Ponatinib Induces Apoptosis in Imatinib-Resistant Human Mast Cells by Dephosphorylating Mutant D816V KIT and Silencing β-Catenin Signaling

Bei Jin1,2, Ke Ding3, and Jingxuan Pan1,2

Abstract

Gain-of-function mutations of membrane receptor tyrosine kinase KIT, especially gatekeeper D816V point mutation in KIT, render kinase autoactivation, disease progression, and poor prognosis. D816V KIT is found in approximately 80% of the patients with systemic mastocytosis, and is resistant to the first and second generations of tyrosine kinase inhibitors (TKI). The purpose of this investigation was aimed at exploring whether ponatinib (AP24534), a novel effective TKI against T315I Bcr-Abl, was active against D816V KIT. We discovered that ponatinib abrogated the phosphorylation of KIT harboring either V560G (sensitive to imatinib) or D816V mutation (resistant to imatinib) and the downstream signaling transduction. Ponatinib inhibited the growth of D816V KIT–expressing cells in culture and nude mouse xenografted tumor. Ponatinib triggered apoptosis by inducing the release of cytochrome c and AIF, downregulation of Mcl-1. Furthermore, ponatinib abrogated the phosphorylation of β-catenin at the site Y654, suppressed the translocation of β-catenin, and inhibited the transcription and DNA binding of TCF and the expression of its targets (e.g., AXIN2, c-MYC, and CCND1). Moreover, ponatinib was highly active against xenografted D816V KIT tumors in nude mice and significantly prolonged the survival of mice with aggressive systemic mastocytosis or mast cell leukemia by impeding the expansion and infiltration of mast cells with imatinib-resistant D814Y KIT. Our findings warrant a clinical trial of ponatinib in patients with systemic mastocytosis harboring D816V KIT.

Mol Cancer Ther; 13(5); 1–14. ©2014 AACR.

Introduction

Mastocytosis is an orphan, mostly sporadic disease, characterized by abnormal accumulation of mast cells and activation of mast cells in various organs, involving bone marrow, skin, liver, and gastrointestinal tract (1). Systemic mastocytosis and cutaneous mastocytosis constitute two major forms of mastocytosis. The clinical course in patients with systemic mastocytosis ranges from indolent and asymptomatic to a highly aggressive disease course, even mast cell leukemia (MCL; ref. 1). Acquired activating mutations in KIT (e.g., D816V, D816Y, and D816F) have been identified in most patients with mastocytosis (1). Currently, IFN-α (2) and cladribine (2CdA) constitute the treatments of choice for the first-line therapy in systemic mastocytosis, however, with inadequate degree and duration of response (3). Imatinib has been approved by the U.S. Food and Drug Administration (FDA) for adult systemic mastocytosis patients without the KIT D816V mutation (4). Other TKIs such as dasatinib (5), midostaurin (PKC412; ref. 6), semaxinib (7), and EXEL-0862 (8) have been reported to exhibit growth inhibitory and apoptosis induction effects in mast cells harboring D816V KIT mutation. Although Ustun and colleagues reported that dasatinib has efficacy in systemic mastocytosis-AML patients bearing D816V KIT, a phase II study from an independent group did not support the potency of dasatinib against D816V KIT (9). Although midostaurin (PKC412) has a synergistic effect with dasatinib to inhibit the growth of neoplastic mast cells (6, 10), the long-term benefit of PKC412 has not yet been determined. Therefore, development of novel effective drugs to treat aggressive systemic mastocytosis and MCL is still needed.

KIT is a transmembrane receptor tyrosine kinase for stem cell factor (SCF; ref. 11). On the stimulation of SCF, KIT undergoes phosphorylation at Y568 and Y570 residues and activates various signaling pathways (12). Gain-of-function mutations in KIT also lead to KIT activation and give rise to uncontrolled cell growth, cell survival,
and apoptosis resistance (13). Imatinib inhibits activation of wild-type KIT and some point mutant KIT (e.g., V560G) but not D816V by competitively occupying a KIT enzymatic ATP-binding pocket. Unfortunately, the kinase domain mutant D816V KIT counting for approximately 80% of all patients with systemic mastocytosis is resistant to imatinib (14). In addition, KIT mediates tyrosine phosphorylation and nuclear localization of β-catenin, which is an important component of the Wnt/β-catenin signaling pathway in mast cell leukemia (15, 16). The β-catenin accumulates in cytoplasm and transfers to the nucleus, where it engages TCF/LEF transcription factors for genes transcription (17).

Ponatinib (AP24534), a potent multitargeted kinase inhibitor (18), has been approved by the FDA for treating imatinib-resistant patients with chronic myelogenous leukemia, including those harboring the gatekeeper mutant T315I Bcr-Abl (19, 20). Besides, ponatinib also manifests inhibitory activity toward PDGFR, KIT, as well as FGFR (21, 22). Although it has been reported that ponatinib is active against BaF3 cells stably expressing Y823D KIT in vitro (22) and D816V KIT-positive mast neoplastic cells (10), the in vivo effect remains to be examined.

In this report, our purpose was to validate the inhibitory effect of ponatinib against the mutant KIT cells and the in vivo antineoplastic efficacy in mouse models bearing cells with mutant KIT. We found that ponatinib inhibited the growth of D816V KIT-expressing cells in culture and nude mouse xenografted tumor and significantly prolonged the survival of mice with aggressive systemic mastocytosis or mast cell leukemia.

Materials and Methods

Reagents and antibodies

Ponatinib (purity > 95%, high-performance liquid chromatography) was synthesized in our laboratory, prepared as a 20 mmol/L stock solution in dimethyl sulfoxide (DMSO) and stored in aliquots at −20°C. Imatinib mesylate was a product of Novartis Pharmaceuticals (23). Z-DEVD-fmk (caspase-3 inhibitor) was purchased from BD Biosciences. MG132 was from EMD Biosciences. Antibodies and their sources were as follows: mouse monoclonal antibodies against KIT (CD117), PARP, Bcl-2, XIAP, active caspase-3, cytochrome c (clone 6H2.B4), and FITC mouse IgG1 isotype control were from BD Biosciences Pharmingen; rabbit polyclonal antibodies against phospho-Stat5 (Y694/Y699; clone 8-5-2), anti-Stat5, and anti-phospho-Akt (Ser473), and Akt were from Cell Signaling Technology; anti-Bim was from Stressgen; antibodies against phospho-KIT (Y568/570) were from Santa Cruz Biotechnology; anti-actin were from Sigma-Aldrich; and anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G horseradish peroxidase-conjugated antibodies were from Pierce Biotechnology.

Cell culture and analysis of mutation in KIT

Imatinib-sensitive HMC-1.1 cells harboring the V560G mutation in the juxtamembrane domain of KIT and imatinib-resistant HMC-1.2 cells harboring both V560G and D816V mutations were kindly provided by Dr. Joseph Butterfield (Mayo Clinic, Rochester, MN) in 2006 (24, 25). Both subclones were grown in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 10% fetal calf serum (FCS; HyClone) and 1.2 mmol/L α-thioglycerol (Sigma) in a 37°C humidified incubator containing 5% CO₂. The murine P815 mastocytoma cell line harboring the D814Y mutation (corresponding to human D816Y; ref. 26) was purchased from the American Type Culture Collection and cultured in DMEM supplemented with 10% FCS and 25 mmol/L HEPES buffer. All the cell lines were tested and authenticated by using short tandem repeat (STR) matching analysis of cells last month. No cross-contamination of other human cells was found in all three lines of cells.

The presence of mutations in KIT was analyzed routinely every month (the last test was performed last week, Supplementary Fig. S1) in our laboratory with the method described previously (27). The primers of KIT are listed in Supplementary Table S1.

Preparation of cell lysates

Total cell lysates were prepared in radioimmunoprecipitation assay buffer. For detection of AIF and cytochrome c, cytosolic fraction was prepared with digitonin extraction buffer (28). Extracts of subcellular cytoplasmic, mitochondrial, and nuclear fractions were separated as previously described (23).

Real-time quantitative RT-PCR

Total mRNA was evaluated by the median-effect method of Chou and Talalay (28). The MTS assay (CellTiter 96 Aqueous One Solution reagent; Promega) was used to evaluate cell viability as described previously (23, 28). Synergism of combinational drugs was evaluated by the median-effect method of Chou and Talalay (28). Apoptosis was measured using Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI;
mouse IgG1 488-conjugated goat anti-mouse IgG. FITC-conjugated after staining with the secondary antibody Alexa Fluor anti-active-
After washing and blocking, the cells were stained with
bilized in prechilled 90% methanol for 30 minutes on ice.

Transfection of plasmids and small interfering RNA
ON-TARGET plus SMART pool siRNA duplexes against human Mcl-1#1 (cat# L-004501-00-0005) and ON-TARGET plus NON-Targeting siRNA (mock siRNA; cat# D-001810-01) were from Dharmacon RNA Technologies, SignalSilence siRNA duplexes against Mcl-1#2 (cat# 6315), β-catenin #1 (cat# 6225), β-catenin #2 (cat# 6238), and control siRNA were from Cell Signaling Technology. pCMV5-flag-human Mcl-1 and human pcDNA3-β-catenin were from Addgene. Plasmids, empty vector, specific siRNA, or mock siRNA were delivered into HMC-1.2 cells with the Cell Line Nucleofector Kit T (Amaxa) and program O-17 following the manufacturer’s instructions (25). The cells were then exposed to ponatinib and subjected to cell death assay and Western blotting analysis.

Immunofluorescence staining
HMC-1.2 cells were grown with 0.3 μmol/L of ponatinib for 24 hours. Immunofluorescence staining was carried out as previously reported (8).

Assay of intracellular active β-catenin with flow cytometer
Ponatinib-treated HMC-1.2 cells were harvested, fixed in 4% formaldehyde for 10 minutes at 37°C, and permeabilized in prechilled 90% methanol for 30 minutes on ice. After washing and blocking, the cells were stained with anti-active-β-catenin, followed by flow cytometry analysis after staining with the secondary antibody Alexa Fluor 488-conjugated goat anti-mouse IgG. FITC-conjugated mouse IgG1 κ isotype control was stained the same way except the secondary antibody and evaluated the background fluorescence (29).

Dual luciferase reporter assay
The TCF/LEF reporter plasmid, pTOP-flash, and its mutant control, pFOP-flash, were purchased from EMD Millipore. The Renilla luciferase reporter construct, pRL-TK, was purchased from Promega. The dual luciferase reporter assay was performed according to the manufacturer’s instructions (Promega; ref. 28).

Electrophoretic mobility shift assay
Electrophoretic mobility shift assay (EMSA) was performed using the LightShift Chemiluminescent EMSA Kit (Pierce Biotechnology) following the manufacturer’s instructions (28). The oligonucleotides for TCF were as follows: forward: 5’- TGGCGGGTTTGTCCCTTG-3’; reverse: 5’- AGCAAAAGATCAAGCCCCG-3’.

In vivo antitumor effect of ponatinib
For tumor xenograft experiments, male nude nu/nu BALB/c mice were purchased from Slac Laboratory Animal Co (Shanghai, China). HMC-1.2 cells (200 μL of 30 million cells in serum-free medium suspension) were implanted subcutaneously into the flanks of 4- to 6-week-old nude mice (30). Tumor volumes were measured every 2 days. When the tumors were palpable (~50 mm³), mice were randomized according to the average volume of the tumors to the placebo (n = 5) or experimental groups (n = 8), and treated every 2 days by oral gavage with placebo (100 μL of NaCl 0.9% solution which contains the same amount of DMSO as in the ponatinib solution) or ponatinib at 20 mg/kg (18). The animals were euthanized after 2 weeks of treatment. The mean tumor weights and mean tumor volume from the last measurement of the 2 groups were compared by a t test. The body weight, feeding behavior, and motor activity of each animal were monitored as indicators of general health.

For the murine model with aggressive systemic mastocytosis (ASM)/mast cell leukemia (MCL; refs. 25, 30), 6- to 8-week-old male DBA/2 mice purchased from Slac Laboratory Animal Co were randomly divided into 3 groups: normal (n = 5), placebo (n = 8; treated with DMSO-containing tissue culture medium), and experimental (n = 8; treated with 20 mg/kg ponatinib orogastric gavage once daily).

Mice were kept under specific pathogen-free conditions in the Sun Yat-sen University animal care facility. All animal studies were conducted with the approval of the Sun Yat-sen University Institutional Animal Care and Use Committee.

Immunohistochemical staining
Formalin-fixed xenografts of HMC-1.2 cells were embedded in the paraffin and sectioned. Immunohistochemical staining of tumor xenograft sections (4 μm) was carried out with Max Vision kit (Maixin Biol) following the manufacturer’s instructions (23).

Detection of mastocytoma cells by flow cytometry
The mononuclear cells were prepared from spleens of ASM/MCL mice as previously described (25, 29). The cells were stained with anti-CD45-FITC and anti-CD117-PE, followed by analysis with a FACSCalibur flow cytometer.

Statistical analysis
All experiments were performed at least three times, and data are exhibited as mean ± 95% confidence intervals. Comparisons between 2 groups were analyzed by the t test while those between more than 2 groups by one-way ANOVA with post hoc comparison by the Tukey test unless otherwise stated. GraphPad Prism 5.0 (GraphPad
Software) was used for statistical analysis. $P < 0.05$ was considered statistically significant.

Results

Ponatinib abolishes the phosphorylation of KIT and its downstream target molecules

We first analyzed the effect of ponatinib (Fig. 1A) on the phosphorylation of KIT at Y568/570 residues and its downstream signaling (Stat3/5, Akt, Erk1/2). Our results showed that ponatinib potently inhibited the phosphorylation of KIT in both dose- and time-dependent manners (Fig. 1B). However, ponatinib did not alter the levels of total protein and mRNA in KIT as detected with Western blotting analysis and qRT-PCR, respectively (Fig. 1B and C). Ponatinib decreased the level of phosphorylated KIT in P815 cells (Fig. 1D). Correspondingly, ponatinib dose-

![Chemical structure of ponatinib](image)

Figure 1. Ponatinib blocks the phosphorylation of KIT and its downstream target molecules. A, chemical structure of ponatinib. B, ponatinib inhibited the phosphorylation of KIT in a dose- or time-dependent manner in HMC cell lines. C, ponatinib did not alter the transcription of KIT. HMC cells were treated with ponatinib for 24 hours and RNAs were extracted and subjected to real-time RT-PCR analysis. D, ponatinib inhibited the phosphorylation of KIT in P815 cells. E, ponatinib inhibited the downstream signaling of KIT. Stat3, Stat5, Akt, and Erk1/2 were analyzed in the cell lysates from cells with the same treatment in Fig. 1B.
and time-dependently inhibited the phosphorylation of Stat3, Stat5, Akt, and Erk1/2, which are downstream targets of KIT (Fig. 1E). Taken together, these results suggest a strikingly inhibitory effect of ponatinib on KIT signaling.

**Ponatinib inhibits the growth of mast cells harboring D816V KIT**

We next determined the effect of ponatinib on the growth of the mast cells. The presence of D816V mutation in KIT leads to resistance to imatinib (14). We confirmed the differential sensitivity of HMC-1.1 cells and HMC-1.2 cells to imatinib, with IC50 values of 1.1 μmol/L versus >20 μmol/L (Fig. 2A). In contrast, ponatinib significantly inhibited the growth of both imatinib-resistant HMC-1.2 and -sensitive HMC-1.1 cells, with IC50 values of 165 and 143 nmol/L, respectively (Fig. 2A). Furthermore, ponatinib inhibited the viability of the murine P815 cells at an IC50 of 72 nmol/L (Fig. 2A).

Because simultaneously inhibiting a specific tyrosine kinase and its downstream targets such as mTOR usually results in synergism (31, 32), we examined the effect of the combination between ponatinib and rapamycin, an mTOR inhibitor, in human mast cells. The results assessed by MTS assay after 72 hours of incubation in a serially diluted mixture at a fixed ratio revealed synergism between ponatinib and rapamycin based on the combination index (CI; Fig. 2B; ref. 28).

To evaluate the impact of ponatinib on anchorage-independent growth of the mast cells, we performed the colony formation assay in methylcellulose. Mast cells were exposed to increasing concentrations of ponatinib for 24 hours and then assayed for colony formation in drug-free culture. Ponatinib potently inhibited the colony formation in methylcellulose in a dose-dependent manner (Fig. 2C).

**Ponatinib induces apoptosis in mast cells harboring D816V KIT by triggering release of cytochrome c and apoptosis-inducing factor**

We evaluated the capability of ponatinib to induce apoptosis with flow cytometry. Ponatinib induced significantly increased cell death in both imatinib-sensitive HMC-1.1 cells and -resistant HMC-1.2 cells to a similar degree (~55% vs. ~65%; Fig. 3A).

Western blotting analysis showed that ponatinib dose- and time-dependently induced specific cleavage of PARP, caspase-8, -9, and -3 in the malignant mast cells (Fig. 3B), further confirming the occurrence of apoptosis. Of note, when detecting cytochrome c and AIF in the cytosolic fraction, their levels were increased as early as 12 hours after 0.3 μmol/L ponatinib treatment (Fig. 3C), which
obviously preceded the activation of caspase-9 and caspase-3. These results suggested that ponatinib might lead to damage of mitochondria and trigger the intrinsic pathway of apoptosis.

Effect of ponatinib on the expression of apoptosis-related proteins

To figure out the mechanism of ponatinib-induced apoptosis, we examined the expression of apoptosis-related proteins.
related proteins. Western blotting analysis in both mast cell lines indicated a decrease in levels of Mcl-1 and Bcl-2 in a dose- and time-dependent fashion, with no alteration in levels of Bcl-XL, Bax, Bim, or Survivin (Fig. 3D).

**Mcl-1 plays a significant role in ponatinib-induced apoptosis**

To define the significance of Mcl-1 during apoptosis induced by ponatinib, HMC-1.2 cells were transfected with empty vector or human Mcl-1 plasmid, followed by treatment of ponatinib. Ectopic overexpression of Mcl-1 significantly attenuated the ponatinib-induced apoptosis as reflected by the specific cleavage of PARP and the cell death assayed by Trypan blue exclusion (Fig. 4A). In a separate approach, HMC-1.2 cells transfected with mock or Mcl-1 siRNA were exposed to the accelerating concentrations of ponatinib for 18 hours. The data showed that silencing Mcl-1 alone was not sufficient to induce apoptosis. However, knockdown of Mcl-1 astonishingly increased the sensitivity of HMC-1.2 cells to the ponatinib-induced apoptosis (Fig. 4A). Our findings were consistent with the report that silencing Mcl-1 by oligonucleotides resulted in reduced survival and increased apoptosis, and showed synergism with PKC412 in HMC-1.2 cells (33). Therefore, these results indicated that Mcl-1 played a significant role in ponatinib-induced apoptosis in HMC-1.2 cells.

**Ponatinib elicits Mcl-1 downregulation through accelerating turnover rate**

To investigate the mechanism that ponatinib decreased Mcl-1, we first ascertained whether ponatinib accelerated the turnover of Mcl-1 protein. To this end, HMC-1.2 cells were exposed to ponatinib or control medium for 12 hours, followed by the addition of cycloheximide and continued incubation for the indicated durations. The chase-experiment results indicated an accelerated turnover rate in Mcl-1 after ponatinib treatment (Fig. 4B).

The degradation of many intracellular proteins in mammalian cells is attributed to the ubiquitin proteasome pathway. We therefore investigated whether the accelerated turnover of Mcl-1 was involved in this pathway. Cells were incubated with ponatinib with or without the proteasome inhibitor MG132, and then Mcl-1 was measured by immunoblotting. The inhibition of the ubiquitin proteasome pathway resulted in the accumulation of Mcl-1 in the absence of ponatinib (Fig. 4C). However, it did not abrogate the ponatinib-mediated decrease of Mcl-1, suggesting that the downregulation of Mcl-1 was proteasome pathway independent.

Upon activation of caspase-3 in the apoptotic process induced by chemotherapeutic agents, Mcl-1 is cleaved into two fragments: 28 kDa Mcl-1128-336 and 17 kDa Mcl-111-127 (34). In contrast to the precursor p42 Mcl-1, p28 Mcl-1128-350 displays a proapoptotic function (34). To further characterize the effect of caspase-3 on Mcl-1, we exposed mast cells to ponatinib with or without Z-DEVD-fmk (a specific inhibitor of caspase-3). Western blotting analysis showed that Z-DEVD-fmk did not block the cleavage of ponatinib-mediated Mcl-1 (Fig. 4D), although the activity of caspase-3 was inhibited, implying that the ponatinib-mediated decrease in Mcl-1 was caspase-3 independent, and might be via a yet unknown mechanism.

**Ponatinib abrogates the Wnt/β-catenin pathway in mast cells harboring D816V KIT**

The Wnt/β-catenin pathway promotes the hematopoietic malignancy (35). Tyrosine residue phosphorylation at Y654 of β-catenin by tyrosine kinases (e.g., Bcr-Abl, KIT, Flt3, and Src) facilitates its activity to start its downstream target genes (e.g., c-MYC, CCND1, and AXIN2) in leukemia cells (15, 36–39). We evaluated the impact of ponatinib on β-catenin and its effector molecules. The Western blotting analysis showed a dose- and time-dependent decrease in total level of β-catenin in ponatinib-treated cells (Fig. 5A). Because the stability of β-catenin is tightly regulated by GSK3β in the canonical Wnt/β-catenin signaling pathway, we examined and discovered that ponatinib inhibited the phosphorylation at S9 in GSK3β (inactive GSK3β) without changing its phosphorylation at Y216 (active GSK3β; Fig. 5A; ref. 40). Therefore, the ponatinib-mediated decline in β-catenin was unlikely to be through GSK3β. This notion was further supported by the finding that proteasome inhibitor MG132 did not reverse the ponatinib-mediated decline in β-catenin (data not shown). Tyrosine kinase Bcr-Abl was reported to control the degree of β-catenin protein stabilization in leukemia cells (36). It remains elusive whether there is similar interaction
between KIT and β-catenin. We did notice a concomitant reduction in the phosphorylation at Y654 of β-catenin in the ponatinib-treated cells (Fig. 5A).

**Ponatinib downregulates the expression of β-catenin by accelerating its turnover rate**

To unveil the underlying regulation of ponatinib in β-catenin, HMC-1.2 cells were incubated with ponatinib for 12 hours, followed by the addition of cycloheximide to the cells for different durations. The expression levels of β-catenin were detected by Western blotting analysis. The data showed that ponatinib accelerated the turnover of β-catenin (Fig. 5B).

**Ponatinib decreases β-catenin both in cytoplasm and nucleus**

The change in total β-catenin in ponatinib-treated cells prompted us to examine whether ponatinib affected the subcellular distribution of β-catenin. Cytoplasmic and nuclear fractions of mast cells treated with ponatinib were obtained for Western blotting analysis. The results showed that ponatinib remarkably reduced β-catenin both in the cytoplasmic and nuclear fractions (Fig. 5C). This effect of ponatinib was further confirmed by immunofluorescence microscopy (Fig. 5D). To quantitatively measure this effect, we analyzed the intracellular activated β-catenin in cells with flow cytometry and found that the percentage of ABC (active-β-catenin)-positive cells was significantly decreased (from ~46% to 23%, control vs. ponatinib, respectively; Supplementary Fig. S2A).

**Ponatinib inhibits expression of β-catenin downstream targets**

Consistent with the decline of β-catenin, the expression of its key downstream targets such as c-Myc and cyclin D1 was also decreased in the ponatinib-treated cells (Fig. 5A). Furthermore, we confirmed this effect by measuring the expression of AXIN2, which is believed to be a relatively exclusive target gene of β-catenin (41). The qRT-PCR analysis indicated that ponatinib dose-dependently decreased the mRNA levels of AXIN2 (Supplementary Fig. S2B).

**Ponatinib inhibits DNA binding of TCF**

A principal function of β-catenin in cancer lies in its ability to bind TCF/LEF family transcription factors to activate gene expression (42). We employed EMSA to evaluate the inhibitory effect of ponatinib on the binding of TCF to DNA. HMC-1.2 cells were incubated with various concentrations of ponatinib for 24 hours or a fixed concentration for various durations. The nuclear fractions were extracted, electrophoresed, and detected with a TCF probe. Ponatinib induced a dose- and time-dependent decline in levels of the TCF–DNA complex (Fig. 5E).

**Ponatinib represses TCF-dependent reporter gene transcription**

To verify the effect of ponatinib on the TCF/LEF transcriptional function, we performed a dual luciferase reporter assay. HMC-1.2 cells were cotransfected with plasmids of pTOPflash (or pFOPflash) and Renilla luciferase, exposed to increasing concentrations of ponatinib, and subjected to the dual luciferase reporter assay. The data showed that ponatinib significantly reduced the luciferase activity relative to control cells (Fig. 5F).

Taken together, ponatinib inhibited the β-catenin signaling pathway, blocked the translocation of β-catenin, disrupted TCF–DNA binding, and suppressed the TCF/LEF transcriptional function.

**β-Catenin is critical in the ponatinib-mediated apoptosis**

To examine the relative contribution of β-catenin in ponatinib-induced apoptosis, HMC-1.2 cells transfected with empty vector or β-catenin plasmid were treated with control culture medium or ponatinib for 24 hours. The cells transfected with empty vector underwent extensive apoptosis, whereas β-catenin–transfected cells exhibited no increase in apoptosis, as indicated by specific cleavage of PARP, activation of caspase-3, and Trypan blue exclusion (Fig. 5G). Conversely, siRNA silencing of β-catenin greatly potentiated the capability of ponatinib to induce apoptosis. However, silencing of β-catenin alone was not sufficient to trigger apoptosis. Therefore, β-catenin might play an important role in apoptosis induced by ponatinib.

**Ponatinib curbs the growth of xenografted HMC-1.2 cells in nude mice**

We assessed the *in vivo* effect of ponatinib with a nude mouse xenograft model. The tumor growth curves (the estimated tumor size calculated from the tumor dimension over time) were significantly inhibited by ponatinib (Fig. 6A). The tumors did not regrow during the drug administration (Fig. 6A). The weights of tumors were...
significantly lower in the treated group than in the placebo group (Fig. 6B). The body weights of mice were stable, with no significant difference between the placebo and treatment groups (data not shown). In addition, immunohistochemical staining showed that the levels of p-KIT, β-catenin, and Mcl-1 were appreciably decreased in the xenografted tumor tissues from ponatinib-treated mice (Supplementary Fig. S3). In addition, the decrease of Ki67 staining revealed that ponatinib prominently depressed the proliferation of xenografted HMC-1.2 cells (Supplementary Fig. S3).

We further detected the in vivo effect of ponatinib on KIT and its downstream molecules. Western blotting analysis indicated that the levels of β-catenin, p-KIT, and its downstream signaling were extensively decreased in ponatinib-treated tumors (Fig. 6C). In aggregate, the results showed that ponatinib inhibited xenografted imatinib-resistant HMC-1.2 cells in vivo.

**Ponatinib prolonged the survival of mice with aggressive systemic mastocytosis or mast cell leukemia**

Injection of murine mastocytoma cell line P815 carrying D816Y KIT into syngeneic DBA/2 mice is a useful model for testing potential drugs against D816V KIT (the homologous D814Y mutation). We employed this model to evaluate the efficacy of ponatinib against D816V KIT. P815 cells (1 x 10⁶) were injected into the vena cava of DBA/2 mice. The next day, mice were randomized into two groups: placebo (dimethyl sulfoxide (DMSO)-containing medium) and ponatinib (20 mg/kg, oral gavage once daily). A group of healthy mice was also examined as a normal control. The survival was monitored for 20 days after cell injection and analyzed by the Kaplan–Meier survival curve (Fig. 6D). The median survival was 10 and 18 days in mice treated with placebo and ponatinib, respectively (P < 0.001, log-rank test).

For a cross-sectional analysis, when the placebo group seemed moribund on day 10 after cell injection, all three groups of mice were sacrificed. The placebo group exhibited a significant increase in the WBC count and decrease in the platelet and RBC counts in the peripheral blood specimens (Supplementary Fig. S4). Compared with the ponatinib group, mice in the placebo group had increased size and weight of liver and spleen (Fig. 6E). To analyze the infiltration of mast cells, nucleated cells obtained from spleens were analyzed by flow cytometry for the proportion of mast cells that expressed both CD45 (pan-leukocyte antigen) and CD117. As shown in Fig. 6F, mice treated with ponatinib had a lower proportion of CD45⁺CD117⁺ cells. The decreased infiltration in the liver and spleen in the ponatinib-treated mice was confirmed by light microscopy (Fig. 6G). The signal pathways were also inhibited after treatment of ponatinib as indicated by Western blotting (Fig. 6H). In summary, our in vivo data suggest that ponatinib may be an important candidate for treating patients with mastocytosis carrying the D816V KIT mutation.

**Discussion**

Previous studies have shown in vitro activity of ponatinib against tyrosine kinases such as Bcr-Abl, FIP1L1-PDGFRe, KIT Y823D, and FGFR1 (22). Here, we validated the in vivo and in vitro activity of ponatinib against human mast cells to support further clinical evaluation of ponatinib in imatinib-resistant systemic mastocytosis patients.

Oncogenic KIT has been reported to promote mast cell growth via downstream targets (e.g., Stat3/5, Akt, and Erk1/2; ref. 12). Abrogation of PI3K signaling cascade was found to critically impair the transformation capability of cells bearing D816V KIT (12). Our results demonstrate that ponatinib decreases the phosphorylation of KIT at tyrosine 568/570 residues and its downstream signaling crucial to cell proliferation and survival, including Stat3/5, Akt, and Erk1/2. Furthermore, ponatinib induces comparable cell death rates in the two cell lines as analyzed by flow cytometry.

Interestingly, two bands were observed when doing immunoblotting with cyclin D1 antibody in ponatinib-treated cells (Fig. 5A). The lower bands became stronger, coinciding with the kinetic apoptosis after treatment, which prompted one to speculate that cyclin D1 might have been cleaved by caspase(s). However, this hypothesis will need testing in the future.

Ponatinib inhibited the growth of D816V KIT expressing cells in culture, and nude mouse xenografted tumor without tumor regrowth during the administration of the drug. This was further supported by the lowered expression in Ki67, phospho-KIT, Mcl-1, and β-catenin in the immunohistochemistry examination of paraffin sections from explanted tumors.

Mcl-1 is a prosurvival protein (43) that promotes the transformation of hematopoietic stem cells and increasing the resistance to chemotherapeutic agents (44). We demonstrated that silencing Mcl-1 by siRNA increases sensitivity of D816V KIT mutation harboring HMC-1.2 mast cell to ponatinib, whereas overexpression of Mcl-1 attenuated apoptosis induced by ponatinib. These results were in accord with the previous reports with TKIs (e.g., PKC412, AMN107, and imatinib; ref. 33) and agents such as triptolide (43) and homoharingtonine (25) in human mast cells. Our results suggest that Mcl-1 was reduced in a ubiquitin proteasome pathway-independent and caspase-3–independent manner in response to ponatinib. These findings leave it an open question the mechanism by which ponatinib decreases Mcl-1. Nevertheless, we and others (25, 33, 43, 44) provided results supporting a critical role of Mcl-1 in ponatinib-induced apoptosis in cells harboring D816V KIT mutation.

Our results indicated that ponatinib decreased β-catenin in vitro and in vivo. Our findings were in line with the previous reports that TKIs such as PKC412 and imatinib induce reduced β-catenin (45, 46). The underlying mechanism is not clear. Our results showed an accelerated turnover rate of β-catenin protein, but not supporting the
involvement of the ubiquitin proteasome pathway. Although we noted a concomitant reduce in the phosphorylation at Y654 in β-catenin in the ponatinib-treated cells, it remains to be investigated whether this effect is involved in the decreased stabilization in β-catenin induced by ponatinib.

Despite the unclear mechanism of lowered β-catenin by ponatinib, several lines of evidence reveals that ponatinib causes a blockade of β-catenin activity: ponatinib abolishes the tyrosine phosphorylation of KIT and β-catenin, blocks the translocation of β-catenin, and decreases the expression of target genes of Wnt signaling (AXIN2, c-MYC, and CCND1), no matter which mutation the cell harbors. Moreover, the decrease in TCF–DNA complexes and luciferase activity of pTOPflash reveals the transcriptional inhibition of ponatinib in mast cells. Furthermore, ectopic overexpression of β-catenin rendered a resistance to ponatinib-induced apoptosis while silencing β-catenin increased the sensitivity. The inhibition of β-catenin by TKIs seems a general phenomenon because PKC412 and imatinib have similar effects (45, 46).

The combined results of our in vitro and in vivo studies suggest that ponatinib exhibits antineoplastic activity against human mast cells harboring imatinib-sensitive and -resistant KIT mutations. Considering the present findings and the approval of ponatinib by FDA for the treatment of refractory patients with chronic myelogenous leukemia (19), a clinical trial of ponatinib for systemic mastocytosis patients with D816V KIT mutation may be warranted.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: B. Jin, J. Pan
Development of methodology: B. Jin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Pan, K. Ding
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): B. Jin, J. Pan
Writing, review, and/or revision of the manuscript: B. Jin, J. Pan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Jin, J. Pan
Study supervision: J. Pan

Grant Support
This study was supported by grants from the National Natural Science Fund of China (nos. 81025021, 81373434, 91213304, 90713036, and U1301226; to J. Pan), the National Basic Research Program of China (973 Program grant no. 2009CB825506; to J. Pan), the Research Foundation of Education Bureau of Guangdong Province, China (grant cxxd 1103; to J. Pan), the Research Foundation of Guangzhou Bureau of Science and Technology, China (grant to J. Pan), the National Hi-Tech Research and Development Program of China (863 Program grant no. 2008A022Z420; to J. Pan). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 16, 2013; revised January 16, 2014; accepted February 3, 2014; published OnlineFirst February 19, 2014.

References
8. Frost MJ, Ferraro PT, Hughes TP, Ashman LK. Juxtamembrane mutant V560GKit is more sensitive to Imatinib (STI571) compared with wild-type c-kit whereas the kinase domain mutant D816VKit is resistant. Mol Cancer Ther 2002;1:1115–24.

www.aacrjournals.org Mol Cancer Ther; 13(5) May 2014


Molecular Cancer Therapeutics

Ponatinib Induces Apoptosis in Imatinib-Resistant Human Mast Cells by Dephosphorylating Mutant D816V KIT and Silencing β-Catenin Signaling

Bei Jin, Ke Ding and Jingxuan Pan

Mol Cancer Ther  Published OnlineFirst February 19, 2014.

Updated version  Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-13-0397

Supplementary Material  Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/02/19/1535-7163.MCT-13-0397.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/early/2014/04/22/1535-7163.MCT-13-0397. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.