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Molecular Cancer Therapeutics

Potent Activity of Ponatinib (AP24534) in Models of FLT3-Driven Acute Myeloid Leukemia and Other Hematologic Malignancies

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Abstract

Ponatinib (AP24534) is a novel multitargeted kinase inhibitor that potently inhibits native and mutant BCR-ABL at clinically achievable drug levels. Ponatinib also has *in vitro* inhibitory activity against a discrete set of kinases implicated in the pathogenesis of other hematologic malignancies, including FLT3, KIT, fibroblast growth factor receptor 1 (FGFR1), and platelet derived growth factor receptor α (PDGFRα). Here, using leukemic cell lines containing activated forms of each of these receptors, we show that ponatinib potently inhibits receptor phosphorylation and cellular proliferation with IC_{50} values comparable to those required for inhibition of BCR-ABL (0.3 to 20 nmol/L). The activity of ponatinib against the FLT3-ITD mutant, found in up to 30% of acute myeloid leukemia (AML) patients, was particularly notable. In MV4-11 (FLT3-ITD$^{+/+}$) but not RS4;11 (FLT3-ITD$^{-/-}$) AML cells, ponatinib inhibited FLT3 signaling and induced apoptosis at concentrations of less than 10 nmol/L. In an MV4-11 mouse xenograft model, once daily oral dosing of ponatinib led to a dose-dependent inhibition of signaling and tumor regression. Ponatinib inhibited viability of primary leukemic blasts from a FLT3-ITD positive AML patient (IC_{50} 4 nmol/L) but not those isolated from 3 patients with AML expressing native FLT3. Overall, these results support the investigation of ponatinib in patients with FLT3-ITD–driven AML and other hematologic malignancies driven by KIT, FGFR1, or PDGFRα. *Mol Cancer Ther; 10(6); 1028–35. ©2011 AACR.*

Introduction

Ponatinib (AP24534) is an oral multitargeted tyrosine kinase inhibitor (TKI) that has been characterized previously for its ability to potently inhibit BCR-ABL (1–3). Importantly, ponatinib inhibits both native and mutant forms of BCR-ABL, including the T315I gatekeeper mutant that is refractory to all approved TKIs. Ponatinib is currently being investigated in a pivotal phase 2 clinical trial in patients with chronic myeloid leukemia (CML, NCT01207440, www.clinicaltrials.gov). We have previously shown that ponatinib exhibits potent *in vitro* inhibitory activity against a discrete subset of additional protein tyrosine kinases including members of the class III/IV subfamily of receptor tyrosine kinases (RTK) FLT3, KIT, FGFR1 (fibroblast growth factor receptor 1), and platelet derived growth factor receptor α (PDGFRα; ref. 2). Dysregulation of these RTKs, for example via genetic alterations that lead to the generation of fusion proteins or activating mutations, has been implicated in the pathogenesis of multiple hematologic malignancies (4, 5).

Translocations affecting the activity of FGFR1 and PDGFRα are found in a subset of rare myeloproliferative neoplasms (ref. 6). Translocations involving the *FGFR1* gene and a range of other chromosome partners such as the *FGFR1OP2* gene are characteristic of 8p11 myeloproliferative syndrome which is an aggressive disease that can rapidly transform to acute myeloid leukemia (AML; ref. 7). The FIP1L1-PDGFRα fusion protein is found in approximately 10% to 20% of patients with chronic eosinophilic leukemia/idiopathic hypereosinophilia and it has been reported that these patients respond well to PDGFR inhibition (6). Activating mutations in KIT and FLT3 are found in AML. KIT mutations are less common and are found in specific cytogenetic subsets of AML with an overall frequency of 2% to 8% (8). Activating mutations in FLT3 are the most common type of genetic alteration in AML, found in up to 30% of newly diagnosed patients (9). The majority of these mutations arise from an internal tandem duplication (ITD) in the juxtamembrane region of the receptor. Activating point mutations in the kinase activation loop also occur, but with

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lower frequency. FLT3-ITD mutations have been associated with a worse prognosis for AML patients, both in terms of relapse and overall survival, when treated with standard therapy (9–11).

AML is the most common myeloid disorder in adults, which has the worst prognosis of all leukemias and lacks effective targeted therapies (12). FLT3-ITD has emerged as an attractive therapeutic target, and consequently a number of small molecule TKIs with activity against FLT3 have now been developed. Many of these compounds have already been evaluated in clinical trials, including CEP-701 (lestaurtinib), PKC412 (midostaurin), sunitinib, sorafenib, MLN-518 (tandutinib), and KW-2449 (13, 14). Overall, however, most of these agents have shown relatively modest clinical activity and the effects have not been durable, suggesting that first-generation FLT3 inhibitors may have limited utility as single agents (13–15). However, FLT3-ITD remains an attractive drug target and new inhibitors such as AC220 (16, 17) have begun to show promising clinical activity.

We evaluated the cellular activity of ponatinib against FLT3, KIT, FGFR1, and PDGFRα in a panel of leukemic cell lines that express these dysregulated RTKs to explore potential applications of ponatinib in hematologic malignancies beyond BCR-ABL–driven CML. We further assessed the potency and selectivity of ponatinib for FLT3-ITD in primary leukemic blasts and the efficacy of ponatinib in a FLT3-ITD–driven xenograft model.

Materials and Methods

Cell lines, antibodies, and reagents

MV4-11, RS4;11, Kasumi-1, and KG1 cells were obtained from the American Type Culture Collection, and EOL1 cells were obtained from DSMZ. Further cell line authentication was not carried out by the authors. Cells were maintained and cultured according to standard techniques at 37°C in 5% (v/v) CO_2 using RPMI 1640 supplemented with 10% FBS (20% FBS for Kasumi-1 cells). The antibodies used included: phospho-PDGFRα, PDGFRα, FLT3, FGFR1, and GAPDH from Santa Cruz Biotechnology; STAT5, KIT, phospho-KIT, phospho-FGFR, and phospho-FLT3 from Cell Signaling Technology; phospho-STAT5 from BD Biosciences. Ponatinib was synthesized at ARIAD Pharmaceuticals, and sorafenib and sunitinib were purchased from American Custom Chemical Corporation. Stock solutions (10 mmol/L) in dimethyl sulfoxide of the above compounds were prepared and used in all *in vitro* studies.

Cell viability assays

Cell viability was assessed using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay. Exponentially growing cell lines were plated into 96-well plates and incubated overnight at 37°C. Twenty-four hours after plating, cells were treated with compound or vehicle (dimethyl sulfoxide) for 72 hours. Absorbance was measured using a Wallac Victor microplate reader (PerkinElmer). Data are plotted as percent viability relative to vehicle-treated cells and the IC_{50} values (the concentration that causes 50% inhibition) are calculated using XLfit version 4.2.2 for Microsoft Excel. Data are shown as mean (±SD) from 3 separate experiments, each tested in triplicate.

Immunoblot analysis

To examine inhibition of RTK signaling, cells were treated with ponatinib over a range of concentrations for 1 hour. Cells were lysed in ice-cold SDS lysis buffer (0.06 mol/L Tris-HCL. 1% SDS, and 10% glycerol) and protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Thermo Scientific). Cellular lysates (50 μg) were resolved by electrophoresis and transferred to nitrocellulose membranes using NuPage Novex reagents (Invitrogen). Membranes were immunoblotted with phosphorylated antibodies and then exposed to Supersignal ELISA femto maximum sensitivity substrate (Thermo Scientific) to generate a chemiluminescent signal. Band intensity was quantified using Quantity One 4.6.7 software (Bio-Rad). Membranes were stripped with Restore Western Blot Stripping Buffer (Thermo Scientific) and immunoblotted with total protein antibodies. The IC_{50} values were calculated by plotting percent phosphorylated protein in ponatinib-treated cells relative to vehicle-treated cells.

Apoptosis assays

For measurement of caspase activity, MV4-11 cells were seeded into black-walled 96-well plates at 1×10^4 cells per well for 24 hours and then treated with ponatinib for the indicated time-points. Apo-One Homogeneous Caspase-3/7 Reagent (Promega) was added according to the manufacturer's protocol, and fluorescence was measured in the Wallac Victor microplate reader. To measure PARP cleavage, MV4-11 cells were plated in 6-well plates and, the following day, were treated for 24 hours with ponatinib. At the end of treatment, cells were lysed with SDS buffer and immunoblotted to measure for both total PARP and cleaved PARP expression (Cell Signaling Technology).

Subcutaneous xenograft model

All animal experiments were carried out under a protocol approved by the Institutional Animal Care and Use Committee. The MV4-11 human tumor xenograft efficacy study was carried out by Piedmont Research Center. Briefly, tumor xenografts were established by the subcutaneous implantation of MV4-11 cells (1×10^7 in 50% matrigel) into the right flank of female CB.17 severe combined immunodeficient mice and dosing was initiated when the average tumor volume reached approximately 200 mm^3. Ponatinib was formulated in aqueous 25 mmol/L citrate buffer (pH = 2.75) and mice were dosed orally once daily for 4 weeks. The tumors were measured in 2 dimensions (length and width) with a caliper in millimeters. Tumor volume (mm^3) was

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calculated with the following formula: tumor volume = (length × width^2)/2. Tumor growth inhibition (TGI) was calculated as follows: TGI = $(1-\Delta T/\Delta C) \times 100$, where ΔT stands for mean tumor volume change of each treatment group and ΔC for mean tumor volume change of control group. The tumor volume data were collected and analyzed with a 1-way ANOVA test (GraphPad Prism) to determine the overall difference among groups. Each ponatinib treatment group was further compared to the vehicle control group for statistical significance using Dunnett's Multiple Comparison Test. A *P*-value less than 0.05 was considered to be statistically significant and a *P*-value less than 0.01 to be highly statistically significant.

Pharmacokinetics and pharmacodynamics

Following MV4-11 xenograft tumor establishment, mice were administered a single oral dose of ponatinib and tumors harvested 6 hours later. Individual tumors were homogenized in ice-cold Phospho-safe (Novagen) and clarified by centrifugation. Samples were resolved by SDS-PAGE, transferred to nitrocelluose membranes, and immunoblotted with antibodies against total and phosphorylated FLT3 and STAT5. Ponatinib concentrations in plasma were determined by an internal standard liquid chromatography/tandem mass spectrometry method using protein precipitation; calibration standards were prepared in blank mouse plasma. The lower limit of quantitation of the assay was 1.2 ng/mL ponatinib. Reported concentrations are the mean values from 4 mice per group.

Treatment of primary AML patient samples *ex vivo*

All patient samples were de-identified and collected with informed consent with approval from the Institutional Review Board of Oregon Health & Science University. Mononuclear cells were isolated from peripheral blood from patients with AML over a Ficoll gradient followed by red cell lysis. Cells were quantitated using Guava ViaCount reagent and a Guava Personal Cell Analysis flow cytometer (Guava Technologies). Cells were plated into 96-well plates (5×10^4 per well) over graded concentrations of ponatinib in RPMI supplemented with 10% FBS, penicillin/streptomycin, L-glutamine, fungizone, and 10^{-4} mol/L 2-mercaptoethanol. After a 72 hour incubation, cells were subjected to an MTS assay (Cell Titer Aqueous One Solution Cell Proliferation Assay, Promega) for assessment of cell viability. All values were normalized to the viability of cells plated without any drug and percent viability was used to determine the ponatinib IC_{50} for each sample. FLT3 status was determined by PCR on genomic DNA from each patient. Briefly, genomic DNA was isolated from white blood cell pellets from patients (5×10^6 cells; Qiagen DNeasy). DNA (20 ng) was amplified using AccuPrime GC-rich DNA Polymerase (Invitrogen) at an annealing temperature of 60°C and a 68°C extension for 30 seconds. After 40 cycles, the FLT3 wild-type band (393 base pairs) was resolved from FLT3-ITD bands (varying lengths) using gel electrophoresis. The following primers were used: forward: 5′-GTGTTTGTCTCCTCTTCATTGTCGT-3′ and reverse: 5′-AAGCACCTGATCCTAGTACCT TCC-3′. PCR products were sequenced to confirm presence or absence of ITDs.

Results

Ponatinib inhibits signaling and proliferation in hematopoietic cell lines driven by mutant, constitutively active FLT3, KIT, FGFR1, and PDGFRα

Previous studies have shown that ponatinib (Fig. 1A) inhibits the *in vitro* kinase activity of FLT3, KIT, FGFR1, and PDGFRα with IC_{50} values of 13, 13, 2, and 1 nmol/L, respectively (2). Here, the activity of ponatinib was evaluated in a panel of leukemic cell lines that harbor activating mutations in FLT3 (FLT3-ITD; MV4-11 cells; ref. 18) and KIT (N822K; Kasumi-1 cells; ref. 19), or activating fusions of FGFR1 (FGFR1OP2-FGFR1; KG-1 cells; ref. 20) and PDGFRα (FIP1L1-PDGFRα; EOL1 cells; ref. 21). Ponatinib inhibited phosphorylation of all 4 RTKs in a dose-dependent manner, with IC_{50} values between 0.3 to 20 nmol/L (Fig. 2A and Table 1). Consistent with these activated receptors being important in driving

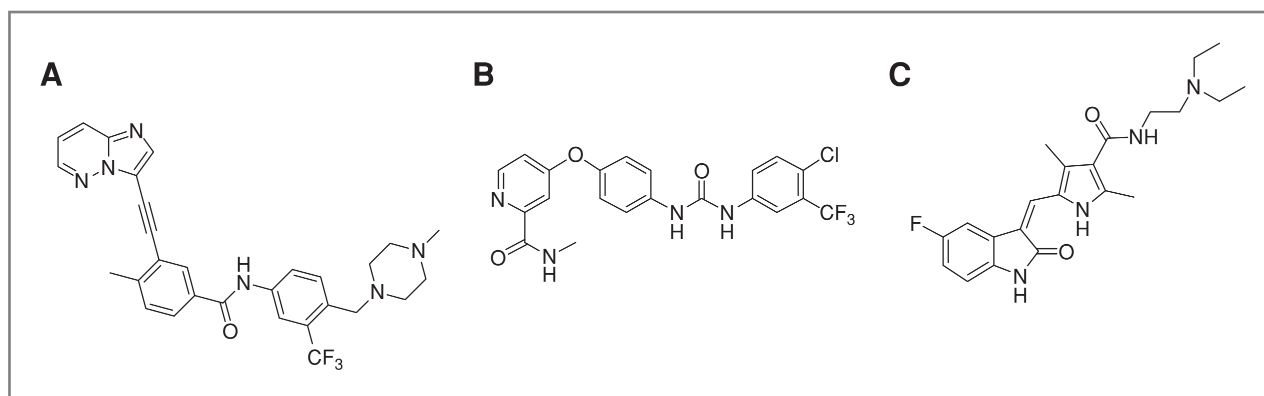

Figure 1. Chemical structure of ponatinib (A), sorafenib (B), and sunitinib (C).

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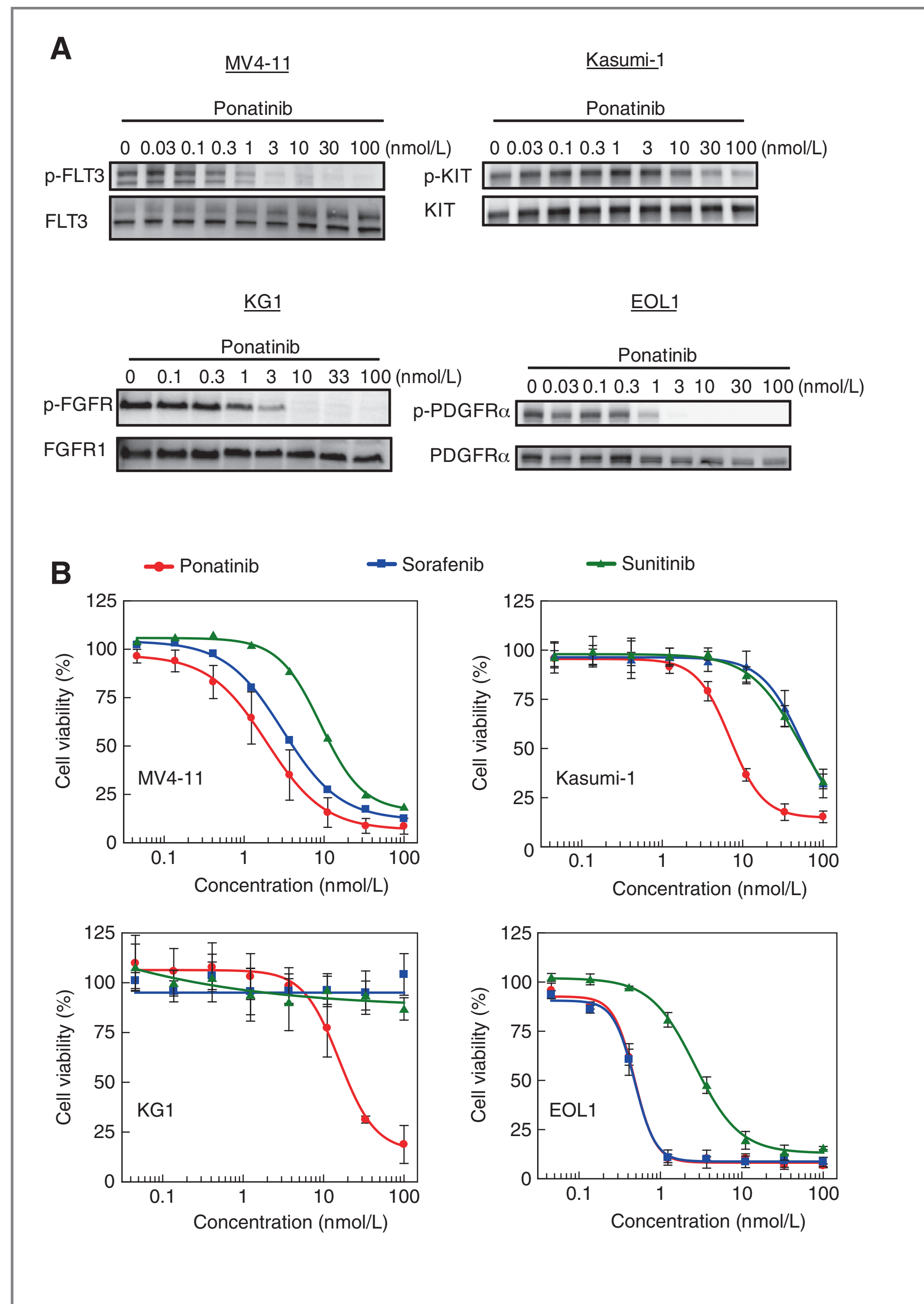

Figure 2. Ponatinib inhibits phosphorylation of activated RTKs and cell viability in AML cell lines. A, MV4-11, Kasumi-1, KG1, and EOL1 cells were incubated with the indicated concentrations of ponatinib for 1 hour. Lysates were prepared and immunoblotted for phosphorylated FLT3, KIT, FGFR, or PDGFRα, respectively. Blots were subsequently stripped and reprobed for total levels of the relevant kinase. Similar results were obtained in 2 independent experiments. B, AML cells were incubated with increasing concentrations of compound for 72 hours, and cell viability was assessed using an MTS assay. Data are presented as mean ± SD from 3 experiments.

leukemogenesis (4) ponatinib also potently inhibited the viability of all 4 cell lines with IC_{50} values of 0.5 to 17 nmol/L (Fig. 2B and Table 1). In contrast, the IC_{50} for inhibition of RS4;11 cells which express native (unmutated) FLT3, (22) was more than 100 nmol/L. These data suggest that ponatinib selectively targets leukemic cells that express one of these aberrant RTKs.

The potency and activity profile of ponatinib was next compared to that of 2 other multitargeted kinase inhibitors, sorafenib (Fig. 1B) and sunitinib (Fig. 1C), by examining their effects on viability of the same panel of cell lines in parallel. While potent inhibitory activity of sorafenib and sunitinib was observed against FLT3 (IC_{50s} of 4 and 12 nmol/L, respectively) and PDGFRα (0.5 and 3 nmol/L), neither compound exhibited high potency against KIT (59 and 56 nmol/L) or FGFR1 (>100 and >100 nmol/L; ref. Fig. 2B and Table 1).

Potent apoptotic effects of ponatinib on MV4-11 cells

Given the major clinical relevance of the FLT3-ITD mutation in AML, subsequent studies focused on the

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Table 1. Ponatinib inhibits proliferation and signaling in AML cell lines driven by activated RTKs

Cell line	Activated RTK status	Assay	IC_{50}, nmol/L		
			Ponatinib	Sorafenib	Sunitinib
MV4–11	FLT3-ITD	RTK phosphorylation	0.3	–	–
		Cell viability	2	4	12
Kasumi–1	c-KIT (N822K)	RTK phosphorylation	20	–	–
		Cell viability	8	59	56
KG1	FGFR1OP2-FGFR1	RTK phosphorylation	3	–	–
		Cell viability	17	>100	>100
EOL1	FIP1L1-PDGFRα	RTK phosphorylation	0.6	–	–
		Cell viability	0.5	0.5	3
RS4;11	Native FLT3	RTK phosphorylation	–	–	–
		Cell viability	>100	>100	>100

Note: Dash (–) indicates not tested. RS4;11 cells express native FLT3 and have not been reported to express activated RTKs.

characterization of ponatinib's activity against this target. To examine the basis for ponatinib's effect on viability of FLT3-ITD–driven MV4-11 cells, its effect on 2 markers of apoptosis was measured. A dose- and time-dependent increase in caspase-3/7 activity was observed, with maximal induction (up to 4-fold) seen with 10 to 30 nmol/L ponatinib and within 16 hours of treatment (Fig. 3A). Similarly, at concentrations of 10 nmol/L or more, ponatinib showed near maximal induction of PARP cleavage and concomitant inhibition of phosphorylation of STAT5 (Fig. 3B), a direct downstream substrate of the mutant FLT3-ITD kinase (23), and important regulator of cell survival. Taken together, these data suggest that inhibition of FLT3-ITD by ponatinib inhibits MV4-11 cell viability through the induction of apoptosis.

In vivo efficacy and pharmacodynamic studies

To examine the effect of ponatinib on FLT3-ITD–driven tumor growth *in vivo*, ponatinib (1–25 mg/kg), or vehicle, was administered orally, once daily for 28 days, to mice bearing MV4-11 xenografts. As shown in Fig. 4A, ponatinib potently inhibited tumor growth in a dose-dependent manner. Administration of 1 mg/kg, the lowest dose tested, led to significant inhibition of tumor growth (TGI = 46%, $P < 0.01$) and doses of 2.5 mg/kg or greater resulted in tumor regression. Notably, dosing with 10 or 25 mg/kg led to complete and durable tumor regression with no palpable tumors detected during a 31-day follow up.

To confirm target modulation *in vivo*, mice bearing MV4-11 xenografts were administered a single oral dose of vehicle or ponatinib at 1, 2.5, 5, or 10 mg/kg. Tumors

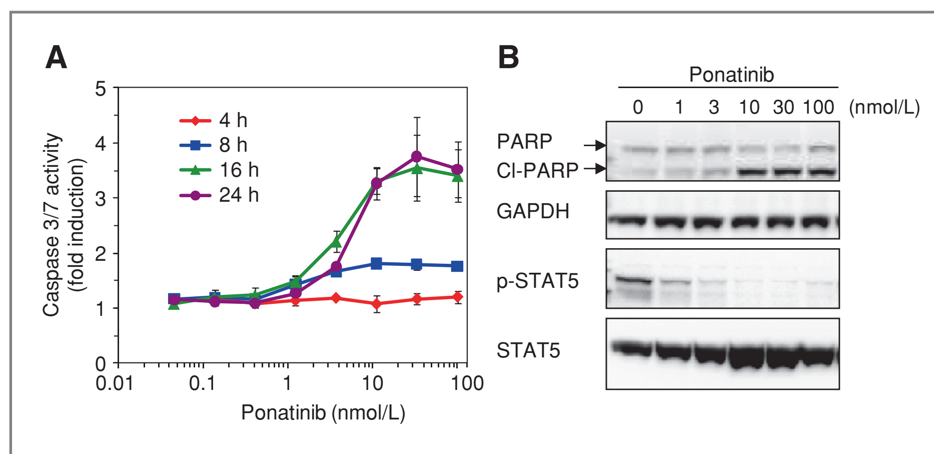

Figure 3. Ponatinib induces apoptosis in MV4-11 cells. A, MV4-11 cells were seeded in 96-well plates, treated with increasing concentrations of ponatinib, and caspase-3/7 activity measured at the indicated times. Data are expressed as fold induction of caspase activity relative to vehicle-treated cells and are presented as mean ± SD from 3 individual experiments. B, MV4-11 cells were treated with the indicated range of ponatinib concentrations for 24 hours. Cells were harvested and immunoblotted for phosphorylated and total STAT5, as well as total and cleaved (Cl)-PARP. GAPDH was included as a loading control.

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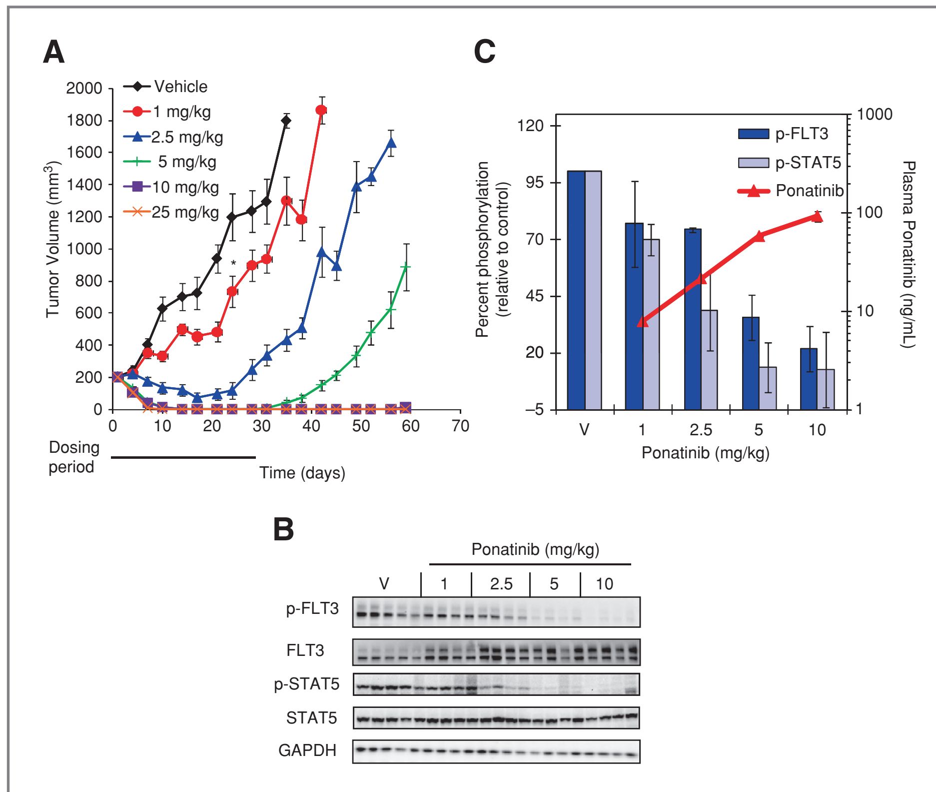

Figure 4. Ponatinib showed dose-dependent efficacy, tumor regression, and target inhibition in MV4-11 xenografts. A, daily oral administration of vehicle or ponatinib for 4 weeks at doses of 1, 2.5, 5, 10, and 25 mg/kg/d was initiated when MV4-11 flank xenograft tumors reached approximately 200 mm^3 (10 mice per group). Mean tumor volumes (±SEM) are plotted. Three of ten animals in the vehicle control group were sacrificed before the last treatment on day 28 due to tumor burden. Therefore, TGI was calculated from days 0 to 24 (as indicated by the *), the next to last time-point for tumor measurement during the dosing phase. B, mice bearing established MV4-11 tumor xenografts were administered a single oral dose of ponatinib (4 mice per group) at the level indicated; control animals received vehicle alone (5 mice). Tumors were harvested 6 hours later and analyzed for levels of phosphorylated and total FLT3 and STAT5 by immunoblotting. GAPDH was examined as a control. Each lane represents a separate animal. C, relative phosphorylation levels of FLT3 and STAT5 are shown as mean (±SEM) from 2 independent experiments (one of which is shown in B). GAPDH was used as a loading control. Plasma ponatinib levels are shown as mean (±SEM) from the same 2 experiments.

were harvested after 6 hours and levels of total and phosphorylated FLT3 and STAT5 were evaluated by immunoblot analysis (Fig. 4B). A single dose of 1 mg/kg ponatinib had a modest inhibitory effect on FLT3 signaling, decreasing levels of p-FLT3 and p-STAT5 by approximately 30% (Fig. 4C). Increased doses of ponatinib led to increased inhibition of signaling with 5 and 10 mg/kg doses inhibiting signaling by approximately 75% and 80%, respectively. Pharmacokinetic analysis showed a positive association between the concentration of ponatinib in plasma and inhibition of FLT3-ITD signaling (Fig. 4C). These data show that inhibition of signaling by ponatinib is associated with the degree of efficacy (Fig. 4A) and strongly suggest that inhibition of FLT3-ITD signaling accounts for the antitumor activity of ponatinib in this model.

Activity of ponatinib in primary AML cells

To assess the activity of ponatinib in primary cells from patients with AML, we obtained peripheral blood blasts from 4 patients; 3 that expressed native FLT3 and 1 that harbored a FLT3-ITD. FLT3 status was confirmed by PCR on genomic DNA from each patient (data not shown). Cell viability was measured following exposure to ponatinib for 72 hours (Fig. 5). Consistent with the results obtained in cell lines, ponatinib reduced viability of FLT3-ITD positive primary blasts with an IC_{50} 4 nmol/L, while blasts expressing native FLT3 showed no reduction in viability at the concentrations tested (up to 100 nmol/L). Taken together, these findings support the hypothesis that ponatinib is selectively cytotoxic to leukemic cells harboring a FLT3-ITD mutant.

Discussion

Ponatinib is an orally active, multitargeted kinase inhibitor that has shown potent activity against BCR-ABL, and all mutant variants tested, in preclinical models of CML (2). Viability of cells driven by native or mutant BCR-ABL, including BCR-ABL^{T315I}, has previously been

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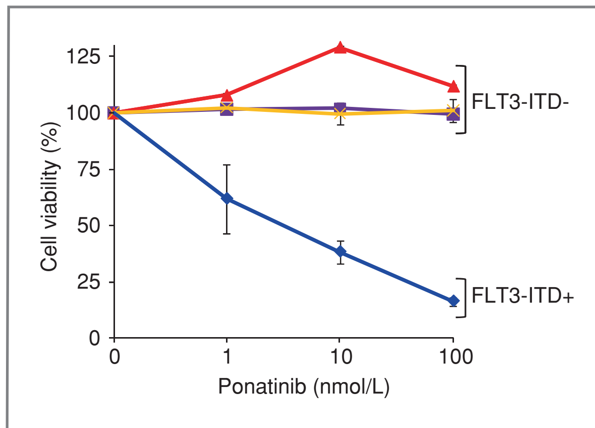

Figure 5. Ponatinib selectively inhibits cell viability of FLT3-ITD primary AML blasts *ex vivo*. Primary leukemic blast cells were isolated from peripheral blood from 4 individual AML patients. FLT3-ITD status was determined by PCR and sequencing. Primary cell cultures were treated with the indicated concentrations of ponatinib for 72 hours, at which time viability was assessed using an MTS assay. All values are normalized to the viability of cells incubated in the absence of drug.

shown to be inhibited with IC_{50} values between 0.5 and 36 nmol/L. Previous studies (2) have also shown potent *in vitro* inhibitory activity against a discrete set of additional kinases, including several implicated in the pathogenesis of other hematologic malignancies (4, 5): FLT3, KIT, and members of the FGFR and PDGFR families. Here, using leukemic cell lines containing activated forms of each of these receptors, we show that ponatinib exhibits activity against each of these kinases with potency similar to that observed for BCR-ABL: IC_{50} values for inhibition of target protein phosphorylation and cell viability ranged from 0.3 to 20 nmol/L and 0.5 to 17 nmol/L, respectively. Other multitargeted kinase inhibitors, such as sorafenib and sunitinib, have previously been shown to have inhibitory activity against a subset of these kinases. However we found that ponatinib was unique in its ability to inhibit activity of all 4 kinases with high potency. Importantly, preliminary results reported from an ongoing phase 1 clinical trial of ponatinib that includes patients with refractory CML show that levels of ponatinib required to functionally inhibit BCR-ABL, and mutant variants, are attainable (24). In the models tested here, ponatinib exhibited potency against FLT3, KIT, FGFR1, and PDGFRα comparable to that observed previously in BCR-ABL–driven models of CML (2), suggesting that inhibition of these additional targets is clinically achievable. Overall these results provide support for clinical testing of ponatinib in diseases in which these kinases play a role.

Myeloproliferative neoplasms with genetic rearrangements of FGFR1 and PDGFRα are considered to be rare; however, it has been shown that the resulting fusion proteins play a major role in the pathogenesis of these diseases (6, 7). The 8p11 myeloproliferative syndrome is an aggressive disease that can rapidly transform to AML in the absence of treatment. We have shown here that ponatinib potently inhibits viability of the AML KG1 cell line, which is driven by an FGFR1OP2-FGFR1 fusion protein, suggesting that ponatinib may have clinical activity in this disease type. HEL/CEL patients with a PDGFRα fusion achieve dramatic hematological responses when treated with the PDGFR inhibitor imatinib (6) and we have shown that ponatinib has potent activity against the FIP1L1-PDGFRα fusion protein as shown in the leukemic EOL cell line. However, the T674I mutant of PDGFRα, which is mutated at the position analogous to the T315I gatekeeper residue in BCR-ABL, has been shown to confer resistance to imatinib in patients (6). Importantly, ponatinib has potent activity against the PDGFRα T674I mutant kinase, with an IC_{50} of 3 nmol/L (2), suggesting that ponatinib may be effective in treating patients who carry this fusion protein. More generally, the unique linker of ponatinib is specifically designed to accommodate mutated gatekeeper residues, suggesting that the ability to inhibit such mutations may also apply to other targets (2, 3). Indeed ponatinib potently inhibits the FGFR1 gatekeeper mutant $FGFR1^{V561M}$ with an IC_{50} of 7 nmol/L (2). The fact that the same isoleucine side chain is shared by $BCR\text{-}ABL^{T315I}$, KIT^{T670I}, and $FLT3^{F691I}$ suggests that ponatinib should also be active against these KIT and FLT3 gatekeeper mutants, based on the molecular interactions observed in the crystal structure of T315I ABL bound with ponatinib (2, 3).

Both the incidence and prognostic significance of FLT3-ITD alterations in AML suggest that this kinase plays a critical role in the pathogenesis of the disease (25) and, as such, represents a major target for therapeutic intervention. In the studies reported here, using the FLT3-ITD expressing cell line MV4-11, we show a close relationship between inhibition of FLT3 activity, both *in vitro* and *in vivo*, and inhibition of tumor cell viability. *In vitro*, low nmol/L concentrations of ponatinib (i.e., <10 nmol/L) led to a decrease in FLT3 phosphorylation, a decrease in viability, and an increase in markers of apoptosis. In an *in vivo* xenograft model, a daily oral dose of 1 mg/kg ponatinib led to significant inhibition of tumor growth and a dose of 5 mg/kg or greater led to tumor regression. Consistent with the effects on tumor growth being due to inhibition of FLT3, a single dose of 1 mg/kg ponatinib led to a partial inhibition of FLT3-ITD and STAT5 phosphorylation, while doses of 5 and 10 mg/kg led to substantial inhibition. Finally, ponatinib potently inhibited viability of primary blasts isolated from a FLT3-ITD positive AML patient (IC_{50} of 4 nmol/L), but not those isolated from 3 FLT3 wild-type patients (IC_{50} > 100 nmol/L).

Multiple compounds with FLT3 activity have been described and several have already been evaluated in patients. Relatively modest clinical activity has been reported to date (11, 13, 14), although AC220 has begun to show promise (16). Based on preclinical studies that show that FLT3 inhibition needs to be sustained to effect

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killing of FLT3-dependent AML cells (26), a view has emerged that to achieve maximum therapeutic benefit, continuous and near-complete inhibition of FLT3 kinase may be required (26). Our *in vitro* studies show that complete inhibition of FLT3 phosphorylation and function can be obtained at 10 nmol/L or more concentrations. Importantly, preliminary analysis of the pharmacokinetic and pharmacodynamic properties of ponatinib show that well-tolerated oral daily doses lead to trough plasma drug levels exceeding 40 nmol/L, and sustained inhibition of BCR-ABL activity in circulating leukemic cells (24). These data suggest that the potency and pharmacologic properties of ponatinib may allow continuous and near-complete inhibition of FLT3 in patients.

In summary, ponatinib is a multitargeted kinase inhibitor that displays potent inhibition of FLT3 and is cytotoxic to AML cells harboring the FLT3-ITD mutation. Importantly, this agent exhibits activity against additional RTKs, FGFR1, KIT, and PDGFRα, which have also been shown to play roles in the pathogenesis of hematologic malignancies. Notably, the potency of ponatinib against these RTKs *in vitro* and plasma levels of ponatinib observed in humans suggest that ponatinib may have clinical activity against these targets. Taken together, these observations provide strong preclinical support for the evaluation of ponatinib as a novel therapy for AML and other hematologic malignancies.

Disclosure of Potential Conflicts of Interest

All authors except for J. Tyner, M. Loriaux, and B. Druker are full-time employees of and have ownership interest in ARIAD Pharmaceuticals, Inc.

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