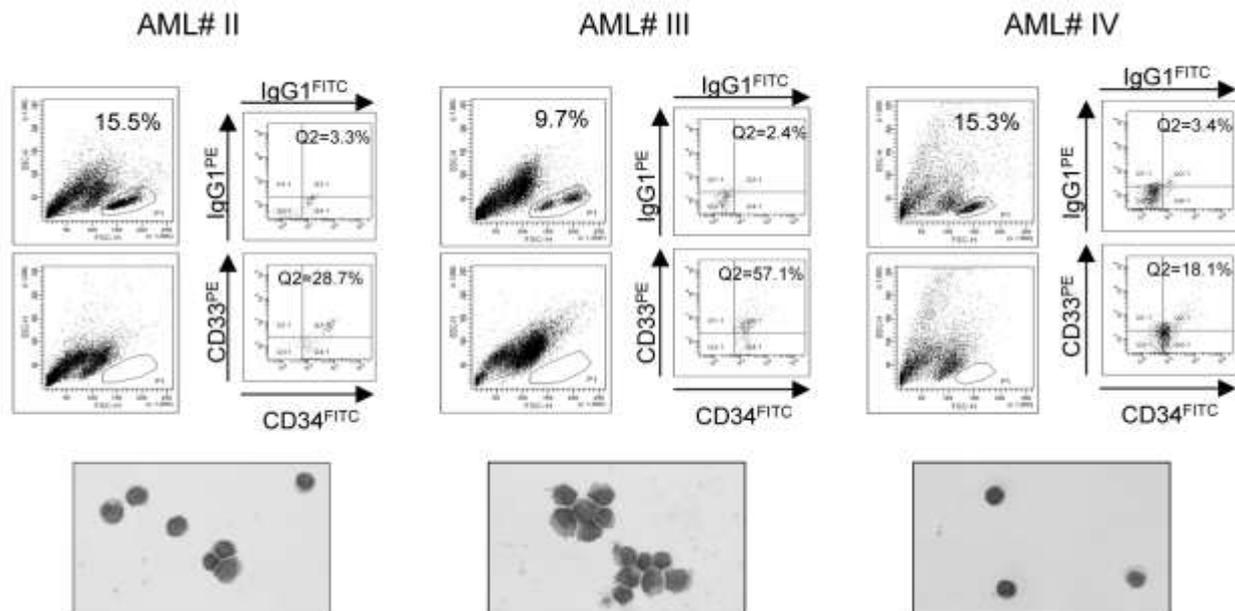


Figure 2. AML cells ablated by canertinib display blast cell appearance and express immature markers

Mononuclear cells from three AML patients (patient II-IV as in Figure 4) were cultured for 24 hours without canertinib, after which the cell population displaying intermediate-high forward and low side scatter (P1 gate), and previously shown to be eradicated by canertinib-treatment at 5 μ M (lower left dot plots), was analysed for expression of CD33 and CD34 by flow cytometry. Numbers indicate double-positive staining cells in upper right quadrants (Q2). The same cells were also FACS-sorted (within the P1 gate) for cytopsin preparations and stained by May-Grunwald-Giemsa.

Canertinib induces apoptosis in human leukaemic cell lines expressing mutated FLT3

Due to the results that canertinib had certain selectivity to FLT3-ITD, we next tested its cytostatic effects on human leukaemic cell lines expressing either FLT3-ITD or wildtype FLT3. Canertinib induced apoptosis at 0.3 μ M in MV4;11, expressing a mutated FLT3-ITD allele (Quentmeier, *et al* 2003), and in MonoMac-6, carrying an activated FLT3-V592A mutation (Spiekermann, *et al* 2003), whereas cell lines expressing wildtype FLT3 (THP-1, NB4, and RS4;11) died only at significantly higher concentrations (Figure 3A). Within 6 hours of treatment with 1 μ M canertinib, the levels of fragments of cleaved caspase-3

(17/19/20 kDa) and the 86-kDa fragment of cleaved poly (ADP-ribose) polymerase (PARP) as characteristic signs of apoptosis increased (Figure 3B). Western blot analysis confirmed that ERBB expression was absent in the cell lines (data not shown).

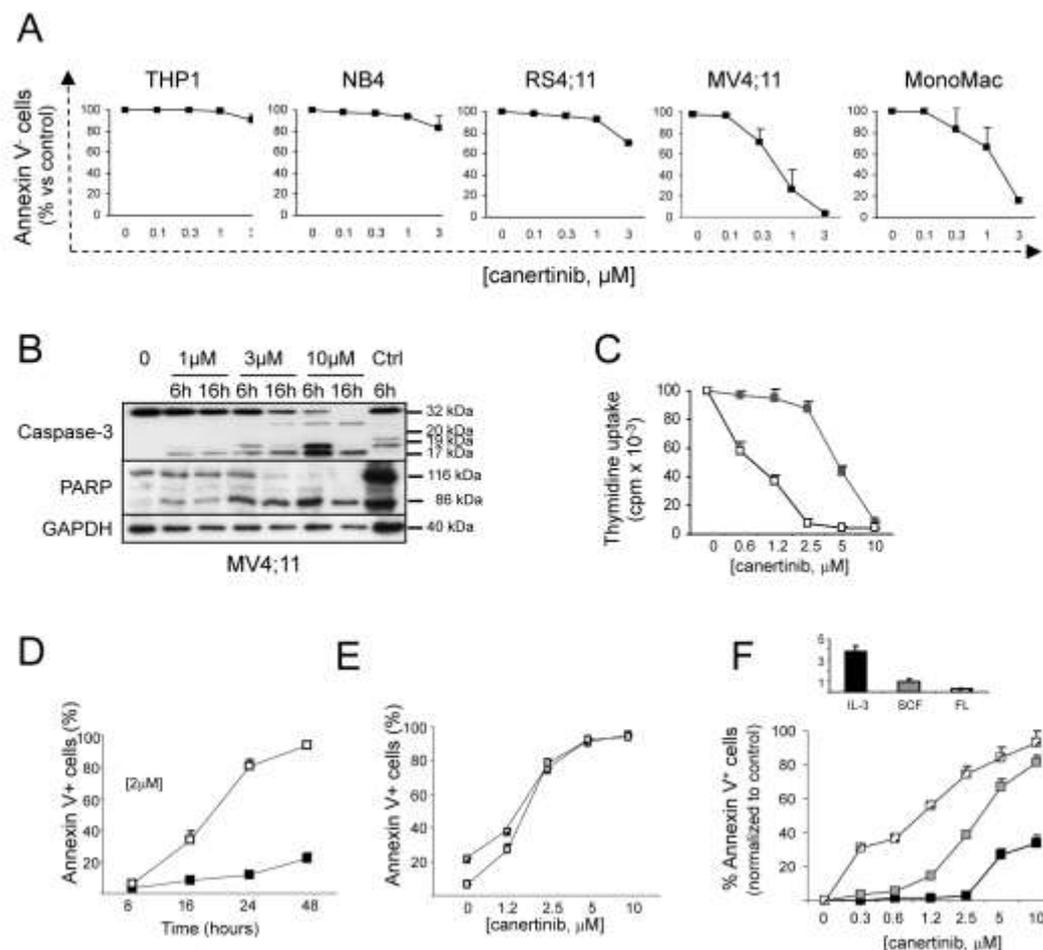


Figure 3. Canertinib induces apoptosis of human leukaemic cell lines expressing mutated FLT3

(A) Leukaemic cells (1×10^5 cells/ml) expressing wildtype FLT3 (THP-1, NB4, RS4;11), or mutated FLT3 (MV4;11, MonoMac-6) were cultured with or without canertinib. Forty-eight hours later cells were analysed for apoptosis by flow cytometry by staining with Annexin V-FITC and propidium iodide. Data shown are mean \pm SD from three experiments. (B) Western blot analysis for cleavage of caspase-3 and PARP in MV4;11 cells treated for 6 and 16 hours with 1, 3, or 10 μ M canertinib. GAPDH was used as a loading control, and Jurkat cells treated 6 hours with camptothecin at 5 μ M as a control for PARP cleavage. (C) FDC-P1 cells (black squares) stimulated with IL-3 or FDC-P1 cells retrovirally transduced with FLT3-ITD (white squares) were cultured with increasing concentrations of canertinib. Proliferation was measured by [3 H]-TdR incorporation after 72 hours. Results are presented as the mean value \pm SD ($n=3$) of percent of control. (D) A time course study was performed on FDC-P1 cells with IL-3 or FDC-P1/FLT3-ITD with a constant concentration of canertinib (2 μ M) and cells were analysed for apoptosis by flow cytometry by Annexin V-PI staining. Data are mean \pm SD from three experiments performed in duplicates. (E) FDC-P1 cells expressing FLT3 were stimulated with FL and analysed for apoptosis after addition of increasing concentrations of canertinib. FDC-P1/FLT3-ITD cells were tested without cytokine (white squares). Numbers of apoptotic cells were analysed after 48 hours by flow cytometry by Annexin V-PI staining. (F) FDC-P1/FLT3 cells stimulated with FL (50 ng/ml; striped squares), SCF (100 ng/ml; grey squares), or IL-3 (10 ng/ml; black squares) with increasing concentrations of canertinib. Forty-eight hours later, cells were analysed for apoptotic cells by flow cytometry. IC₅₀-value for sensitivity to canertinib of FDC-P1/FLT3 cells stimulated with either FL, SCF, or IL-3 as estimated by MTT assays performed in triplicates for 72 hours by titration of canertinib (0.01-10 μ M). Results are presented as mean \pm SD ($n=3$).

Canertinib induces cell cycle block and apoptosis in hematopoietic progenitor cells expressing either wild-type or FLT3-ITD

By using mouse FDC-P1 progenitor cells expressing FLT3 or FLT3-ITD we could confirm canertinib's inhibiting activity. At low concentrations, a 50% reduction of proliferation of FDC-P1/FLT3-ITD cells was seen at 0.8 μM , whereas FDC-P1 cells stimulated with IL-3 were affected only at higher concentrations (Figure 3C). Canertinib led to pronounced cell cycle arrest and exit from S-phase in FDC-P1/FLT3-ITD cells but not in FDC-P1 cells with IL-3 (not shown). Treatment at 5 μM resulted in time-dependent increase of apoptotic FLT3-ITD-expressing cells, whereas IL-3 stimulated cells were essentially unaffected (Figure 3D). Furthermore, canertinib inhibited ligand-dependent activation of WT FLT3, and after 48 hours of FLT3 ligand stimulation, equivalent number of cells became apoptotic with increasing amounts of canertinib as compared to FLT3-ITD (Figure 3E). To test the selectivity of canertinib, FDC-P1/FLT3 cells known also to express c-KIT were used. When stimulated with FLT3 ligand cell viability decreased after 48 hours at low canertinib concentrations (0.3-1.2 μM), whereas cells stimulated with SCF (KIT ligand) or IL-3 were unaffected (Figure 3F). However, at higher concentrations ($> 5 \mu\text{M}$) more than 50% of the cells stimulated with SCF stained positive for Annexin V. The inhibitory concentration of canertinib to reduce proliferation by 50% (IC_{50}) was determined by MTT assay to 0.27 μM for FL-engaged wildtype FLT3 and mutated FLT3-ITD, 3.8 μM for IL-3, and 1.0 μM for SCF (inserted in Figure 3F). The results therefore suggest that cells expressing activated FLT3 are significantly more sensitive to canertinib inhibition.

Canertinib inhibits FLT3 autophosphorylation and downstream targets AKT and ERK

To further clarify the selectivity of canertinib, we analysed receptor autophosphorylation in Ba/F3 cells expressing FLT3, FLT3-ITD, or FLT3-TKD/D835Y. Canertinib inhibited both

FLT3 ligand-induced and constitutive receptor tyrosine phosphorylation in a dose-dependent manner. Inhibition was apparent at concentrations $>2.5 \mu\text{M}$ (Figure 4A). A simultaneous decrease in kinase activity of FLT3 receptor was seen in all three cell lines when tested for phosphorylation of myelin basic protein as an *in vitro* target (Figure 4A). Treatment with canertinib for 1 hour led to dose-dependent dephosphorylation of both AKT and ERK1/2 in the three cell lines at 0.6-1.2 μM (Figure 4B). In contrast, when parental Ba/F3 cells were stimulated with IL-3, both AKT and ERK1/2 remained phosphorylated even at 10 μM of canertinib treatment for 1 hour, although a tendency to less phosphorylated ERK1/2 was seen (Figure 4C).

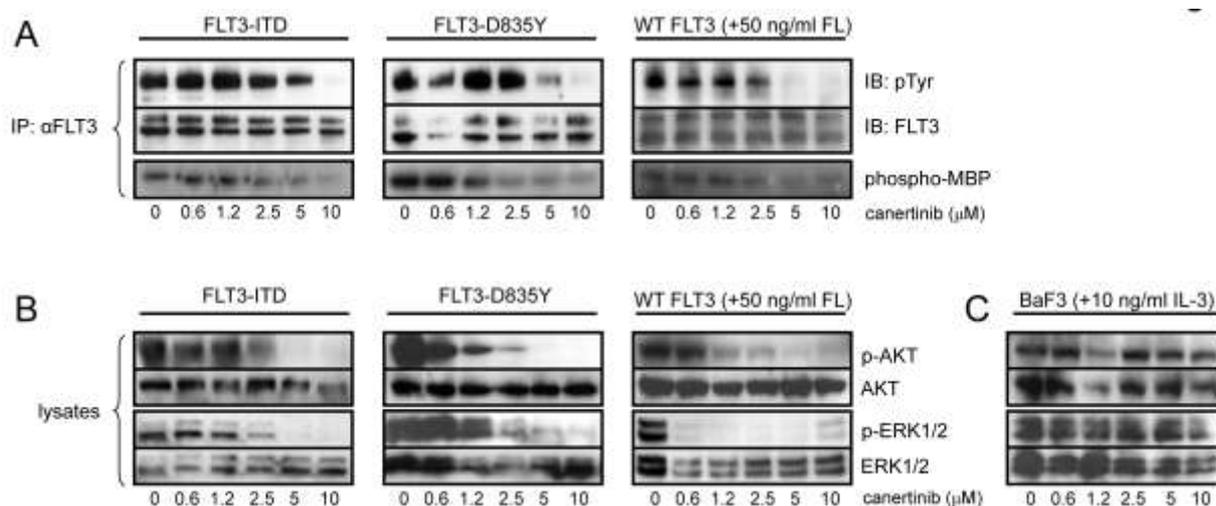


Figure 4. Canertinib inhibits FLT3-driven phosphorylation and signalling

Ba/F3 cells expressing FLT3, FLT3-ITD, or FLT3-D835Y were incubated with canertinib for 2 hours. Ba/F3-FLT3 cells were starved before receiving canertinib for 2 hours and FL for 30 minutes. (A) Cell lysates were subjected to immunoprecipitation with anti-human-FLT3 antibody, followed by immunoblotting for p-Tyr or total FLT3. Immunoprecipitated FLT3 was also subjected to *in vitro*-kinase assays for phosphorylation of myelin basic protein (MBP). (B-C) Western blot analysis for p-AKT and p-ERK1/2 in Ba/F3 cells with FLT3, FLT3-ITD, or FLT3-D835Y (B) or non-transduced Ba/F3 cells stimulated with IL-3 for 15 minutes (C). Blots were reprobed with antibody to AKT and ERK1/2 to ensure equal loading. Experiments were repeated twice.

Apoptosis induced by canertinib involves downregulation of phosphorylated FOXO3a and accumulation of proapoptotic BCL2L1/BIM

FOXO3a is a downstream target of AKT, and is phosphorylated and inactivated by FLT3 signalling (Jönsson, *et al* 2004, Scheijen, *et al* 2004). We therefore investigated whether FOXO3a was activated by canertinib treatment. FDC-P1/FLT3-ITD cells treated with increasing concentrations of canertinib displayed dose-dependent FOXO3a dephosphorylation at concentrations above 0.6 μ M (Figure 5A).

The BH3-only protein gene BCL2L11 (also *BIM*) is a transcriptional target of dephosphorylated FOXO3, which is known to initiate apoptosis (Dijkers, *et al* 2000, Essafi, *et al* 2005) and is upregulated in FLT3-ITD cells treated with FLT3 selective inhibitors (Nordigården, *et al* 2009). In addition, previous reports have described the ability of ERBB1 inhibitors such as erlotinib and gefinitib to execute apoptosis via BCL2L11 (Costa, *et al* 2007, Cragg, *et al* 2007, Gong, *et al* 2007). We first tested if introduction of FLT3-ITD could prevent BCL2L11 induction in cytokine-deprived FDC-P1 cells. As shown in Figure 5B, BCL2L11 was induced when FDC-P1 cells were starved of IL-3, and this induction was prevented by FLT3-ITD. Inhibition of FLT3-ITD phosphorylation by canertinib treatment led to strong induction of BCL2L11 (Figure 5B), indicating that activation of BCL2L11 is a potential mechanism by which canertinib executes apoptosis. In agreement with this notion, we could demonstrate upregulation of *BCL2L11* RNA expression by quantitative PCR in MV4;11 cells treated with canertinib, whereas *BCL2L11* was induced to a lesser degree in FLT3-wildtype THP-1 cells (Figure 5C). By gene silencing experiments, we established that BCL2L11 was required for canertinib-mediated apoptosis. Thus, the number of viable FDC-P1/FLT3-ITD cells (Figure 5D) increased to $60.0 \pm 10.9\%$ ($p < 0.001$) with *BCL2L11* siRNA compared to $27.8 \pm 11.6\%$ in cells with control siRNA and treated with 1 μ M canertinib or 5 μ M of AG1295, previously shown to inhibit FLT3 (Levis, *et al* 2001). Because FOXO3a

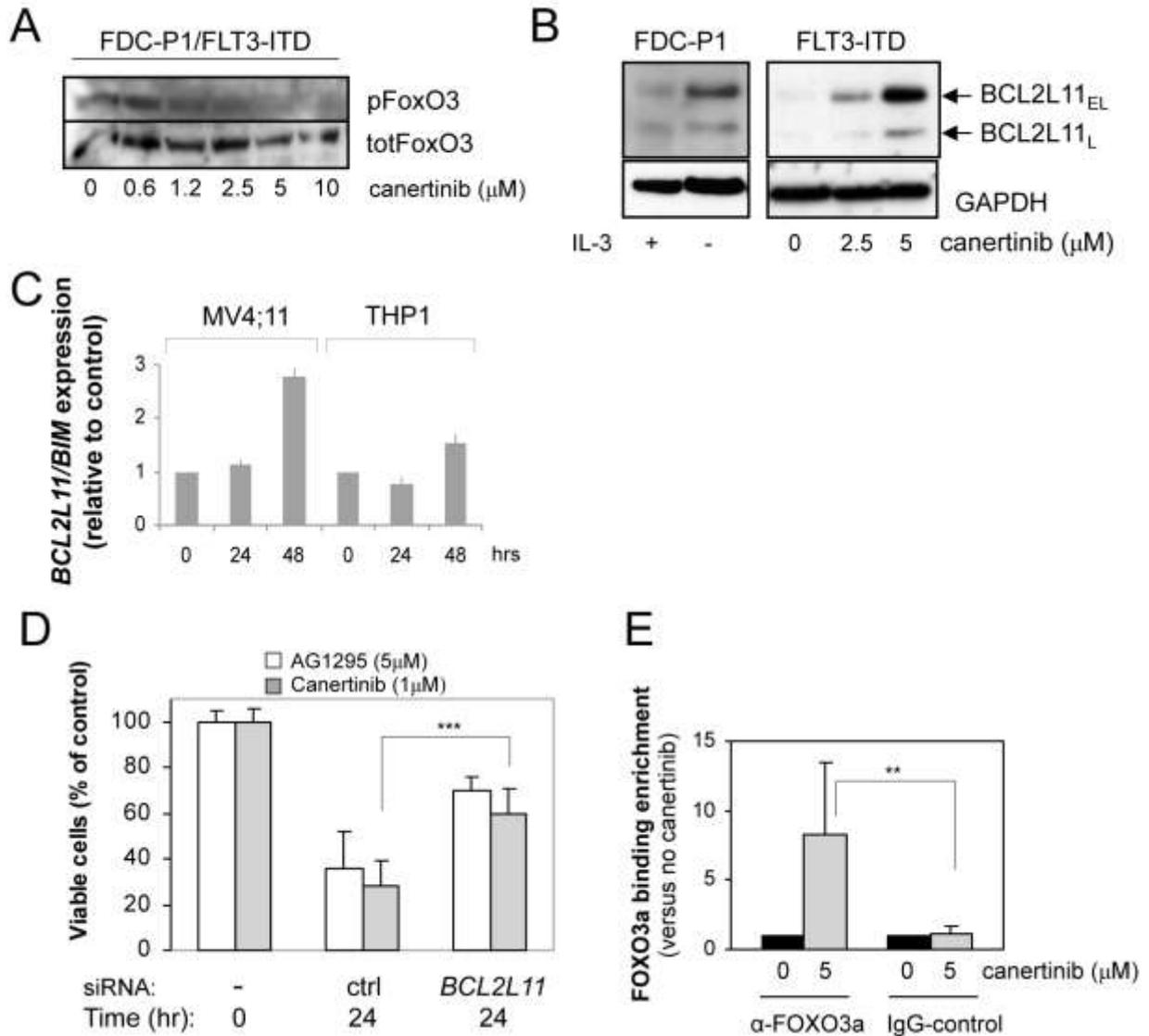


Figure 5. BH3-only protein *BCL2L11/BIM* is critical in apoptotic induction of *FLT3* receptor inhibition by canertinib

(A) FDC-P1/FLT3-ITD cells treated for 2 hours with canertinib were immunoblotted with antibodies to p-FOXO3a. (B) FDC-P1 cells starved from IL-3 for 18 hours and FDC-P1/FLT3-ITD cells treated with canertinib for 18 hours were immunoblotted with antibodies to *BCL2L11*. (C) Relative RNA expression of *BCL2L11* in MV4;11 and THP-1 cells were measured by real-time PCR after 24 and 48 hours of treatment (5 μM). Results are from triplicates of three independent experiments. (D) FDC-P1/FLT3-ITD cells were nucleofected with siRNA oligonucleotides against mouse *BCL2L11* (0.7 μg) or with a control siRNA (pmaxGFPTM; 1.5 μg). Four hours post-transfection, AG1295 (5 μM) or canertinib (1 μM) was added. Cells were harvested after 24 hours and analysed for apoptosis by flow cytometry by Annexin V-PI staining. Data are mean \pm SEM from three experiments. *** Significant difference ($p < 0.001$). (E) ChIP-qPCR analysis for FOXO3a binding to the *BCL2L11* promotor. Sonicated DNA from FDC-P1/FLT3-ITD cells treated with canertinib at 5 μM for 5 hours was immunoprecipitated with control rabbit IgG or anti-FOXO3a and amplified by qPCR using primers for the *BCL2L11* promotor. Relative expression was normalised to the input value and then compared to the corresponding untreated samples. Error bars represent SEM of triplicate reactions from four separate analyses. ** Significant difference ($p < 0.01$).

activates *BCL2L1* transcription we wanted to confirm that FOXO3a binds to the promoter in canertinib-treated cells. Indeed, chromatin immunoprecipitations with anti-FOXO3a on the Forkhead responsive element (FHRE) in the *BCL2L1* promoter of FOXO3a-DNA complexes from FDC-P1/FLT3-ITD cells treated with 5 μ M canertinib revealed that canertinib induces apoptosis by direct binding of FOXO3a to the *BCL2L1* promoter. Thus, a 8-fold increase in activity ($p < 0.01$) compared to untreated cells was seen (Figure 3E).

Canertinib induces regression of intravenously injected FLT3-ITD cells in mice

To evaluate the *in vivo* anti-leukaemic activity of canertinib, 2×10^6 FDC-P1 cells expressing FLT3-ITD were injected intravenously into syngenic DBA/2 mice according to the scheme in Figure 6A. We used a retroviral vector containing the same FLT3-ITD as in the previous experiments but EYFP as a marker to enable detection in peripheral blood of mice (Bagrintseva, *et al* 2005). Mice were injected for five consecutive days with either canertinib at two doses or vehicle alone (0.1% DMSO in PBS). Inoculation of mice produced rapid increase of EYFP⁺ cells in peripheral blood within the first week (range: 13.7-23.4%). In mice not injected with canertinib, this continued to increase to reach an average of $50.9 \pm 6.6\%$ EYFP⁺ cells on day 14 (Figure 6B). Necropsy of mice revealed splenomegaly and infiltration of EYFP⁺ cells (not shown). However, in mice treated with canertinib at a dose of 80 mg/kg, the numbers were significantly decreased to $13.5 \pm 7.8\%$, corresponding to 74% reduction ($p < 0.001$), and spleens were dramatically reduced (not shown). An intermediate response ($40.2 \pm 4.8\%$; $p < 0.01$) was seen in mice receiving 40 mg/kg.

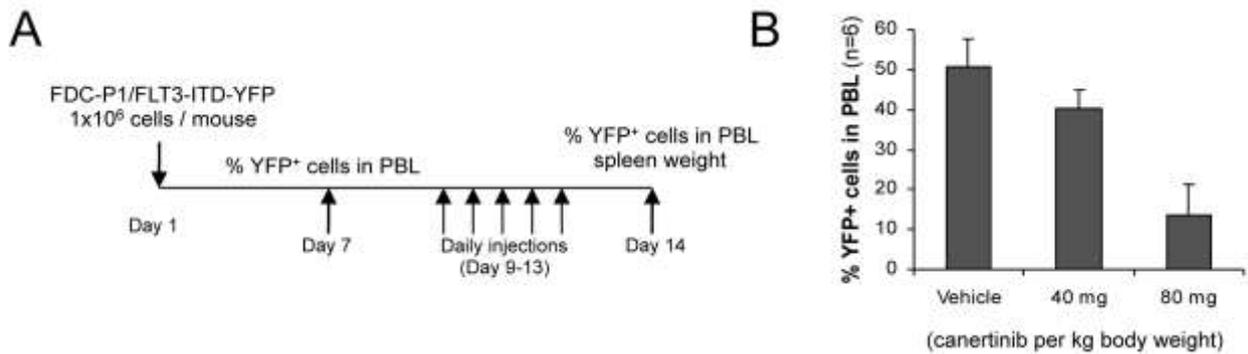


Figure 6. Canertinib induces regression of intravenously injected FDC-P1/FLT3-ITD cells in transplanted mice

(A) Experimental outline: FDC-P1 cells (2×10^6) transduced with FLT3-ITD-EYFP were tail-injected into syngenic DBA/2 mice. Engraftment in peripheral blood was determined by flow cytometry on day 7. Treatment was initiated on day 9 by intraperitoneal injections of 0.5 mL canertinib and continued for four days. On day 14, the experiment was terminated and engraftment was analysed again. **(B)** Level of EYFP-engraftment of FLT3-ITD cells in peripheral blood from mice treated with PBS or canertinib at two doses (40 or 80 mg/kg bodyweight). Results are mean \pm SEM (n=6). ** Significant difference ($p < 0.01$) versus control; *** Significant difference ($p < 0.001$) versus control.

Discussion

Recent studies have demonstrated that TKI such as gefitinib and erlotinib, selectively inhibiting the ERBB1/EGF receptor, have anti-proliferative effects in an ERBB receptor-independent manner on leukaemic cell lines in the micromolar range (5-10 μM) (Boehrer, *et al* 2008, Stegmaier, *et al* 2005). We have reported on similar results with the pan-ERBB inhibitor canertinib, in which treatment with 2 μM canertinib promoted cell cycle arrest of the human leukaemic cell lines HL-60 and U-937, whereas doses of 5 μM or more induced apoptosis (Trinks, *et al* 2010). In this study, we demonstrate that at lower concentrations (0.1-0.3 μM) canertinib acts as an inhibitor of activated FLT3 receptor in human leukaemic cell lines expressing mutated FLT3 as well as progenitor cell lines expressing either wildtype FLT3 ligand stimulated receptor or mutated receptor. Canertinib inhibited autophosphorylation and kinase activity of wildtype FLT3, FLT3-ITD, and FLT3-TKD/D835Y, and downstream signalling via PI3-kinase and RAS/MAP kinase were inactivated. Apoptosis was a consequence of canertinib-mediated activation of the transcription factor FOXO3a leading to upregulation of BH3-only protein BCL2L11/BIM. This is similar to recent studies in which the BCR-ABL1 protein in chronic myelogenous leukaemia and FLT3-ITD in AML support cell survival through downregulation of *BCL2L11* expression by a FOXO3a-dependent mechanism (Brandts, *et al* 2005, Kuribara, *et al* 2004, Scheijen, *et al* 2004). Moreover, the BCR-ABL1-selective drug imatinib elicits apoptotic responses via BCL2L11 (Essafi, *et al* 2005), similar to apoptosis induced by the selective FLT3 inhibitor PKC412 (Nordigården *et al*, 2009). Several recent studies also suggest that BCL2L11 executes apoptosis of solid tumor cells treated with ERBB1/EGF inhibitors (Costa, *et al* 2007, Cragg, *et al* 2007, Gong, *et al* 2007). Our results that RNAi-mediated *BCL2L11* knockdown protects FDC-P1/FLT3-ITD cells from apoptosis, and that FOXO3a binds directly to the *BCL2L11* promotor upon canertinib-treatment, provide one possible mechanistic insight to how canertinib is executing its cytotoxic effects in leukaemic cells

expressing FLT3. This is to our knowledge the first study demonstrating the mechanism by which an ERBB inhibitor causes apoptosis of leukaemic cells and exemplifies that a selective blocking agents developed to target a specific group of receptors may be useful for treatment of other pathways. Since canertinib is a pan-ErbB inhibitor acting on all members of the family and not only ERBB1/EGFR, it is likely to display a wider inhibition. In addition, covalent binding of canertinib to specific cysteines in the kinase domain results in irreversible suppression of ATP-binding and catalytic activity (Slichenmyer *et al*, 2001; Smaill *et al*, 2000), features that may provide canertinib with a therapeutic advantage relative to other inhibitors. It is possible that canertinib displays a similar effect on FLT3 by binding to important cysteine residues in the two kinase domains of the FLT3 receptor, for instance Cys-694 in the ATP-binding site (Mahboobi, *et al* 2006).

Compared to some other FLT3 inhibitors canertinib appears to exert its effects at higher concentrations. The micromolar range of canertinib necessary to reach a decrease of viability of primary AML blasts varied between patient samples and was significantly higher than the doses used for cell lines (0.3-1.2 μM versus 5-20 μM). This is not surprising, however, due to the heterogeneous nature of the disease. Previous studies with other FLT3 kinase inhibitors, e.g., CEP701 and PKC412/midostaurin, have shown that the cytotoxic response *in vitro* varies between AML blast samples and occurs at higher drug concentrations than in FLT3-transformed cell lines (Knapper, *et al* 2006). PKC412 inhibits the proliferation of Ba/F3-FLT3-ITD cells with an IC_{50} of less than 10 nM and displays potent inhibition of FLT3 tyrosine autophosphorylation with 0.01-1 μM . Using a tyrosine kinase inhibition assay, PKC412 was demonstrated to have an IC_{50} of approximately 0.5 μM (Weisberg, *et al* 2002). When applied on primary AML cells, even higher concentrations were needed to inhibit growth. In bone marrow transplantation assays, PKC412 at 100 mg/kg exerted antitumor

effects. This is similar to the dose at 80 mg/kg by which canertinib in our study eradicated FLT3-ITD-transformed FDC-P1 cells in peripheral blood of syngenic mice.

Recent clinical trials suggest that many cancer patients can benefit from ERBB-targeted agents. While side effects such as skin rashes and diarrhea are found in some patients assigned to canertinib-treatment, haematological parameters are seldom affected and limited to a certain degree of thrombocytopenia in some patients (Janne, *et al* 2007, Zinner, *et al* 2007). Thus, the effects of canertinib on leukaemic cell lines and primary AML samples presented here are promising with respect to this drug as an anti-leukaemic regimen. Moreover, the ability of canertinib to inhibit both mutated FLT3 and ligand-stimulated wildtype FLT3 may provide an advantage for treatment of leukaemia patients overexpressing non-mutated FLT3. It has been shown that canertinib is less specific for the ERBB family than previously concluded (Fabian, *et al* 2005). The specificity profile of canertinib against a large panel of kinases demonstrated that the drug is among the more promiscuous compounds. Therefore it is not surprising that canertinib was not truly selective for FLT3 and also inhibited c-KIT signaling, although at significantly higher concentrations. In addition, we have recently found that canertinib inhibits other tyrosine kinases such as LCK and ZAP70 associated to the T-cell receptor complex (Trinks, *et al* 2011). This adds the possibility that canertinib besides some degree of FLT3 selectivity could also act by kinase multi-targeting, providing the drug with further therapeutic advantages.

If targeted therapy will prove possible with canertinib in AML patients, combining the drug with other small molecules could suppress the disease more efficiently. It will also be of interest to study if canertinib can overcome resistance to FLT3-selective drugs in AML cells in an equivalent manner as its ability to inhibit tumor cells resistant to gefinitib or erlotinib (Steeghs, *et al* 2007, Wang, *et al* 2006), thereby offering an alternative rationale for future AML treatment.

Authorship

J.I.J.: Conception and design, collection and assembly of data, data analysis, and drafted the manuscript; K.L. and T.M.W.: conception, data analysis; A.N. and J.Z.: collection and assembly of data, data analysis; C.T., H.G., P.E., and P.D.: collection of data, data analysis; L.R.: provided material, data analysis. All authors have participated in evaluation of art work, and have participated in writing the manuscript.

Competing interest

The authors declare no competing interests.

Acknowledgements

The authors thank Dr. Donnie W. Owens (Pfizer Global Research & Development, Ann Arbor, Michigan) for the generous supply of canertinib. We also thank Drs. G. Gilliland, J. Griffin, E. Lam, and F. Öberg for reagents and cell lines. We are grateful to K. Ellnebo Svedlund and P. Hammar for technical help. This work was supported by grants from the Swedish Cancer Foundation, the Swedish Children's Cancer Foundation, the Swedish Research Council, the County Council of Östergötland, the Cancer Foundation of Östergötland, and the Ollie and Elof Ericssons Foundation.

References

- Ako, E., Yamashita, Y., Ohira, M., Yamazaki, M., Hori, T., Kubo, N., Sawada, T. & Hirakawa, K. (2007) The pan-erbB tyrosine kinase inhibitor CI-1033 inhibits human esophageal cancer cells in vitro and in vivo. *Oncol Rep*, **17**, 887-893.
- Bagrintseva, K., Geisenhof, S., Kern, R., Eichenlaub, S., Reindl, C., Ellwart, J.W., Hiddemann, W. & Spiekermann, K. (2005) FLT3-ITD-TKD dual mutants associated with AML confer resistance to FLT3 PTK inhibitors and cytotoxic agents by overexpression of Bcl-x(L). *Blood*, **105**, 3679-3685.
- Boehrer, S., Ades, L., Braun, T., Galluzzi, L., Grosjean, J., Fabre, C., Le Roux, G., Gardin, C., Martin, A., de Botton, S., Fenaux, P. & Kroemer, G. (2008) Erlotinib exhibits anti-neoplastic off-target effects in AML and MDS: a preclinical study. *Blood*, **111**, 2170-2180.
- Brandts, C.H., Sargin, B., Rode, M., Biermann, C., Lindtner, B., Schwable, J., Buerger, H., Muller-Tidow, C., Choudhary, C., McMahon, M., Berdel, W.E. & Serve, H. (2005) Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. *Cancer Res*, **65**, 9643-9650.
- Campos, S., Hamid, O., Seiden, M.V., Oza, A., Plante, M., Potkul, R.K., Lenahan, P.F., Kaldjian, E.P., Varterasian, M.L., Jordan, C., Charbonneau, C. & Hirte, H. (2005) Multicenter, randomized phase II trial of oral CI-1033 for previously treated advanced ovarian cancer. *J Clin Oncol*, **23**, 5597-5604.
- Costa, D.B., Halmos, B., Kumar, A., Schumer, S.T., Huberman, M.S., Boggon, T.J., Tenen, D.G. & Kobayashi, S. (2007) BIM mediates EGFR tyrosine kinase inhibitor-induced apoptosis in lung cancers with oncogenic EGFR mutations. *PLoS Med*, **4**, 1669-1679; discussion 1680.
- Cragg, M.S., Kuroda, J., Puthalakath, H., Huang, D.C. & Strasser, A. (2007) Gefitinib-induced killing of NSCLC cell lines expressing mutant EGFR requires BIM and can be enhanced by BH3 mimetics. *PLoS Med*, **4**, 1681-1689; discussion 1690.
- Dijkers, P.F., Medema, R.H., Lammers, J.W., Koenderman, L. & Coffey, P.J. (2000) Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol*, **10**, 1201-1204.
- Essafi, A., Fernandez de Mattos, S., Hassen, Y.A., Soeiro, I., Mufti, G.J., Thomas, N.S., Medema, R.H. & Lam, E.W. (2005) Direct transcriptional regulation of Bim by FoxO3a mediates STI571-induced apoptosis in Bcr-Abl-expressing cells. *Oncogene*, **24**, 2317-2329.
- Fabian, M.A., Biggs, W.H., 3rd, Treiber, D.K., Atteridge, C.E., Azimioara, M.D., Benedetti, M.G., Carter, T.A., Ciceri, P., Edeen, P.T., Floyd, M., Ford, J.M., Galvin, M., Gerlach, J.L., Grotzfeld, R.M., Herrgard, S., Insko, D.E., Insko, M.A., Lai, A.G., Lelias, J.M., Mehta, S.A., Milanov, Z.V., Velasco, A.M., Wodicka, L.M., Patel, H.K., Zarrinkar, P.P. & Lockhart, D.J. (2005) A small molecule-kinase interaction map for clinical kinase inhibitors. *Nat Biotechnol*, **23**, 329-336.
- Gong, Y., Somwar, R., Politi, K., Balak, M., Chmielecki, J., Jiang, X. & Pao, W. (2007) Induction of BIM is essential for apoptosis triggered by EGFR kinase inhibitors in mutant EGFR-dependent lung adenocarcinomas. *PLoS Med*, **4**, e294.
- Green H, Lotfi K, Zackrisson AL, Peterson C. Spontaneous reversal of p-glycoprotein expression in multidrug resistant cell lines. (2003) *Pharmacol Toxicol*, **93**, 297-304.
- Janne, P.A., von Pawel, J., Cohen, R.B., Crino, L., Butts, C.A., Olson, S.S., Eiseman, I.A., Chiappori, A.A., Yeap, B.Y., Lenahan, P.F., Dasse, K., Sheeran, M. & Bonomi, P.D. (2007)

- Multicenter, randomized, phase II trial of CI-1033, an irreversible pan-ERBB inhibitor, for previously treated advanced non small-cell lung cancer. *J Clin Oncol*, **25**, 3936-3944.
- Jönsson, M., Engström, M. & Jönsson, J.I. (2004) FLT3 ligand regulates apoptosis through AKT-dependent inactivation of transcription factor FoxO3. *Biochem Biophys Res Commun*, **318**, 899-903.
- Kelly, L.M., Liu, Q., Kutok, J.L., Williams, I.R., Boulton, C.L. & Gilliland, D.G. (2002) FLT3 internal tandem duplication mutations associated with human acute myeloid leukemias induce myeloproliferative disease in a murine bone marrow transplant model. *Blood*, **99**, 310-318.
- Kindler, T., Lipka, D.B. & Fischer, T. (2010) FLT3 as a therapeutic target in AML: still challenging after all these years. *Blood*, **116**, 5089-5102.
- Knapper, S., Mills, K.I., Gilkes, A.F., Austin, S.J., Walsh, V. & Burnett, A.K. (2006) The effects of lestaurtinib (CEP701) and PKC412 on primary AML blasts: the induction of cytotoxicity varies with dependence on FLT3 signaling in both FLT3-mutated and wild-type cases. *Blood*, **108**, 3494-3503.
- Kuribara, R., Honda, H., Matsui, H., Shinjyo, T., Inukai, T., Sugita, K., Nakazawa, S., Hirai, H., Ozawa, K. & Inaba, T. (2004) Roles of Bim in apoptosis of normal and Bcr-Abl-expressing hematopoietic progenitors. *Mol Cell Biol*, **24**, 6172-6183.
- Lee, B.H., Williams, I.R., Anastasiadou, E., Boulton, C.L., Joseph, S.W., Amaral, S.M., Curley, D.P., Duclos, N., Huntly, B.J., Fabbro, D., Griffin, J.D. & Gilliland, D.G. (2005) FLT3 internal tandem duplication mutations induce myeloproliferative or lymphoid disease in a transgenic mouse model. *Oncogene*, **24**, 7882-7892.
- Levis, M., Tse, K.F., Smith, B.D., Garrett, E. & Small, D. (2001) A FLT3 tyrosine kinase inhibitor is selectively cytotoxic to acute myeloid leukemia blasts harboring FLT3 internal tandem duplication mutations. *Blood*, **98**, 885-887.
- Li, L., Piloto, O., Nguyen, H.B., Greenberg, K., Takamiya, K., Racke, F., Huso, D. & Small, D. (2008) Knock-in of an internal tandem duplication mutation into murine FLT3 confers myeloproliferative disease in a mouse model. *Blood*, **111**, 3849-3858.
- Mahboobi, S., Uecker, A., Sellmer, A., Cenac, C., Hocher, H., Pongratz, H., Eichhorn, E., Hufsky, H., Trumpler, A., Sicker, M., Heidel, F., Fischer, T., Stocking, C., Elz, S., Bohmer, F.D. & Dove, S. (2006) Novel bis(1H-indol-2-yl)methanones as potent inhibitors of FLT3 and platelet-derived growth factor receptor tyrosine kinase. *J Med Chem*, **49**, 3101-3115.
- Nordigården, A., Kraft, M., Eliasson, P., Labi, V., Lam, E.W., Villunger, A. & Jönsson, J.I. (2009) BH3-only protein Bim more critical than Puma in tyrosine kinase inhibitor-induced apoptosis of human leukemic cells and transduced hematopoietic progenitors carrying oncogenic FLT3. *Blood*, **113**, 2302-2311.
- Ocana, A. & Amir, E. (2009) Irreversible pan-ErbB tyrosine kinase inhibitors and breast cancer: current status and future directions. *Cancer Treat Rev*, **35**, 685-691.
- Quentmeier, H., Reinhardt, J., Zaborski, M. & Drexler, H.G. (2003) FLT3 mutations in acute myeloid leukemia cell lines. *Leukemia*, **17**, 120-124.
- Razumovskaya, E., Masson, K., Khan, R., Bengtsson, S. & Rönstrand, L. (2009) Oncogenic Flt3 receptors display different specificity and kinetics of autophosphorylation. *Exp Hematol*, **37**, 979-989.
- Rocha-Lima, C.M., Soares, H.P., Raez, L.E. & Singal, R. (2007) EGFR targeting of solid tumors. *Cancer Control*, **14**, 295-304.
- Scheijen, B., Ngo, H.T., Kang, H. & Griffin, J.D. (2004) FLT3 receptors with internal tandem duplications promote cell viability and proliferation by signaling through Foxo proteins. *Oncogene*, **23**, 3338-3349.

- Slichenmyer, W.J., Elliott, W.L. & Fry, D.W. (2001) CI-1033, a pan-erbB tyrosine kinase inhibitor. *Semin Oncol*, **28**, 80-85.
- Smaill, J.B., Rewcastle, G.W., Loo, J.A., Greis, K.D., Chan, O.H., Reyner, E.L., Lipka, E., Showalter, H.D., Vincent, P.W., Elliott, W.L. & Denny, W.A. (2000) Tyrosine kinase inhibitors. 17. Irreversible inhibitors of the epidermal growth factor receptor: 4-(phenylamino)quinazoline- and 4-(phenylamino)pyrido[3,2-d]pyrimidine-6-acrylamides bearing additional solubilizing functions. *J Med Chem*, **43**, 1380-1397.
- Spiekermann, K., Dirschinger, R.J., Schwab, R., Bagrintseva, K., Faber, F., Buske, C., Schnittger, S., Kelly, L.M., Gilliland, D.G. & Hiddemann, W. (2003) The protein tyrosine kinase inhibitor SU5614 inhibits FLT3 and induces growth arrest and apoptosis in AML-derived cell lines expressing a constitutively activated FLT3. *Blood*, **101**, 1494-1504.
- Steehls, N., Nortier, J.W. & Gelderblom, H. (2007) Small molecule tyrosine kinase inhibitors in the treatment of solid tumors: an update of recent developments. *Ann Surg Oncol*, **14**, 942-953.
- Stegmaier, K., Corsello, S.M., Ross, K.N., Wong, J.S., Deangelo, D.J. & Golub, T.R. (2005) Gefitinib induces myeloid differentiation of acute myeloid leukemia. *Blood*, **106**, 2841-2848.
- Taussig, D.C., Pearce, D.J., Simpson, C., Rohatiner, A.Z., Lister, T.A., Kelly, G., Luongo, J.L., Danet-Desnoyers, G.A. & Bonnet, D. (2005) Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia. *Blood*, **106**, 4086-4092.
- Trinks, C., Djerf, E.A., Hallbeck, A.L., Jönsson, J.I. & Walz, T.M. (2010) The pan-ErbB receptor tyrosine kinase inhibitor canertinib induces ErbB-independent apoptosis in human leukemia (HL-60 and U-937) cells. *Biochem Biophys Res Commun*, **393**, 6-10.
- Trinks, C., Severinson, E.A., Holmlund, B., Green, A., Green, H., Jönsson, J.I., Hallbeck, A.L., & Walz, T.M. (2011) The pan-ErbB tyrosine kinase inhibitor canertinib induces caspase-mediated cell death in human T-cell leukemia (Jurkat) cells. *Biochem Biophys Res Commun*, **410**, 422-427.
- Vardiman, J.W., Thiele, J., Arber, D.A., Brunning, R.D., Borowitz, M.J., Porwit, A., Harris, N.L., Le Beau, M.M., Hellström-Lindberg, E., Tefferi, A. & Bloomfield, C.D. (2009) The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood*, **114**, 937-951.
- Wang, S.E., Narasanna, A., Perez-Torres, M., Xiang, B., Wu, F.Y., Yang, S., Carpenter, G., Gazdar, A.F., Muthuswamy, S.K. & Arteaga, C.L. (2006) HER2 kinase domain mutation results in constitutive phosphorylation and activation of HER2 and EGFR and resistance to EGFR tyrosine kinase inhibitors. *Cancer Cell*, **10**, 25-38.
- Weisberg, E., Boulton, C., Kelly, L.M., Manley, P., Fabbro, D., Meyer, T., Gilliland, D.G. & Griffin, J.D. (2002) Inhibition of mutant FLT3 receptors in leukemia cells by the small molecule tyrosine kinase inhibitor PKC412. *Cancer Cell*, **1**, 433-443.
- Zinner, R.G., Nemunaitis, J., Eiseman, I., Shin, H.J., Olson, S.C., Christensen, J., Huang, X., Lenehan, P.F., Donato, N.J. & Shin, D.M. (2007) Phase I clinical and pharmacodynamic evaluation of oral CI-1033 in patients with refractory cancer. *Clin Cancer Res*, **13**, 3006-3014.