Essential Requirement for PP2A Inhibition by the Oncogenic Receptor c-KIT Suggests PP2A Reactivation as a Strategy to Treat c-KIT+ Cancers

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Abstract

Oncogenic mutations of the receptor tyrosine kinase c-KIT play an important role in the pathogenesis of gastrointestinal stromal tumors, systemic mastocytosis, and some acute myeloid leukemias (AML). Although juxtamembrane mutations commonly detected in gastrointestinal stromal tumor are sensitive to tyrosine kinase inhibitors, the kinase domain mutations frequently encountered in systemic mastocytosis and AML confer resistance and are largely unresponsive to targeted inhibition by the existing agent imatinib. In this study, we show that myeloid cells expressing activated c-KIT mutants that are imatinib sensitive (V560G) or imatinib resistant (D816V) can inhibit the tumor suppressor activity of protein phosphatase 2A (PP2A). This effect was associated with the reduced expression of PP2A structural (A) and regulatory subunits (B55α, B56δ, B56γ, and B56δ). Overexpression of PP2A-Aα in D816V c-KIT cells induced apoptosis and inhibited proliferation. In addition, pharmacologic activation of PP2A by FTY720 reduced proliferation, inhibited clonogenic potential, and induced apoptosis of mutant c-KIT+ cells, while having no effect on wild-type c-KIT cells or empty vector controls. FTY720 treatment caused the dephosphorylation of the D816V c-KIT receptor and its downstream signaling targets pAkt, pSTAT5, and pERK1/2. Additionally, in vivo administration of FTY720 delayed the growth of V560G and D816V c-KIT tumors, inhibited splenic and bone marrow infiltration, and prolonged survival. Our findings show that PP2A inhibition is essential for c-KIT–mediated tumorigenesis, and that reactivating PP2A may offer an attractive strategy to treat drug-resistant c-KIT+ cancers. Cancer Res; 70(13); OF1–10.

Introduction

c-KIT is a type 3 receptor tyrosine kinase that is characterized by five extracellular immunoglobulin-like domains and an intracellular split tyrosine kinase domain (1). Binding of stem cell factor to c-KIT activates multiple signaling pathways important for cellular proliferation, differentiation, and survival (2). Gain-of-function c-KIT mutations have been documented in core-binding factor acute myeloid leukemia (CBF-AML; ref. 3), systemic mastocytosis (4), gastrointestinal stromal tumors (5), testicular seminoma (6), and melanoma (7). In gastrointestinal stromal tumor samples ~85% of c-KIT aberrations are located in the juxtamembrane domain (e.g., V560G; ref. 5), whereas the majority of CBF-AML and systemic mastocytosis patients express kinase domain mutations (e.g., D816V). In most cases, the presence of an activating c-KIT mutation is associated with a higher relapse rate and reduced survival compared with patients expressing the wild-type (WT) receptor (8).

The tyrosine kinase inhibitor imatinib has shown remarkable success in treating metastatic gastrointestinal stromal tumor patients that harbor juxtamembrane c-KIT mutations (9). In contrast, mutations involving the kinase domain are resistant to imatinib inhibition, and as such, c-KIT+ CBF-AML and systemic mastocytosis patients are unresponsive to imatinib therapy (10, 11). Recent clinical trials with the second generation tyrosine kinase inhibitor dasatinib (SPRY-CEI, Bristol-Myers Squib) have also reported disappointing results (12). To improve treatment outcomes, a greater understanding of the signaling pathways activated downstream of c-KIT is required.

Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase that negatively regulates numerous signal transduction pathways that are involved in cell cycle progression,
DNA replication, and apoptosis (13). PP2A is emerging as an important tumor suppressor that is mutated or downregulated in several cancers including breast, lung, and colon (14–16). It is also inactivated by the oncogenic tyrosine kinase, BCR/ABL, in chronic myeloid leukemia (17). As BCR/ABL and c-KIT activate similar oncogenic pathways, we hypothesized that PP2A may also be regulated by c-KIT. In this study, we report that PP2A activity is inhibited in myeloid precursors expressing imatinib-sensitive (V560G) and imatinib-resistant (D816V) mutant c-KIT. Using in vitro and in vivo models of c-KIT tumorigenesis, we show that overexpression of PP2A-Aα, or reactivation of PP2A with FTY720 (fingolimod; Novartis; refs. 18, 19), inhibits c-KIT-mediated growth and survival. Furthermore, FTY720 exhibits selectivity for activating c-KIT mutations over the WT c-KIT receptor. As mutant c-KIT is the driving force behind multiple cancer types, these data suggest that PP2A activation may be a powerful strategy for the treatment of c-KIT+ malignancies.

Materials and Methods

Cell lines, retroviral infection, and patient samples

The FDC-P1 mouse growth factor–dependent myeloid cell line (20) expressing empty vector (EV), WT, and the oncogenic V560G and D816V mutant forms of human c-KIT have been previously described (21). The expression of c-KIT was routinely monitored by flow cytometry (21). The Kasumi-1 myeloid leukemia cell line (22) and HMC-1 human mast cell lines (23) expressing c-KIT mutations have been previously described (21). The human AML cell lines MV4-11 (24) and THP-1 (25) were a kind gift from Dr. Kyu-Tae Kim (University of Newcastle, Callaghan, New South Wales, Australia). Retroviral expression vectors pMIG EV and pMIG PP2A-Aα (Addgene plasmid 9044 and 10884; deposited by Prof. William C. Hahn, Dana Faber Cancer Institute, Boston, MA) have been previously reported (26). FDC-P1 D816V c-KIT cells expressing pMIG EV or PP2A-Aα were generated by retroviral transduction followed by fluorescence-activated cell sorting–mediated sorting of green fluorescent protein–positive cells (27).

A bone marrow sample was obtained from a 71-year-old male CBF-AML patient according to institutional guidelines. Studies were approved by the Royal Adelaide Hospital Human Ethics Committee. Mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation, washed, and resuspended at 2 × 10^5/mL in Iscove’s modified Dulbecco’s medium/0.5% fetal bovine serum. c-KIT mutation analysis was performed using high-resolution melt analysis and direct sequencing as described (28).

Drug treatments

Imatinib was provided by Prof. Bruce Lyons (University of Tasmania, Hobart, Australia) and resuspended in milli-Q water at 10 mmol/L for cell culture or 10 mg/mL for in vivo administration. For cell culture, FTY720 (Cayman Chemicals) and FTY720-P (Echelon Biosciences, Inc.) were dissolved in DMSO at 50 mmol/L stock. For in vivo administration, FTY720 was prepared daily in normal saline (0.9% w/v NaCl) at 1 mg/mL. Okadaic acid (Sigma) was dissolved in DMSO to 1 mmol/L.

Phosphatase activity assay

PP2A activity was determined using the PP2Ac immunoprecipitation phosphatase assay kit, as per the manufacturer’s instructions (Millipore). The percentage of phosphatase activity was determined by dividing the free phosphate (PO_4) of the test cells by that of the nontreated EV cells.

Immunoblotting and immunoprecipitation

Cells were lysed in 1% Triton X-100, 50 mmol/L HEPES (pH 7.4), 0.5% sodium deoxycholate, 0.1% SDS and 5 mmol/L EDTA supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, 50 mmol/L NaF, 1 mmol/L NaN_3, 20 μg/mL leupeptin, 20 μg/mL aprotinin, and 25 μL/mL complete protease inhibitor cocktail (Sigma). Total protein (50 μg) was separated by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare). For immunoblotting, the antibodies used were as follows: anti-PP2A-A (Calbiochem); anti-PP2A-B56α, anti-PP2A-B56γ3, anti–PP2A-B56δ, and anti–PP2A-B56ε (Novus Biochemicals); anti-PP2Ac Y307 (Epitomics), anti-PP2Ac Y320 (Epitomics), anti-PP2Ac Y397 (Epitomics), anti-Bad S112, anti–pAktT308, anti–pERK1/2T202/Y204, anti–pMEK1/2T222/Y223, anti–pp38MAPK T180/Y192, and anti–pSTAT3 T203/Y204 (Cell Signaling Technology, Inc.); and anti-actin (Sigma). Affinity-purified rabbit polyclonal antibodies were raised against a PP2A-B50α peptide (CESQVKGAVDVDV; ref. 29) and a PP2Ac peptide (PHVTRTPDYFL; ref. 30). Membranes were incubated with horseradish peroxidase–conjugated secondary antibodies and protein-antibody complexes visualized by enhanced chemiluminescence (GE Healthcare). Images were captured with a FUJIFILM LAS-3000 detection system and analyzed using Multi Gauge v3.0. Immunoprecipitation of c-KIT and detection of tyrosine phosphorylation was performed as previously described (31).

Immunofluorescent staining

Cells were cytoseparated onto a glass slide, fixed in 3.7% formaldehyde/PBS for 10 minutes at room temperature, and permeabilized in 0.1% Triton/PBS for 3 minutes. The slides were blocked in 10% FCS/PBS for 20 minutes, incubated with either 1:500 anti–PP2A-Aα (clone 6F9; Covance) or anti–cleaved caspase-3 (clone Asp175; Cell Signaling Technology, Inc.) for 1 hour, then with 1:1,000 dilution of the appropriate Alexa Fluor-555–conjugated secondary antibody (Invitrogen), followed by 4′,6-diamidino-2-phenylindole (DAPI) mounting (Invitrogen). Images were obtained using a FlouView FV1000 confocal microscope (Olympus) and the FV10-ASW v2.0 software (Olympus).

Cell proliferation, apoptosis, and cell cycle

FDC-P1 and human AML cell lines (2 × 10^5/well) were seeded in a 96-well plate with appropriate factors and the indicated concentrations of drug for 48 hours. Proliferation was assessed using the CellTiter-Blue Cell Viability Assay (Promega). The concentration of drug that reduces cell viability by 50% (ID_{50}) was analyzed using cubic spline regression (32). Assays
were plated in quadruplicate and repeated at least three times. Apoptosis was measured using the Annexin V FITC apoptosis detection kit (BD Biosciences). Samples were run on a FACSCalibur flow cytometer, and data were analyzed using the CellQuest software (BD Biosciences). For cell cycle analysis, cells ($5 \times 10^5$) were resuspended in 0.5 mL 0.1% glucose/PBS with 2.5 mL 70% ethanol for 1 hour at 4°C. Cells were washed in PBS then incubated with 50 μg/mL propidium iodide (BD Biosciences) and 0.2 mg/mL RNase (Fermentas) for 30 minutes at 37°C. Samples were analyzed on a FACSARia (BD Biosciences), and histograms were fitted for cell cycle ratios using ModFit LT v3.2 (Verity Software House). Four independent experiments were performed for each assay. For the AML patient sample, cells were treated with 0, 0.1, 0.3, 1, and 10 μmol/L FTY720, and AML blast survival was measured at 24 hours by containing with Annexin V FITC (Roche). The ID$_{50}$ of apoptosis induction by FTY720 was calculated as above.

**Clonogenic assay**

Cells ($2 \times 10^5$) were plated into 1% methylcellulose medium (MethoCult; Stem Cell Technologies, Inc.) in the presence of appropriate factors and indicated drug concentrations. Colonies (>50 cells) were scored 7 days later. Assays were performed in triplicate and repeated three times.

**Syngeneic mouse model**

Eight- to 10-week-old female DBA/2J mice (Animal Resources Centre) were s.c. injected on both flanks with $5 \times 10^5$ FDC-P1 V560G c-KIT or $3.5 \times 10^5$ FDC-P1 D816V c-KIT cells in 200 μL 1:1 PBS/Matrixel (Trevigen). When tumors reached a volume of ~200 mm$^3$ (day 5), mice were randomized into two groups that received daily i.p. injections of either saline or 10 mg/kg FTY720. Tumor volume was determined using the formula: $0.5 \times \text{length} (\text{mm}) \times \text{width}^2 (\text{mm})$. On day 14, three mice from FDC-P1 V560G c-KIT and six mice from FDC-P1 D816V c-KIT–injected groups were sacrificed. The remaining mice were used for survival studies and culled when tumor volumes reached ~2,100 mm$^3$. The tumors and organs were fixed in formalin and paraffin embedded. Immunohistochemical staining of anti-human c-KIT (CD117; DAKO) was detected with the Vectastain Elite ABC kit and diamobenzidine (Vector Laboratories, Inc.), and stained with a ColorView II camera and analysys software (Olympus Soft Imaging Systems GmbH). Apoptosis was assessed by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining using an *In situ* Cell Death Detection kit (Roche). Images were acquired with a Axioplan 2 imaging system and merged with Axio Vision v4.7 (Carl Zeiss). All animal studies were performed with approval of The University of Newcastle Animal Care and Ethics Committee.

**Statistical analysis**

Statistical significance between samples was assessed using an unpaired two-tailed Student’s *t* test. Survival probabilities between groups were determined by the Kaplan-Meier method, and differences in survival distributions were evaluated by the log-rank test. Values shown are the mean ± SEM. All analyses were performed using the GraphPad Prism software.

**Results**

**c-KIT inhibits PP2A activity and alters the expression of PP2A subunits**

To evaluate whether PP2A is regulated by c-KIT, we measured the activity of PP2Ac immunoprecipitates extracted from FDC-P1 mouse myeloid cells expressing EV, WT c-KIT, the oncogenic imatinib-sensitive juxtamembrane V560G c-KIT mutant, or the imatinib-resistant kinase domain D816V c-KIT mutant (21). The phosphatase activity of PP2A was significantly reduced to 72% and 65% in cells expressing V560G and D816V c-KIT, respectively, compared with EV controls (Supplementary Table S1). Thus, activating mutations of c-KIT reduce PP2A activity.

The cellular localization and substrate specificity of PP2A is regulated by posttranslational modification of the catalytic subunit and binding of regulatory B subunits (33, 34). To investigate the mechanism by which constitutive c-KIT activation inhibits PP2A, expression of PP2A subunits were examined by immunoblotting. There was no change in the total expression or phosphorylation of PP2Ac in cells expressing WT or mutant c-KIT (Supplementary Fig. S1; Fig. 1). In contrast, protein levels of PP2A-A, B55α, B56α, and B56β were significantly decreased in mutant c-KIT+ cells. Furthermore, B56γ was barely detectable in mutant c-KIT+ cells, whereas WT c-KIT cells expressed a lower molecular weight isoform (Supplementary Fig. S1; Fig. 1). No change was observed in B56ε. B55β and B53γ are neuronal specific isoforms and are not expressed in the FDC-P1 cells (data not shown). Quantitative real-time PCR showed no significant difference in PP2A subunit mRNA levels between EV controls and mutant c-KIT+ cells (Supplementary Fig. S2). Interestingly, total expression of the PP2A inhibitory protein, SET, was elevated in the mutant c-KIT+ cells, together with the presence of a lower molecular weight isoform (Fig. 1).

**Overexpression of PP2A-Aα reverses the tumorigenic phenotype**

To investigate the functional importance of reduced PP2A activity in oncogenic c-KIT+ cells, the pMIG vector encoding the PP2A-Aα subunit or corresponding EV control was introduced into the FDC-P1 D816V c-KIT cells by retroviral transduction. After 24 hours (day 0), the top 10% of GFP+ positive cells increased further in the PP2A-Aα injected groups compared with the EV controls (Fig. 2B). Furthermore, the percentage of cleaved caspase-3–positive cells increased from 4% in the EV controls to 16% in the PP2A-Aα cells.
 Accordingly, overexpression of PP2A-α inhibited the proliferation of D816V c-KIT cells (Fig. 2C), supporting the hypothesis that the functional loss of PP2A contributes to c-KIT–mediated tumorigenesis.

Reactivation of PP2A inhibits the proliferation of mutant c-KIT+ cells

To determine the biological relevance and potential therapeutic implications of PP2A loss-of-function in c-KIT+ malignancies, we used the pharmacologic activator, FTY720. PP2A activity was enhanced 1.7-fold by FTY720 (2.5 μmol/L; 6 h) in cells expressing V560G c-KIT and 2.4-fold in FTY720-treated D816V c-KIT cells compared with nontreated controls (Fig. 3A). In contrast, minimal effect on PP2A activity was seen in WT c-KIT cells or EV controls (Fig. 3A).

FTY720 is phosphorylated in vivo by sphingosine kinase 2 to yield FTY720-P, which binds to sphingosine-1-phosphate–specific G protein–coupled receptors (S1PR) and induces signaling that regulates a variety of cellular processes (35). To determine whether reactivation of PP2A is dependent on S1PR-induced signaling, we incubated the cells with FTY720-P (2.5 μmol/L; 6 h). In all cell lines, PP2A activity did not increase (Fig. 3A), suggesting that nonphosphorylated FTY720 is responsible for PP2A reactivation.

The effect of FTY720 on viable cell number was determined using a colorimetric assay. Importantly, the ID₅₀ values for V560G and D816V c-KIT cells were significantly reduced compared with WT c-KIT and EV cell lines (Supplementary Fig. S3A; Table 1A). Treatment with a chemically distinct PP2A activator, forskolin (17), showed similar results, whereby mutant c-KIT cells were more sensitive to reactivation of PP2A than EV controls (Table 1A). Incubation with FTY720-P up to 20 μmol/L had no effect on the growth of FDC-P1 cell lines (Table 1A). That FTY720 and forskolin have distinct mechanisms of action, yet both activate PP2A, strongly suggests that the cytotoxicity observed in mutant c-KIT+ cells is due to the enhanced activation of PP2A. Furthermore, this effect is not mediated through S1PR signaling.

We then tested the efficacy of FTY720 on a panel of human AML cell lines. The human mast cell line HMC-1.1 expresses a V560G c-KIT mutation, and HMC-1.2 expresses an additional D816V c-KIT mutation (23). The Kasumi-1 CBF-AML cell line harbors a t(8;21) translocation together with a N822K c-KIT mutation (22). Each of these cell lines displayed increased sensitivity to FTY720 than human AML cell lines expressing WT c-KIT (MV4-11 and THP-1; Supplementary Fig. S3B; Table 1B; refs. 24 and 25). Furthermore, blasts isolated from a CBF-AML patient with inv(16) and D816V c-KIT displayed remarkable sensitivity to apoptosis induction by FTY720 with an ID₅₀ of 0.6 μmol/L (Table 1C). These data show that myeloid malignancies characterized by mutant c-KIT are sensitive to FTY720.

FTY720 induces apoptosis and inhibits the clonogenic potential of mutant c-KIT+ cells

The ability of FTY720 to induce apoptosis in c-KIT–expressing myeloid precursors was investigated by staining with Annexin V (Fig. 3B). In both the mutant c-KIT cell lines, FTY720 (2.5 μmol/L) markedly increased the percentage of Annexin V+ cells at 24 hours compared with nontreated controls (Supplementary Fig. S4; Fig. 3B) and induced a shift into the sub-G₀ phase at 36 hours (Fig. 3C). Importantly, FDC-P1 EV or WT c-KIT cells remained unaffected by the presence of 2.5 μmol/L FTY720 (Fig. 3B and C) up to 48 hours (data not shown). Accordingly, the clonogenic potential of FDC-P1 cells expressing V560G c-KIT or D816V c-KIT was significantly inhibited by 2.5 μmol/L FTY720 (Fig. 3D). In contrast, no difference was observed between nontreated and FTY720-treated WT c-KIT cells or EV controls (Fig. 3D).

To confirm that the observed cytotoxic effects were a consequence of PP2A reactivation by FTY720, FDC-P1 D816V c-KIT cells were cotreated with the serine-threonine phosphatase inhibitor okadaic acid (0.25 nmol/L) at a concentration that specifically inhibits the activity of PP2A (36). Importantly, the addition of okadaic acid rescued the colony formation of FTY720-treated cells (Fig. 3D). Furthermore, the clonogenic potential of V560G and D816V c-KIT
cells treated with 2.5 μmol/L FTY720-P resembled that of nontreated controls (Fig. 3D). Together these data indicate that FTY720 mediates its effects on FDC-P1 mutant c-KIT cells by specifically reactivating PP2A.

Reactivation of PP2A dephosphorylates the c-KIT receptor and its downstream signaling targets

c-KIT activity is regulated by tyrosine phosphorylation (37). To determine if PP2A reactivation affects c-KIT phosphorylation, the receptor was immunoprecipitated from FDC-P1 cells treated with FTY720 (2.5 μmol/L; 6, 12, and 24 h). FTY720 caused the marked inhibition of V560G and D816V c-KIT phosphorylation at 6 hours (Fig. 4A). In contrast, phosphorylation of the WT c-KIT receptor remained stable, consistent with these cells being less responsive to FTY720 (Fig. 4A).

We next analyzed the effect of FTY720 on known PP2A and c-KIT signaling targets. Phosphorylation of Akt, STAT5, extracellular signal-regulated kinase 1/2 (ERK1/2), p38MAPK, and BAD in WT and V560G c-KIT–expressing cells was undetectable (data not shown), which is consistent with previous reports (21, 38, 39). As such, our investigations focused on the D816V c-KIT cells, which showed reduced phosphorylation of pAkt, pSTAT5, pERK1/2, pp38MAPK, and pBAD with FTY720 treatment (2.5 μmol/L; 12 h; Supplementary Fig. S5; Fig. 4B). No change in pMEK was observed. Although the D816V cells expressed increased total levels of several proteins, the expression did not alter with FTY720 (Supplementary Fig. S6; Fig. 4B). Importantly, FTY720 did not affect signaling through the phosphoinositide 3-kinase (PI3K), signal transducers and activators of transcription (STAT), or mitogen-activated protein kinase (MAPK) pathways in the EV controls (Fig. 4B). These findings suggest that PP2A inhibition is required for sustained c-KIT activation, and that enhancing the activity of PP2A results in the dephosphorylation of D816V c-KIT and its downstream targets.

FTY720 delays mutant c-KIT tumor growth in vivo

The efficacy of FTY720 against established tumors expressing either the V560G or D816V mutant c-KIT was evaluated in a syngeneic mouse model. For mice harboring V560G c-KIT tumors, FTY720 treatment prolonged their survival compared with nontreated controls (21 d versus 24 d; P < 0.05; Fig. 5A). Similarly, for tumors expressing D816V c-KIT, the median survival for FTY720-treated mice was 22 days, a significant increase from 18 days for saline-treated mice.
As expected, imatinib was effective against V560G c-KIT tumors only (Fig. 5A; Supplementary Fig. S7). At day 14, a subset of mice from each group were sacrificed. Compared with saline-treated mice, FTY720 significantly reduced the tumor mass in the V560G c-KIT and D816V c-KIT groups (Fig. 5B). Consistent with these observations, a significant increase in TUNEL-positive cells was observed in tumors treated with FTY720 compared with saline controls (Supplementary Figs. S8 and S9; Fig. 5C).

The D816V c-KIT tumors grew at a faster rate than those expressing V560G c-KIT (Supplementary Fig. S7). Consistent with this observation, saline-treated mice from the D816V c-KIT group developed splenomegaly by day 14, as indicated by an increase in splenic weight compared with age-matched controls (Fig. 5D). Immunohistochemical analysis revealed a disruption of splenic architecture and intense c-KIT–positive staining in saline-treated mice only (Fig. 5D). Importantly, the spleen size of FTY720-treated mice was significantly lower than saline-treated mice; the histopathology resembled that of age-matched controls; and the presence of c-KIT+ cells was almost nondetectable. Similar results were observed in the bone marrow (Fig. 5D). It should be noted that splenomegaly was not observed at day 14 in mice injected with V560G c-KIT cells (Supplementary Fig. S10). No signs of acute or delayed toxicity were observed with FTY720 treatment (data not shown).

**Discussion**

This study has shown that inhibition of PP2A is a crucial mediator of c-KIT tumorigenesis. Constitutive activation of c-KIT through the juxtamembrane domain mutation V560G or the kinase domain mutation D816V impairs the activity

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**Figure 3.** Reactivation of PP2A induces apoptosis and suppresses the clonogenic potential of mutant c-KIT cells. A, FDC-P1 cells were treated with FTY720 or FTY720-P (2.5 μmol/L, 6 h). PP2A activity was determined by incubating the isolated PP2Ac complex with a PP2A-specific phosphopeptide and measuring free phosphate release using a colorimetric assay. PP2A activity is normalized to nontreated EV controls. B, FDC-P1 cells were treated with FTY720 (2.5 μmol/L, 24 h) and assessed for Annexin V by flow cytometry. C, FDC-P1 cells were treated with FTY720 (2.5 μmol/L; 36 h) and stained with propidium iodide (PI) to analyze DNA content. Plots are a representative of four independent experiments. D, FDC-P1 cells were grown in methylcellulose for 7 d in the presence of indicated drugs. Columns, mean colony number (n = 4); bars, SEM. *, P < 0.05; **, P < 0.01, Student's t test compared with nontreated.
of the tumor suppressor PP2A. The mechanism of PP2A inhibition is associated with the decreased expression of PP2A structural and regulatory subunits. Furthermore, pharmacologic reactivation of PP2A specifically reduces the growth and survival of both imatinib-sensitive and imatinib-resistant c-KIT+ cells. Thus, specific activation of PP2A provides a unique target for therapeutic intervention in cancers expressing mutant c-KIT.

Table 1. Cytotoxicity of PP2A activators in mutant c-KIT+ myeloid cells

<table>
<thead>
<tr>
<th>A. FDC-P1 cell line</th>
<th>FTY720 (μmol/L)</th>
<th>Forskolin (μmol/L)</th>
<th>FTY720-P (μmol/L)</th>
</tr>
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<tbody>
<tr>
<td>ID50*</td>
<td>Sensitivity†</td>
<td>ID50</td>
<td>Sensitivity</td>
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<tr>
<td>EV</td>
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<td>WT c-KIT</td>
<td>4.4 ± 0.4</td>
<td>1.25</td>
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<tr>
<td>V560G c-KIT</td>
<td>2.8 ± 0.3</td>
<td>1.96‡</td>
<td>19.4 ± 6.7</td>
</tr>
<tr>
<td>D816V c-KIT</td>
<td>2.4 ± 0.2</td>
<td>2.20‡</td>
<td>10.6 ± 6.2</td>
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<tr>
<th>B. Human cell lines</th>
<th>FTY720 ID50 (μmol/L)*</th>
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<tbody>
<tr>
<td>HMC 1.1 (V560G c-KIT)</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>HMC 1.2 (V560G/D816V c-KIT)</td>
<td>3.4 ± 0.1</td>
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<tr>
<td>Kasumi-1 (N822K c-KIT)</td>
<td>4.9 ± 0.4</td>
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<tr>
<td>MV4-11 (WT c-KIT)</td>
<td>7.5 ± 0.3</td>
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<tr>
<td>THP-1 (WT c-KIT)</td>
<td>5.5 ± 0.1</td>
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<th>C. Patient sample</th>
<th>Age, y/sex</th>
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<th>c-KIT</th>
<th>FTY720 ID50 (μmol/L)§</th>
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<tr>
<td>CBF-AML-1</td>
<td>71/M</td>
<td>69</td>
<td>Inv(16)</td>
<td>D816Y</td>
<td>0.62 ± 0.2</td>
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</table>

Abbreviation: N/A, not available.
*ID50 is the concentration (μmol/L) of drug required to reduce cell viability by 50% at 48 h and was calculated using fit-spline lowess regression. Data are presented as the mean of at least three independent experiments performed in quadruplicate ± SEM.
†Sensitivity was determined by dividing the ID50 of the FDC-P1 EV cells by the ID50 of the FDC-P1 c-KIT cell lines.
‡P < 0.01 compared with EV, Student’s t test.
§Primary AML cells were treated with 0, 0.1, 0.3, 1, and 10 μmol/L FTY720. Induction of apoptosis was measured at 24 h by Annexin V binding, and ID50 was calculated as above.

Figure 4. Reactivation of PP2A dephosphorylates the c-KIT receptor and its downstream signaling targets. A, c-KIT was immunoprecipitated from FDC-P1 cells treated with 2.5 μmol/L FTY720 (0, 6, 12, 24 h). Levels of tyrosine phosphorylated (pY) and total c-KIT were determined by immunoblotting (top). Bottom, quantitation of three independent experiments. B, immunoblots detecting phosphorylated or total Akt, STAT5, ERK1/2, p38MAPK, MAP/ERK kinase, and BAD after FTY720 treatment (2.5 μmol/L; 12 h).
Regulation of PP2A activity and specificity is complex. Translation of the catalytic subunit is tightly controlled (13), and indeed, no changes in the total protein expression or phosphorylation of PP2Ac in cells with or without c-KIT was found (Fig. 1). However, inhibition of PP2A in the mutant c-KIT+ cells was associated with reduced expression of the structural and several regulatory PP2A subunits (Fig. 1). Downregulation of PP2A subunits has been reported in human gliomas, breast cancers, and B-cell chronic lymphocytic leukemia primary samples (15, 40, 41), and in melanoma and lung cancer cell lines (29, 42). Furthermore, suppression of PP2A-Aα or B56γ using short hairpin RNA induces tumorigenicity in transformed HEK293T cells (26, 29). That overexpression of the PP2A-Aα subunit induces apoptosis and inhibits the proliferation of FDC-P1 D816V c-KIT cells (Fig. 2) suggests that functional loss of PP2A is required for c-KIT-mediated tumorigenesis and further implicates PP2A as a tumor suppressor.

We show that impaired PP2A activity can be restored in mutant c-KIT+ myeloid cells using the pharmacologic agent, FTY720 (Fig. 3A). Importantly, treatment with FTY720 induces c-KIT tyrosine dephosphorylation on both imatinib-sensitive and imatinib-resistant activating mutants (Fig. 4A). The mechanisms underlying this are unclear. As PP2A is a serine/threonine phosphatase, dephosphorylation of c-KIT most likely involves the recruitment of additional proteins that act directly on the receptor, such as the c-KIT targeting tyrosine phosphatase SHP-1 (17, 43). Furthermore, FTY720 specifically inhibited components of the PI3K, MAPK, and Janus-activated kinase/STAT pathways in the D816V c-KIT cells (Fig. 4B). Thus, the cytotoxicity in these cells can be explained by the fact that FTY720...
inhibits both the D816V c-KIT receptor and its downstream signals.

Consistent with the dephosphorylation of oncogenic c-KIT and its downstream targets, reactivation of PP2A by FTY720 reduced the proliferation (Table 1), induced apoptosis (Fig. 3B), and suppressed the clonogenic potential of cells expressing V650G or D816V c-KIT (Fig. 3D). Importantly, at the same concentration of FTY720, these effects were not observed in granulocyte macrophage colony-stimulating factor–dependent EV cells or WT c-KIT cells grown in stem cell factor. This highlights that the growth inhibition with FTY720 treatment is dependent on the functional status of PP2A, whereby cells expressing activating c-KIT mutations are more sensitive due to impaired PP2A activity. The increased sensitivity of c-KIT mutants compared with WT c-KIT suggests that FTY720 could target malignant cells at low doses without disrupting normal stem cell factor/c-KIT signaling.

We show that daily administration of FTY720 markedly delays the in vivo growth of tumors expressing activating c-KIT mutations through the induction of apoptosis at the cellular level (Fig. 5C). Importantly, the reduction in tumor mass resulted in prolonged survival of FTY720-treated mice compared with saline-treated controls (Fig. 5A). Moreover, FTY720 inhibited the engraftment of FDC-P1 D816V c-KIT tumor cells into secondary lymphoid organs including the spleen and bone marrow (Fig. 5D). FTY720 also inhibited the proliferation of mutant c-KIT human cell lines and induced apoptosis of primary blasts from a CBF-AML patient with a D816Y c-KIT mutation (Table 1). Taken together, these results show that cancers harboring human c-KIT mutations are sensitive to FTY720 treatment.

FTY720 is a water-soluble, nontoxic drug with high oral bioavailability that is currently being evaluated as an immunomodulator in phase III trials for multiple sclerosis patients (35, 44). FTY720 becomes phosphorylated in vitro by sphingosine kinase 2 and FTY720-P binds to S1PR, which induces its internalization (45). Although the mechanism of action is not currently known, FTY720 has also been shown to activate purified PP2A in vitro (18), and its ability to activate PP2A and induce cytotoxicity has been shown in a variety of leukemic models (19, 46). In agreement with the mechanism whereby FTY720 induces apoptosis of BCR/ABL+ myeloid and lymphoid progenitors (19), we have shown that PP2A reactivation does not require FTY720 phosphorylation (Fig. 3A). Accordingly, incubation with FTY720-P has no effect on cell viability or the clonogenic potential of mutant c-KIT+ cell lines (Table 1; Fig. 3). Furthermore, the induction of apoptosis by FTY720 specifically requires activation of PP2A, as inhibition of FTY720-induced PP2A activation by okadaic acid restores the clonogenic potential of mutant c-KIT+ cells (Fig. 3D). We also show that a chemically distinct PP2A activator, forskolin, inhibits the growth of c-KIT+ cells (Table 1). The hypersensitivity of oncogenic c-KIT+ cells to two distinct PP2A activators strongly indicates that reactivation of PP2A is the essential mechanism of action.

In summary, our data show for the first time that activating c-KIT mutations inhibit the tumor suppressor, PP2A, and that reactivating PP2A effectively suppresses the in vitro and in vivo growth of imatinib-sensitive and imatinib-resistant c-KIT+ cells. As kinase domain mutations, such as D816V, are most commonly detected in nonresponsive patients, and there is currently no effective inhibitor against these mutants, PP2A reactivation represents a unique and powerful strategy for treating drug-resistant patients. Furthermore, our study together with those related to the interplay between PP2A and BCR/ABL (17) indicate that functional inactivation of PP2A tumor suppressor activity represents a key step in the induction and maintenance of leukemias and, perhaps, other solid tumors characterized by the aberrant activation of oncogenic tyrosine kinases.

Disclosure of Potential Conflicts of Interest

A provisional patent has been filed for the use of PP2A activators to treat c-KIT cancers based on these data.

Acknowledgments

We thank Prof. William Hahn for providing the PP2A antibodies, Lauren Watt (University of Newcastle, Australia) for the assistance with confocal microscopy, and Angela Tan and David Westerman (Peter MacCallum Cancer Centre, Melbourne, Australia) for the c-KIT mutation screening of AML samples.

Grant Support

Grants from the Cancer Council NSW, Anthony Rothe Memorial Trust, Cure Cancer Australia Foundation, and Hunter Medical Research Institute (N.M. Verrills); the National Cancer Institute CA095512, NIH, Bethesda, MD, and the US Army, CML Research Program, W81XWH-07-1-0270 (D. Perrotti); the American-Italian Cancer Foundation (P. Neviani); an Australian Postgraduate Award and a Cancer Institute NSW Research Scholar Award (K.G. Roberts); an Australian Postgraduate Award and an Arrow Bone Marrow Transplant Foundation Scholarship (A.M. Smith); and scholarship from The Leukemia and Lymphoma Society (D. Perrotti), N.M. Verrills is a National Health and Medical Research Council Peter Doherty Fellow. 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 07/09/2009; revised 05/06/2010; accepted 05/07/2010; published OnlineFirst 06/15/2010.

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OF10 Cancer Res; 70(13) July 1, 2010

Cancer Research


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Cancer Res  Published OnlineFirst June 15, 2010.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-09-2544

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