Simultaneous Inhibition of PDK1/AKT and Fms-Like Tyrosine Kinase 3 Signaling by a Small-Molecule KP372-1 Induces Mitochondrial Dysfunction and Apoptosis in Acute Myelogenous Leukemia

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Abstract

Phosphoinosit-3-kinase (PI3K)/protein kinase B (AKT) and Fms-like tyrosine kinase 3 (FLT3) signaling are aberrantly activated in acute myelogenous leukemia (AML) cells. Constitutively activated AKT and FLT3 regulate leukemia cell survival and resistance to chemotherapy. In this study, we investigated the effects of the novel multiple kinase inhibitor KP372-1 on the survival of AML cell lines and primary AML samples. KP372-1 directly inhibited the kinase activity of AKT, PDK1, and FLT3 in a concentration-dependent manner. Western blot analysis indicated that KP372-1 decreased the phosphorylation of AKT on both Ser473 and Thr308, abrogated the phosphorylation of p70S6 kinase, BAD, and Foxo3a via PI3K/AKT signaling, and down-regulated expression of PIM-1 through direct inhibition of FLT3. Treatment of AML cell lines with KP372-1 resulted in rapid generation of reactive oxygen species and stimulation of oxygen consumption, followed by mitochondrial depolarization, caspase activation, and phosphatidylserine externalization. KP372-1 induced pronounced apoptosis in AML cell lines and primary samples irrespective of their FLT3 status, but not in normal CD34+ cells. Moreover, KP372-1 markedly decreased the colony-forming ability of primary AML samples (IC50 < 200 nmol/L) with minimal toxicity to normal progenitor cells. Taken together, our results show that the simultaneous inhibition of critical prosurvival kinases by KP372-1 leads to mitochondrial dysfunction and apoptosis of AML but not normal hematopoietic progenitor cells.

Introduction

The phosphoinosit-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway has been shown to be aberrantly activated in a variety of malignancies. Activation of the lipid kinase activity of PI3K results in the generation of the second messenger phosphatidylinositol-3,4,5-triphosphate and subsequent recruitment of AKT to the plasma membrane. Plasma membrane–bound PKD1 in turn phosphorylates Thr308 in the pleckstrin homology domain of AKT, which results in activation of AKT (1). However, AKT activity is ~10-fold enhanced when the protein is phosphorylated on COOH-terminal Ser473 by PDK2, integrin-linked kinase, or via autophosphorylation (2). AKT promotes cell growth, proliferation, and survival through direct phosphorylation of downstream substrates, including Bcl-2 family member BAD (3, 4), Forkhead family of transcription factors (FKHRL1 or Foxo3a; ref. 5), p70S6K (6), and glycogen synthase kinase (GSK) 3β (7). In leukemias, the PI3K/AKT/mTOR pathway is frequently activated downstream of a variety of transforming oncogenes, including breakpoint-cluster region/Abelson leukemia, activated Ras, and platelet-derived growth factor receptor β fusion proteins (8–10). Results from our group (3) and others have shown that AKT is constitutively phosphorylated on Ser473 and Thr308 in the majority of samples from patients with acute myelogenous leukemia (AML; refs. 11, 12), and the p110δ isoform of PI3K was recently reported to be overexpressed in high percentage of AML cases (13). Hence, inhibitors of PI3K/AKT would be effective chemotherapeutic agents either alone or in combination with other oncogene-specific inhibitors, such as tyrosine kinase–specific inhibitors.

Fms-like tyrosine kinase 3 (FLT3), a member of the class III receptor tyrosine kinase family, is constitutively expressed at high levels in AML. Approximately 30% of AML patients have gain-of-function mutations in FLT3, including either FLT3-ITD (internal tandem duplication) mutations (24%) or FLT3 activation loop mutations, such as D835 (7%). Patients harboring FLT3-ITD mutations have a relatively poor prognosis (14), especially when the other allele is mutated and/or lost (15).

Both FLT3 mutations result in constitutive activation of the FLT3 kinase, which gives a proliferative and survival advantage to malignant hematopoietic progenitors (16, 17). Activated FLT3 directly up-regulates signal transducer and activator of transcription 5 (STAT5) and the proto-oncogene PIM-1, likely downstream of STAT5 (18, 19). Furthermore, constitutive activation of AKT with concomitant phosphorylation of Foxo3a (19, 20) and activation of RAS/mitogen-activated protein kinase (MAPK) pathway (18, 21) have been observed in response to FLT3 activation (16). Specific FLT3 inhibitors, such as CEP701 (22), CT53518 (23), and SU5414 (24), have been shown to inhibit the autophosphorylation of FLT3/ITD and are currently under development as therapeutic agents for AML. PKC412, a multikinase inhibitor (25, 26) with activity against FLT3, induces mitochondrial dysfunction and apoptosis in AML cell lines irrespective of their FLT3 status, but not in normal CD34+ cells. Moreover, PKC412 markedly decreased the colony-forming ability of primary AML samples (IC50 < 200 nmol/L) with minimal toxicity to normal progenitor cells. Taken together, our results show that the simultaneous inhibition of critical prosurvival kinases by KP372-1 leads to mitochondrial dysfunction and apoptosis of AML but not normal hematopoietic progenitor cells.
In this study, we examined the antileukemic effects of the novel small-molecule kinase inhibitor KP372-1. We present evidence that KP372-1 potently inhibits pathways critical for AML survival, including PDK1/AKT and FLT3. The combined effects of inhibiting these pathways using agent results in mitochondrial dysfunction and apoptosis in cell lines and primary AML samples, including those with FLT3 mutations. These data support the notion of targeting multiple signaling pathways to induce apoptosis in leukemia cells and warrant further investigation into the potential clinical utility of this strategy for the therapy of AML.

Materials and Methods

Reagents. KP372-1, a mixture of two isomers present in approximately equal amounts (29), was provided by QLT/Kinetek Pharmaceuticals (Vancouver, BC, Canada; patent no. US6,514,972 B2). KP372-1 was identified in a high-throughput screen of ~70,000 compounds in a kinase assay using recombinant PKB/AKT enzyme and a specific peptide substrate (QLT, Inc., Vancouver, BC, Canada), and KP372-1 has at least a 10-fold selectivity against a limited number of kinase targets, including Cdk1, CK2, CSK, DNAPK, ERK1, GSK3b, JCK, MEK1, PIM-1, PKA, PKC, and S6K. A stock solution of KP372-1 for enzyme or cellular assays was prepared in DMSO and then diluted in the medium. The final concentration of DMSO in the incubation mixture did not exceed 0.1% v/v. L32/29002 (2-[4-morpholinyl]-8-phenyl-[4H]-benzopyran-4-one) and DMSO were purchased from Sigma Chemical Company (St. Louis, MO).

Cell lines and primary AML samples. KG-1, U937, and TF-1 cells were purchased from the American Type Culture Collection (Rockville, MD). NB4 cells were obtained from Dr. M. Lanotte (INSERM, Paris, France; ref. 30). Murine parental BaF3 cells and BaF3 cells transfected with wild-type (WT) FLT3 (BaF3/FLT3) or with a mutated FLT3 (FLT3/ITD and FLT3/D835G) were kindly provided by Dr. Donald Small (John Hopkins School of Medicine, Baltimore, MD). Cells were maintained in RPMI 1640 containing 10% FCS (Gemini Bio-Products, Woodland, CA) and 1% penicillin-streptomycin (Life Technologies Laboratories, Grand Island, NY). In all experiments, cells were plated at an initial density of 0.5 × 10^5/mL, and exposed to KP372-1 for the indicated time period. Cell viability was determined by trypan blue exclusion.

Bone marrow or peripheral blood samples were obtained for in vitro studies from patients with newly diagnosed or recurrent AML. Samples were obtained during routine diagnostic procedures after informed consent, obtained in accordance with regulations and protocols sanctioned by the Human Subjects Committee of The University of Texas MD. Anderson Cancer Center. All samples contained >70% blasts after Ficoll-Hypaque separation. The clinical features of the patients are listed in Table 1. Cells were either used for colony assays, as described below, or cultured in AIM-V medium (Life Technologies Laboratory) supplemented with 5% fetal bovine serum (FBS), 1 mM L-glutamine, and 50 μg/mL penicillin/streptomycin. Normal bone marrow or apheresis samples were isolated from samples of hematologically healthy transplant donors under informed consent. In some experiments, low-density bone marrow cells were separated by Ficoll-Hypaque density-gradient centrifugation, and the CD34+ progenitor cells were isolated by use of the magnetic-activated cell sorting method.

**AKT kinase assay.** The AKT kinase assay kit was obtained from Cell Signaling (Beverly, MA) and used according to the instructions of the manufacturer. Briefly, cell lysates (200 μg) were incubated for 2 hours with immobilized AKT monoclonal antibody. After extensive washing, the immobilized protein was exposed to different concentrations of KP372-1 for 30 minutes, and an AKT kinase assay was done in the presence of ATP and GSK-3 as a substrate. The phosphorylation of GSK-3 was measured by Western blot analysis in which an anti-phospho-GSK-3 antibody (Cell Signaling) was used.

**PDK1 kinase assay.** This in vitro assay was done using a PDK1 kinase assay kit (Upstate, Lake Placid, NY) according to the instructions of the vendor. This cell-free assay is based on the ability of recombinant PDK1, in the presence of DMSO vehicle or the test agent, to activate its downstream kinase serum- and glucocorticoid-regulated kinase (SGK) which, in turn, phosphorlates the AKT/SGK–specific peptide substrate RPRAATF with [γ-32P]ATP. The 32P-phosphorylated peptide substrate was then separated from the residual [γ-32P]ATP using phosphocellulose paper and quantified by a scintillation counter (Perkin-Elmer, Downers Grove, IL). The reported values represent the means of two independent determinations.

**Small interfering RNA transfection.** MCF-7 cells were cultured in six-well plates in 2 mL DMEM supplemented with 5% FBS. When cells reached ~50% to 60% confluency, small interfering RNA (siRNA) RPS6KB1 purchased from Dharmaco (Lafayette, CO) was transfected using Oligofectamine reagent (Invitrogen, Carlsbad, CA). Briefly, 7 μL of 20 μM/L stock solution of siRNAs was added into each well to give a final concentration of 140 nmol/L. After 48-hours transfection, cells were treated with different concentrations of KP372-1 for 8 hours and lysed in phosphoprotein lysis buffer. Western blot was used to detect the effects on target proteins.

**Flow cytometric analysis of apoptosis.** Apoptosis was determined by the flow cytometric measurement of phosphorylation of DNA-PK and GSK-3 using Annexin V FITC. Briefly, cells were washed twice with binding buffer [10 mM/L HEPES, 140 mM/L NaCl, and 5 mM/L CaCl2 (pH 7.4); all from Sigma Chemical] and stained with FITC-conjugated Annexin V for 15 minutes at room temperature. Annexin V fluorescence was determined with a Becton Dickinson FACScan flow cytometer, and the membrane integrity of the cells was simultaneously assessed by the propidium iodide exclusion method. Annexin V binds to those cells that express phosphatidylserine on the outer layer of their membrane, and propidium iodide stains the cellular DNA of those cells with a compromised membrane. The mitochondrial membrane potential (ΔΨm) in intact cells was evaluated in cells loaded with CMXROS (300 nmol/L). MitoTracker Green (100 μmol/L) was used concomitantly to monitor mitochondrial mass. CMXROS and MitoTracker Green were purchased from Molecular Probes (Eugene, OR). The ΔΨm was determined by measuring CMXROS retention (red fluorescence) while simultaneously adjusting for the mitochondrial mass (green fluorescence). Active caspases were detected by flow cytometry using CaspaTag (Intergen, Gaithersburg, MD), as described elsewhere (31).

**Cytochrome c release.** Cells were permeabilized with digitonin extraction buffer [0.25 mol/L sacrose, 70 mM/L LiCl, 137 mM/L NaCl, 4.3 mM/L Na2HPO4, 1.4 mM/L KH2PO4 (pH 7.2), 200 μg/mL digitonin, and protease inhibitors] for 5 minutes on ice, then centrifuged at 1,000 × g for 5 minutes at 4°C, after which the supernatant was separated by 10,000 × g centrifugation for 10 minutes at 4°C. The supernatants were cytosolic fractions, and their cytochrome c content was determined by Western blot analysis using specific antibodies to cytochrome c.

**Western blot analysis.** For the Western blot analyses, cells were lysed in either protein lysis buffer (0.25 mol/L Tris-HCl 2% SDS, 4% β-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue; 0.2 mg/mL ribonucleic acid, 0.5 μL/L lysophosphatidylcholine). Lysis buffers were supplemented with a protease inhibitor cocktail (Roche Diagnostic Co., Indianapolis, IN). Lysates were then separated on a 12% polyacrylamide gel, transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Buckinghamshire, England), probed with the appropriate antibodies, and visualized by using ECL plus kit (Amersham Pharmacia Biotech). Western blots were analyzed using a STORM-860 system by using Imagequant software (Molecular Dynamics, Sunnyvale, CA). Antibodies to human AKT, Thr308, or Ser473-phosphorylated AKT, BAD, S189-phosphorylated BAD, pp70S6K, Foxo3a, pERK, and anti-phospho FLT3 antibodies were purchased from Cell Signaling. Anti-Flt-3/FK-2 and PIM-1 were from Santa Cruz Biotechnology (Santa Cruz, CA), Rel-2 antibody was from DAKO (Glostrup, Denmark), and Fasl antibody from BD PharMingen.

5 Unpublished data communicated by Dr. A. Stewart from QLT.
For quantitation, gels were analyzed using the Scion Image software. Integrated absorbance of each lane (band obtained with the phosphospecific antibody) was recorded and compared with the absorbance of the band obtained with the total antibody of the same sample. The activation status of FLT3 was also confirmed by immunoprecipitation, as previously described (32). Briefly, BaF3/FLT3 and BaF3/ITD cells were treated with different concentrations of KP372-1 for 45 minutes, washed once in cold PBS, and lysed in a phosphoprotein lysis buffer. Clarified lysates were incubated with anti-FLT3 antibodies for 16 hours at 4°C, then mixed with 20 μL recombinant protein A/G Plus agarose beads solution (Santa Cruz, Biotechnology) for 2 hours at 4°C. Immunocomplexes were washed in TBST [10 mmol/L Tris-HCl (pH 7.4), 100 mmol/L NaCl, and 0.1% Tween 20] twice, boiled in sample buffer, and resolved by SDS-PAGE. Proteins were then immunoblotted with antiphosphotyrosine antibodies.

### Colony-forming assays.
Bone marrow mononuclear cells (1 × 10^5–2 × 10^6) containing >80% blasts from patients with AML were plated in methylcellulose (Methocult; Stem Cell Technologies, Vancouver, BC, Canada) supplemented with human recombinant growth factors [3 units/mL erythropoietin, 20 ng/mL interleukin (IL)-3, 20 ng/mL granulocyte macrophage (GM) colony-stimulating factor, 20 ng/mL granulocyte-stimulating factor, and 50 ng/mL stem cell factor]. KP372-1 at 0.1, 0.5, 1.0, or 2.0 μmol/L or DMSO alone was added at the beginning of the cultures. Duplicate cultures were grown in 35 mm Petri dishes and incubated at 37°C in a humidified atmosphere with 5% CO2. Light microscopy was done on days 8 to 10 to assess AML blast colony formation. A blast colony was defined as a cluster of 40 or more cells. In three experiments, 2 × 10^5 CD34^+ cells isolated from normal bone marrow or granulocyte colony-stimulating factor–stimulated peripheral blood samples were plated in 0.8% methylcellulose with Iscove’s modified Dulbecco’s medium, 1 unit/mL human erythropoietin (Terry Fox Laboratories, Vancouver, BC, Canada), and 50 ng/mL recombinant hGM colony-stimulating factor. KP372-1 was added at the initiation of cultures at concentrations of 0.5 to 5 μmol/L. All cultures were evaluated after 14 days for the number of colony-forming unit (CFU)-GM colonies, defined as a cluster of 40 granulocytes, monocyte-macrophages, or both.

### Table 1. Clinical data for patients

#### A. Samples used for in vitro suspension assay

<table>
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<th>Patient no.</th>
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<th>ITD</th>
<th>D835</th>
<th>Cytogenetics</th>
<th>Status</th>
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</tr>
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#### B. Samples used for colony assays

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<th>Patient no.</th>
<th>Source</th>
<th>Blast%</th>
<th>FAB</th>
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Abbreviations: UNK, unknown (outside diagnosis); BM, bone marrow; PB, peripheral blood; NA, not applicable; FAB, French-American-British; D835, substitution of the aspartic acid 835.
Measurement of reactive oxygen species generation. For measurement of intracellular reactive oxygen species (ROS), 0.5 × 10^6 cells were resuspended in fresh RPMI 1640 and incubated with 5 μmol/L dichlorodihydrofluorescein diacetate (Molecular Probes) dissolved in the culture medium at 37°C for 30 minutes, followed by treatment with KP372-1, DMSO, or 50 μmol/L H_2O_2 for the indicated times. The cells were washed in RPMI 1640 and immediately analyzed by flow cytometry.

Measurement of oxygen consumption in cultured cells. Oxygen consumption of intact cells was measured as described previously (33). Briefly, cells were pretreated with 1 μmol/L KP372-1 or DMSO for the indicated time period, resuspended in 1 mL fresh culture medium, and placed in sealed respiration chamber equipped with thermostat control and microstirring device (Oxytherm, Hansathach Instrument, England). Oxygen consumption was measured at 37°C using Clark-type oxygen electrode. The oxygen content in the medium was constantly monitored, and the signals were integrated using the software supplied by manufacturer. The oxygen contents in the starting medium were standardized assuming an O_2 concentration of 220 μmol/L in air-saturated medium at 37°C. The oxygen consumption rate is expressed as nanomoles of O_2 consumed as a function of time (μmol/L/min).

Statistical analysis. Statistical analysis consisted of the two-tailed paired t test. A P value of < 0.05 was considered to indicate statistical significance. Unless otherwise indicated, average values were expressed as the mean ± SE. All experiments were repeated at least thrice.

Results

KP372-1 inhibits PDK1/AKT signaling in AML. We first investigated the effects of KP372-1 on the phosphorylation status of AKT on Ser^473 and Thr^308 in leukemic cells. U937 cells were treated with increasing concentrations of KP372-1 for 16 hours. KP372-1 inhibited the phosphorylation of AKT on both Thr^308 and Ser^473, which was associated with a dose-dependent decrease in the phosphorylation of AKT downstream targets p70S6 kinase, BAD, and Foxo3a but did not affect the phosphorylation of ERK or levels FasL protein (Fig. 1A). As it is well documented that the phosphorylation status on Ser^473 and Thr^308 is a prerequisite for the catalytic activity of AKT, we next assessed effects of KP372-1 on AKT kinase activity. AKT protein immunoprecipitated from extracts of U937 cells exposed to 0.5 μmol/L KP372-1 for 30 minutes displayed ~50% less kinase activity as measured in vitro by the phosphorylation of GSK, a putative substrate of AKT (Fig. 1B). At 1 μmol/L KP372-1, AKT activity was completely abrogated. These data suggest that loss of phosphorylation of both Ser^473 and Thr^308 is associated with KP372-1-induced inactivation of AKT.

AKT is activated by extracellular stimuli and intracellular signal molecules, such as Ras and Src, in a PI3K-dependent manner. Therefore, KP372-1 inhibition of AKT could result from targeting molecule(s) upstream of AKT. Because PI3K and PDK1 are direct upstream regulators of AKT, we next examined whether KP372-1 inhibits PDK1. In the recombinant PDK1 enzyme assay, KP372-1 inhibited >50% of PDK1 activity at 0.25 μmol/L, and almost completely abrogated its activity at 1 μmol/L (Fig. 1C). Moreover, it was observed that inhibition of AKT Thr^308 phosphorylation was not associated with decreased phosphorylation of PDK1, indicating that KP372-1 does not directly inhibit PI3K phosphotransferase activity (Fig. 1D). These data suggest that KP372-1 directly inactivates PDK1, which likely contributes to the
observed inhibition of AKT phosphorylation at Thr308 in the activation loop.

mTOR is a well-documented downstream target of AKT. Although AKT itself was reported to function as PDK2 and autophosphorylate on Ser473 (34), recent studies indicate that mTOR, when bound to rictor instead of raptor, can phosphorylate AKT in the hydrophobic motif at Ser473 (35). Compelling evidence suggests that a negative feedback loop enables the mTOR pathway, through S6K1, to suppress PI3K activation (for review, see ref. 36). In turn, inhibition of mTOR signaling, or inhibition of S6K1 by siRNA, rescues activation of AKT (37). To investigate the possible effect of KP372-1 on mTOR and its substrates, MCF-7 cells were transfected with siRNA targeting S6k, a direct substrate of mTOR, and treated with increasing concentrations of KP372-1 (Fig. 2 A).

As expected, phosphorylation of AKT at Ser473 was increased after knockdown of S6k; however, KP372-1 inhibited GSK3 phosphorylation in transfected cells in a concentration-dependent fashion similar to untransfected cells, suggesting that KP372-1 does not modulate the mTOR pathway to inhibit AKT activation.

Taken together, these results implicate KP372-1 as a dual PDK1/AKT inhibitor, which induces potent inactivation of AKT signaling in leukemic cells, independent of mTOR.

**KP372-1 induces mitochondrial-mediated apoptosis.** Inhibition of AKT signaling reportedly induces apoptosis and/or cell cycle arrest in cancer cells with constitutively activated AKT signaling (38). To investigate the molecular determinants of KP372-1-induced growth inhibition, we conducted time course studies using 1 μmol/L KP372-1. In U937 cells, KP372-1 treatment profoundly decreased viability, with a nearly complete loss of viability at 24 hours (Fig. 2 C). The loss of viability was accompanied by decrease in ΔΨm, increased caspase activation, and phosphatidylserine externalization, all biochemical hallmarks of apoptosis (Fig. 2 B). Immunoblot analysis (Fig. 2 D) showed that treatment of U937 cells with 1 μmol/L KP372-1 resulted in mitochondrial release of cytochrome c within 3 hours, which was partially inhibited by a pharmacologic inhibitor of caspases zVAD-fmk. In contrast, no significant changes in cell cycle distribution were noted (data not shown), suggesting that induction of apoptosis via the mitochondrial pathway is the major cellular response to PDK1/AKT inhibition. Because generation of ROS is one of the hallmarks of the mitochondrial dysfunction (39), we investigated the levels of ROS in AML cells treated with 1 μmol/L KP372-1 for 30 minutes. Results presented in Fig. 3 A show that KP372-1 markedly induced the generation of ROS in U937, but not in KG-1 cells as measured by oxidation of CM(H)2DCF. Notably, KG-1 cells are lacking constitutive AKT activation (see Fig. 3 C). Consistent with rapid ROS generation, oxygen consumption, a surrogate indicator of mitochondrial function, was increased by ~4.7-fold in U937, but not in KG-1 cells after treatment with 1 μmol/L KP372-1 for 25 minutes (Fig. 3 B). Coadministration of the potent ROS scavenger, reduced glutathione, significantly reduced the KP372-1-induced apoptosis (P = 0.02, data not shown), suggesting that ROS generation mediates, at least in part, the primary cytotoxic effects of KP372-1 in AML cells harboring activated AKT.

**KP372-1 induces apoptosis in leukemic cell lines with activated AKT signaling and in FLT3-mutated leukemic cells.** We compared growth-inhibitory potency of KP372-1 in a variety of AML cell lines. KP 372-1 (at 1 μmol/L) induced significantly more apoptosis in NB4 and U937 (68% and 51%, respectively) compared with KG-1 (30%) and TF-1 (28%) cells (Fig. 3 C).

**Figure 2.** KP372-1-induced apoptosis is associated with rapid loss mitochondrial ΔΨm and cytochrome c release. A, MCF-7 cells were transfected with S6K siRNA for 48 hours and exposed to the indicated concentrations of KP372-1. Changes in the protein expression of phospho-AKT at Ser473 and its direct downstream substrate GSK3β were examined by Western blot. B, cells were treated with KP372-1 at the indicated times, after which Annexin V positivity, CaspaTag activity, and mitochondrial depolarization were determined by flow cytometry. C, U937 cell growth curve after KP372-1 (1 μmol/L) treatment. D, cytochrome c was isolated from cytosolic fraction and identified by Western blot.
analysis showed comparable levels of total and phospho-Thr\textsuperscript{308} AKT in these cells. In contrast, AKT was additionally phosphorylated on Ser\textsuperscript{473} in NB4 and U937 cells, suggesting that AKT phosphorylation at Ser\textsuperscript{473} but not at Thr\textsuperscript{308} determines sensitivity to KP372-1.

Because gain-of-function FLT3 mutation confers a proliferative and/or survival advantage to hematopoietic progenitors via, at least in part, activation of PI3K/AKT and Ras/MAPK signaling pathways and their downstream targets (16), we compared effects of KP372-1 on BaF3 cells transfected either with WT or mutated FLT3 (ITD or D835G). In contrast to published results, where inhibition of PI3K was insufficient to induce apoptosis in FLT3/ITD–transduced BaF3 cells (40), KP372-1 at 0.5 μmol/L drastically reduced viability of both BaF3/FLT3 and BaF3/ITD or BaF3/D835G via induction of apoptosis (Fig. 4A). Because these results suggest the possibility that KP372-1 may directly affect FLT3 signaling, and recent reports showed that the oncogene PIM-1 is overexpressed in patients with AML with FLT3/ITD mutations, we examined effects of KP372-1 on FLT3/PIM signaling. Although no FLT3 phosphorylation was apparent in BaF3/FLT3 cells, BaF3/ITD cells displayed robust expression of phospho-FLT3, which was inhibited by KP372-1 starting as early as 40 minutes and reaching almost complete loss of expression at 4 hours (Fig. 4B and C). In agreement with these results, KP372-1 inhibited phosphotyrosine content of the immunoprecipitated mutant FLT3 (data not shown). Furthermore, KP372-1 down-regulated levels of PIM-1 expression in FLT3/ITD but not in FLT3-transfected BaF3 cells (Fig. 4D). Altogether, these findings support the notion of inactivation of FLT3 in FLT3 mutant cells, resulting in down-regulation of PIM-1 expression and apoptosis.

**KP372-1 induces apoptosis in primary AML with WT or mutated FLT3.** We compared the ability of KP372-1 to induce apoptosis in leukemic and normal hematopoietic progenitor cells. KP372-1 induced apoptosis in a dose-dependent manner in 21 of 25 primary AML samples with over 70% blast, as determined by Annexin V staining. Notably, 0.25, 0.5, and 1.0 μmol/L KP372-1 induced apoptosis in 39.47%, 50.5.31%, and 57.5.04% of CD34\textsuperscript{+} AML progenitor cells. In contrast, the same concentration of KP372-1 did not affect CD34\textsuperscript{+} cell-enriched samples from hematologically healthy donors (Fig. 5A). This differential induction of apoptosis in AML but not in normal progenitor cells was highly significant ($P = 0.001$).

To confirm that AKT was one of the KP372-1 targets in primary AML samples, we tested the effect of KP372-1 on the kinase activity of AKT immunoprecipitated from a primary sample that contained >80% blasts and exhibited >80% apoptotic cells after 24 hours of treatment with 1 μmol/L of this agent (Fig. 5B). After
30-minute incubation with 0.5 μmol/L KP372-1, AKT activity was reduced by >50% and was completely abrogated at the dose of 1 μmol/L. Similar results were obtained in three additional AML samples (not shown). In one of the two samples that were resistant to KP372-1, we were unable to detect AKT protein by Western blot analysis (not shown).

Because KP372-1 inhibits FLT3/PIM signaling in cell lines, we compared the sensitivity of primary AML with WT or mutant FLT3 to KP372-1. The presence of FLT3/ITD or FLT3-D836 mutation (routinely assessed by hematopathology at diagnosis) was found in 4 and 2 of 25 cases, respectively, and both mutations were detected in two samples (Table 1A). In six samples tested by Western blot analysis, five expressed phosphorylated (activated) AKT (three of three with WT FLT3 and two of three with mutant FLT3; not shown). The average IC50 for KP372-1 in 17 AML samples without FLT3 mutations was 1.05 ± 0.28 μmol/L, which did not differ significantly from the average IC50 of eight samples with a FLT3/ITD or D835 (1.91 ± 0.5 μmol/L; P = 0.12). Curiously, two samples with FLT3 double mutations were extremely sensitive to KP372-1 with IC50 values of 0.43 and 0.17 μmol/L, respectively (Fig. 5C).

**KP372-1 abrogates clonogenic growth.** We further examined the effects of KP372-1 on the clonogenic growth of primary AML samples in the CFU-blast assay (Fig. 5D). KP372-1 inhibited colony formation in a dose-dependent manner, significantly reducing clonogenicity at 0.1 μmol/L (57% ± 8% of control; P = 0.001) and at 0.5 μmol/L KP372-1 (27% ± 9.53%; P < 0.001) and almost completely abrogating colony formation at 1 μmol/L KP372-1 in all five samples. In contrast, 63.5 ± 18.68% of GM-CFU from total normal bone marrow survived treatment with 0.5 μmol/L KP372-1, and only a minimal decrease in clonogenicity was observed in one of three samples at 0.1 μmol/L. Taken together, our results show that KP372-1 abrogates AML clonogenic growth at a concentration as low as 0.25 μmol/L, while sparing normal hematopoietic progenitor cells.

**Discussion**

In this report, we have described the characterization of KP372-1 as a novel dual inhibitor of PDK1/AKT and FLT3 kinase. The description here of a nominal Flt3 inhibitor with activity on other survival kinases, including PDK1, is not entirely new (26). However, to our knowledge, none of the other FLT3 inhibitors also inhibits AKT. Furthermore, our report is the first to show that simultaneous inhibition of PDK1/AKT and FLT3 signaling is associated with profound alterations in mitochondrial homeostasis.

KP372-1 causes inhibition of leukemia cell growth associated with decreased AKT activation, assessed by decreased Thr308 and Ser473 phosphorylation, decreased enzymatic activity, and inhibition of downstream AKT targets pp70S6K, phospho-Foxo3a, and phospho-Bad. This was associated with a rapid transient increase in oxygen consumption leading to the generation of ROS, mitochondrial depolarization, cytochrome c release, caspase activation, and apoptosis. Several earlier reports showed a major role of AKT in the regulation of the mitochondrial homeostasis (41), possibly via direct phosphorylation of the mitochondrial β-subunit of ATP synthase and GSK-3β (42) or by controlling localization of hexokinase to the outer mitochondrial membrane (43). Whereas the exact mechanism of KP372-1-induced perturbations of mitochondrial homeostasis was not investigated, KP372-1 causes significantly more ROS generation and increased oxygen consumption than LY294002, a PI3K inhibitor (data not shown), suggesting that directly targeting PDK1 and activated AKT is more effective in inducing mitochondrial dysfunction than inhibiting PI3K.

Based on our results, we propose several mechanisms underlying the molecular basis for inhibition of AKT by KP372-1 (Fig. 6). PDK1 is one kinase upstream of AKT that phosphorylates Thr308. Measurements of the recombinant activated PDK1 in the presence

**Figure 4.** KP372-1 inhibits FLT3/PIM signaling pathway. A, BaF3/FLT3, BaF3/ITD, and BaF3/D835G were treated with 0.5 μmol/L KP372-1 for the indicated time period, and induction of apoptosis was determined by Annexin V flow cytometry. B and C, the effects of KP372-1 on phosphorylated FLT3 in BaF3/FLT3 or BaF3/ITD was detected by Western blot after treatment with 0.5 or 1 μmol/L KP372-1 for 40 minutes, 60 minutes (B), or 4 hours (C). Densitometric analysis was done as described in Materials and Methods. Numbers represent ratios of phospho-FLT3 to total FLT3. D, BaF3/FLT3 or BaF3/ITD were treated with 0.5 μmol/L of KP372-1 for 1 hour, and expression of total PIM was detected by Western blot analysis. Note that mouse PIM-1 also expresses a 44 kDa isoform.
of KP372-1 strongly suggest that it directly inhibits PDK1 phosphotransferase activity, which likely accounts for the ability of KP372-1 to suppress Thr308 phosphorylation. However, inhibition of Thr308 phosphorylation is not sufficient to induce apoptosis, as KG-1 and TF-1 cells lacking baseline Ser473 phosphorylation were resistant to KP372-1, consistent with well-established requirement of Ser473 phosphorylation in the hydrophobic motif for full AKT activation. The mechanism of KP372-1-induced inhibition of Ser473 phosphorylation is not completely clear, but several possibilities arise. In a recent report, it was suggested that the mTOR-rictor complex fulfills the role of PDK2 (35). Although we did not directly examine effects of KP372-1 on mTOR/rictor complexes, KP372-1 inhibited GSK3 phosphorylation in cells in which down-regulation of S6K by siRNA resulted in increased phosphorylation of AKT at Ser473, perhaps through the recently characterized negative feedback loop (36). These results suggest that mTOR or S6K are not direct KP372-1 targets and that inhibition of Ser473 phosphorylation most likely results from inhibiting AKT autophosphorylation (34). Alternative possible mechanisms that were not investigated in the present study include inhibition of integrin-linked kinase or unidentified PDK2, or activation of the novel PP2C-type phosphatase PHLLP that specifically dephosphorylates the hydrophobic motif in AKT (44). Nevertheless, our in vitro experiments favor a scenario in which KP372-1 directly inhibits both PDK1 and AKT kinase activities.

Mutations in the FLT3 receptor tyrosine kinase are documented in ~30% of AML cases and confer poor prognosis. FLT3/ITD might play a crucial role in leukemogenesis by constitutive signaling through multiple signaling pathways, including PI3K/AKT. However, inhibition of PI3K alone by LY294002 was not sufficient to induce apoptosis in FLT3/ITD–transduced cells (40). Recent evidence suggests that FLT3 downstream target kinase PIM-1 may be important in FLT3/ITD–mediated leukemogenesis (45, 46), and PIM-1 was recently identified as one of the discriminative genes in samples from patients with AML with FLT3/ITD mutations (47). Previous work has shown that PIM and AKT contribute to the
mechanistically distinct but functionally overlapping control of hematopoietic cell proliferation, and that ectopic PIM expression shares several properties with oncogenic AKT, such as phosphorylation of proapoptotic protein BAD, phosphorylation of 4EBP1 and GSK3β, and nuclear factor-κB activation [for review, see ref. (48)]. This redundancy provides a plausible explanation for the reported resistance of leukemic cells with mutated FLT3 and activated PIM to growth inhibitory effects of PI3K inhibitor. In contrast to PI3K inhibitors, both cell lines and primary AML samples harboring mutated FLT3 (either ITD or D835 point mutation) underwent apoptosis upon KP372-1 treatment. This observation prompted us to study the effects of KP372-1 on FLT3 signaling. Time course analysis revealed indeed that KP372-1 inhibited FLT3 signaling and suppressed expression of the downstream PIM kinase. Notably, KP372-1 down-regulated PIM expression only in cells transduced with FLT3/ITD but not with WT FLT3, suggesting that this effect is a result of the inhibition of the constitutively active FLT3/ITD and not the inhibition of PIM itself. We noted extreme sensitivity of two cases with double-mutant receptor. Although the biochemical basis between different types of FLT3 mutations is not well defined, recent work showed significant signal transduction differences between ITD mutations and point mutations in the tyrosine kinase domain, the latter being a log more sensitive to FLT3 inhibitor PKC412 (49). Although only 1% to 2% of patients carry both mutations, FLT3-ITD-TKD dual mutants were reported to induce drug resistance toward FLT3 inhibitors and cytotoxic agents (50). Preliminarily, these data suggest that concomitant inhibition of PDK1/AKT and FLT3/PIM signaling results in profound apoptosis in primary AML samples with FLT3-ITD-TKD dual mutations.

In summary, results presented here provide evidence that an agent or combination of agents, which simultaneously target PDK1/AKT and FLT3/PIM signaling cascades, are potent inducers of cell death in leukemias, including chemoresistant FLT3-mutated AML. Our observations suggest that activation of AKT and PIM kinases provide critical survival signals for AML, and therapeutics that directly target these pathways could have a major effect on the treatment of AML. Future clinical trials are required, however, to establish a therapeutic window that may be compromised by impairment of glucose metabolism shown in preclinical models with AKT inhibitors (51). Indeed, we observed mild transient hyperglycemia in mice gavaged with KP372-1 (not shown). Strikingly, inhibition of these two signaling cascades was minimally toxic to normal hematopoietic progenitor cells, including cells with clonogenic potential, suggesting that AML cells are “addicted” to

Figure 6. Effects of KP372-1 on critical prosurvival signaling pathways in AML. For details, please refer to discussion of the data.
the recurrent activation of these signaling modules. Furthermore, data suggest selective effect on mitochondrial homeostasis via inhibition of activated Akt present in malignant, but not in normal cells. Although additional studies are required to elucidate the mechanisms of KP372-1-induced ROS generation and mitochondrial dysfunction, our results support further investigation into the potential of KP372-1 alone or in combination with existing therapies to overcome drug resistance in patients with AML.

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References

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