Targeting acute myeloid leukemia by dual inhibition of PI3K signaling and Cdk9-mediated Mcl-1 transcription

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Key Points

- Simultaneous inhibition of Cdk9 and PI3K in human AML cells liberates Bak from both Mcl-1 and Bcl-xL, inducing Bak-dependent apoptosis.
- Dual inhibitors of Cdk9 and PI3K, such as PIK-75, have broad activity against malignant cells including human AML cells.

Introduction

Acute myeloid leukemia (AML) is an aggressive bone marrow (BM) malignancy characterized by diverse genetic lesions that give rise to the clonal expansion and accumulation of immature blasts.1 The mainstay of AML treatment includes cytotoxic drugs such as cytarabine and anthracyclines; however, the 5-year overall survival (OS) in adults remains <40%.2 While AML cells from most patients retain the ability to activate apoptosis via intrinsic pathways, deregulation of cell survival programs may render AML cells resistant to cytotoxic therapies.3 Thus, drugs that selectively antagonize pro-survival programs in AML may lead to improved therapeutic outcomes.

The Bcl-2 family of proteins is a central regulator of cell survival and apoptosis.4 Overexpression of the prosurvival protein Mcl-1 and closely related family members Bcl-xL, Bcl-w, Mcl-1, and A1 have been widely observed in human cancers and are frequently associated with poor clinical outcomes.5 Although the concept of targeting deregulated cell survival in cancer has long been proposed, it has proved remarkably difficult to implement. One promising approach has been the development of ABT737, a small molecule BH3 mimetic that targets Bcl-2, Bcl-xL, and Bcl-w and has activity against human AML cells.5-8 Insensitivity to ABT737, however, may result from tumors that are dependent on Mcl-1.9

The potential importance of Mcl-1 as a therapeutic target has been underscored by studies showing that amplification of the
MCL-1 locus is one of the most frequent somatic events in human cancer and that overexpression of Mcl-1 is linked to inferior patient prognosis in certain malignancies such as AML.10,11 Furthermore, Xiang et al have reported that Mcl-1 transcripts were consistently expressed at higher levels in de novo AML patient samples compared with other Bcl-2 prosurvival family members.12 Importantly, in a Myc-induced mouse model of leukemia, the same investigators showed that while AML cells were highly sensitive to genetic deletion of a single Mcl-1 allele, heterozygous loss of Mcl-1 in nontransformed hematopoietic cells had minimal impact on hematopoesis, indicating a potential therapeutic window for drugs that target Mcl-1.12 More recently, we have shown that Mcl-1 is critical for the initiation and sustained in vivo growth of mouse AMLs that harbor diverse genetic lesions.13 Thus, the development of drugs capable of targeting Mcl-1 represents a highly desirable therapeutic goal that may improve clinical outcomes in the treatment of AML.14

In a screen for inhibitors that target Mcl-1 expression, we identified the kinase inhibitor PIK-75. We show that PIK-75 blocks 2 independent and critical cell survival pathways in human AML: 1 through the inhibition of the transcriptional kinases, Cdk7 and Cdk9, leading to the rapid depletion of Mcl-1, and 1 through the inhibition of the p110α isoform of phosphatidylinositol 3-OH kinase (PI3K), resulting in the dissociation of Bcl-xL with Bak. Importantly, PIK-75 was significantly more potent at inducing apoptosis of primary human AML cells compared with nontransformed hematopoietic progenitors. Furthermore, PIK-75 enhanced the survival of mice with established AML in the absence of detectable toxicity. Our studies not only establish a biological rationale for the dual blockade of Cdk9 and PI3K in cancer therapy but also validate a new class of anticancer drug that is distinguished by its ability to simultaneously target 2 critical signaling nodes, underpinning oncogenic cell survival in AML.

Materials and methods

Cell lines and culture

Mouse embryonic fibroblasts (MEFs) from knockout mice were generated and cultured as described previously.15 Interleukin (IL)-3 factor-dependent myeloid (FDM) cells from wild-type (wt) and knockout mice were generated and cultured as previously described.15 AML cell lines were maintained in either Dulbecco’s modified Eagle medium (DMEM) or Iscove modified Dulbecco medium (IMDM) and 10% fetal calf serum (FCS; JRH Laboratories).17,19,20 For colony assays, BM cells from either normal donors or AML patients’ cells were seeded in duplicate 35-mm plates (105 cells/plate) with H4230 Stem Cell Technologies methyl cellulose medium and either PIK-75 or PI-103 and placed in a humidified CO2 incubator. AML colonies (leukemic colony-forming unit [CFU]-L) were counted after 7 days: granulocytic-macrophage CFU (CFU-GM) and macrophage CFU were counted after 14 days. Colony assays were also performed on mouse BM by plating 66 000 cells/mL in soft agar containing 50 ng/mL stem cell factor, 8 U/mL erythropoietin, and 50 ng/mL IL-3.

Primary AML samples

Apheresis product, BM, or peripheral blood samples were obtained from patients diagnosed with AML. AML patient samples were collected after informed consent was received in accordance with the Declaration of Helsinki and according to institutional guidelines and studies that were approved by the Royal Adelaide Hospital and the Alfred Hospital Human Research ethics committees. In addition, AML samples were also obtained from the Hémopathies malignes Institute National de la Santé et de la Recherche Médicale Mêl-Epimétrie collection after approval by the Comité de Protection des Personnes Sud-Ouest et Outremer II Ethics Committee. Mononuclear cells were isolated by Ficoll-Hypaque density-gradient centrifugation and resuspended in phosphate-buffered saline containing 0.1% human albumin (CSL).17 FACS purification of primary human CD34+ CD38–CD123+ leukemic stem and progenitor cells was performed as previously described.17 Primary human AMLs were transfected using Lipofectamine RNAiMAX (Invitrogen) and 50 nM BLOCK-iT fluorescent oligo (Invitrogen) together with 50 to 100 nM of either ON-TARGET plus control siRNA or siRNAs targeting Mcl-1, Bcl-2, Bcl-xL, CD9, or p110α (supplemental Methods, available on the Blood Web site).

AML engraftment

Animal studies were performed under the institutional guidelines approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee, University Health Network/Princess Margaret Hospital Animal Care Committee and the Animal Care and Ethics Committee of the University of New South Wales, and the Adelaide University Ethics Committee. Human AML or normal BM cells were incubated with PIK-75 or vehicle (dimethylsulfoxide [DMSO]) for 3 hours in RPMI/10% FCS following which 5 to 10 × 10^6 cells were intravenously injected into sublethally irradiated (250 to 350 cGy) nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice as previously described.17 Engraftment was measured at 4 to 6 weeks by quantifying the percentage of hCD45+ cells in the BM by flow cytometry.18 HL-60 or MV4;11 cells (1 to 5 × 10^6) were engrafted into sublethally NOD/SCID/Lyl2−mmt mice; after 2 weeks, mice were intraperitoneally (IP) injected with either PIK-75 or vehicle. In some studies, PIK-75 was dissolved in 10% DMSO, 5% Tween-80, 85% NaCl, 70% (v/v) PEG400 vehicle; or 60% (wt/vol) (2-hydroxypropyl)-β-cyclodextrin. Blood counts were determined using a SYSMEX XE-2100 hematology analyzer.

Cell survival and colony assays

Cell survival was determined by trypan blue exclusion, annexin V-fluorescein isothiocyanate or annexin V-Alexa 568 (Roche) negativity, propidium iodide exclusion or flow cytometric enumeration of viable cells in reference to Flow Count Fluorospheres (BD Biosciences) using a FACSCalib (Becton Dickinson).17,19,20 For colony assays, BM cells from either normal donors or AML patients’ cells were seeded in duplicate 35-mm plates (105 cells/plate) with H4230 Stem Cell Technologies methyl cellulose medium and either PIK-75 or PI-103 and placed in a humidified CO2 incubator. AML colonies (leukemic colony-forming unit [CFU]-L) were counted after 7 days: granulocytic-macrophage CFU (CFU-GM) and macrophage CFU were counted after 14 days. Colony assays were also performed on mouse BM by plating 66 000 cells/mL in soft agar containing 50 ng/mL stem cell factor, 8 U/mL erythropoietin, and 50 ng/mL IL-3.

Results

A screen for small molecule inhibitors of Mcl-1 expression identified PIK-75 as a potent inducer of apoptosis in human AML cells

To identify candidate compounds able to downregulate Mcl-1 protein, a diverse array of 96 kinase inhibitors (supplemental Table 1) was screened in apoptosis-resistant Bax−/−/Bak−/− double-knockout MEFs and FDM cells. Importantly, this compound library contained flavopiridol and SNS-032, which have been previously identified as transcriptional repressors of Mcl-1. Apart from flavopiridol and SNS-032, the only other drug that downregulated Mcl-1 in both MEFl (Figure 1A) and FDM (Figure 1B) cells was the imidazoline derivative PIK-75 (supplemental Figure 1A). An additional screen was performed using an independent panel of 24 kinase inhibitors (that also included PIK-75; supplemental Table 2) in the MV4;11 human AML cell line that expresses the FLT3-ITD oncogene.21 Reduction of Mcl-1 was most marked with PIK-75, and some
reduction also observed with CEP-701 (FLT3 inhibitor) and GF109203X (PKC inhibitor; Figure 1C; supplemental Figure 1B). In addition to its ability to downregulate Mcl-1, PIK-75 also had potent cytotoxic activity in both MV4;11 cells (50% inhibition/ inhibitory concentration [IC50] = 4 nM) as well as OCI-AML3 cells (IC50 = 220 nM; supplemental Table 2). Furthermore, PIK-75 was found to have substantial activity against both AML cell lines and tumor lines representing multiple myeloma, breast cancer, and glioblastoma (supplemental Table 3; 16 cell lines tested).

To ascertain the clinical potential of PIK-75, a panel of 46 primary AML patient samples (supplemental Table 4; AML12-57) was screened to determine the IC50 of PIK-75 in both bulk AML blasts and FACS-purified primary human CD34+CD38-CD123+ leukemic stem and progenitor cells (supplemental Figure 1C). The majority of AML cases were lethally susceptible to nanomolar concentrations of PIK-75; this is in contrast to CD34+ normal BM progenitors that were significantly less sensitive (Figure 1D). Of clinical importance, the proapoptotic potency of PIK-75 was not lessened in samples from patients with adverse cytogenetic risk karyotype, FLT3-ITD, or relapsed disease (Table 1), indicating that PIK-75 may have utility against diverse clinicopathologic factors typically associated with chemotherapy failure in patients.

PIK-75 induced apoptosis in a manner distinct from other inhibitors of the PI3K pathway

PI3K inhibitors have been shown to induce apoptosis in AML cells.22,23 However, although PIK-75 was originally described as an inhibitor of the p110α isoform of PI3K (supplemental Figure 2A),24 blockade of PI3K or its downstream targets, Akt and mTOR, using multiple independent inhibitors in MV4;11 cells or primary human CD34+CD38-CD123+ leukemic stem and progenitor cells failed to recapitulate the potent cell killing observed with PIK-75 (Figure 2A-B; supplemental Figure 2B). Such findings would suggest that the potent ability of PIK-75 to induce apoptosis in AML cells may be due to the inhibition of targets other than PI3K.

We therefore examined whether PIK-75–induced downregulation of Mcl-1 was mediated by inhibition of PI3K signaling. While the p110α/mTOR dual inhibitor, PI-103,24 was able to inhibit Akt phosphorylation in multiple primary human AML samples to a similar extent to that for PIK-75, only PIK-75 was also able to completely suppress Mcl-1 (Figure 2C). Similar results were obtained for the class I PI3K inhibitor, GDC-0941, in MV4;11 cells (supplemental Figure 2C). In fact, none of the PI3K pathway

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**Table 1. Sensitivity of AML cells to Ara-C and PIK-75**

<table>
<thead>
<tr>
<th>AML cell population</th>
<th>Ara-C IC50 (nM)</th>
<th>PIK-75 IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk AML</td>
<td>8400 (n = 20)</td>
<td>414 (n = 44)</td>
</tr>
<tr>
<td>CD34+CD38-CD123+</td>
<td>156 000 (n = 20)</td>
<td>431 (n = 44)</td>
</tr>
<tr>
<td>AML patient sample</td>
<td></td>
<td></td>
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<tr>
<td>De novo AML</td>
<td>Nd</td>
<td>284 (n = 7)</td>
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<tr>
<td>Relapsed AML</td>
<td>Nd</td>
<td>346 (n = 7)</td>
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<tr>
<td>Cytogenetic risk group</td>
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<tr>
<td>Favorable†</td>
<td>3000 (n = 5)</td>
<td>444 (n = 6)</td>
</tr>
<tr>
<td>Intermediate‡</td>
<td>4350 (n = 10)</td>
<td>367 (n = 28)</td>
</tr>
<tr>
<td>Adverse‡</td>
<td>30 500 (n = 5)</td>
<td>726 (n = 9)</td>
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<td>FLT3-ITD and NPM status</td>
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</tr>
<tr>
<td>FLT3-ITD+</td>
<td>111 000 (n = 6)</td>
<td>360 (n = 17)</td>
</tr>
<tr>
<td>FLT3-ITD−</td>
<td>4800 (n = 14)</td>
<td>428 (n = 27)</td>
</tr>
<tr>
<td>NPM+</td>
<td>5950 (n = 6)</td>
<td>367 (n = 16)</td>
</tr>
<tr>
<td>NPM−</td>
<td>10 400 (n = 14)</td>
<td>444 (n = 8)</td>
</tr>
</tbody>
</table>

Nd, not determined.

†Favorable is defined as t(8;21)/(q22; q22) or inv(16)/(p13.1 q22) or t(16;16)/(p13.1; q22).
‡Intermediate is defined as normal karyotype or t(9;11)/(p22; q23) or cytogenetic abnormalities not classified as favorable or adverse.
†Adverse is defined as inv(3)/(q21q26.2) or t(3;3)/(q21q22.6) or t(6;9)/(p23;q34) or t(11)/(q23) or –5 or del(5q) or –7 or del(17p) or complex karyotype.
inhibitors used in our screens, which included 8 pan-specific or isoform-selective PI3K inhibitors (LY294002, Wortmannin, GDC-0941, BEZ-235, PIK-90, PI-103, TGX-221, IC87114, AS252424), nor inhibitors against downstream PI3K targets (rapamycin and AKT-1 I) employing 3 cell types (DFM, MEF, MV4;11) had significant effects on Mcl-1 levels (Figure 1A-C; supplemental Figure 1B). Furthermore, PI3K inhibition using LY294002 or PI-103 failed to downregulate Mcl-1 in U251 (supplemental Figure 2D) or U87 (data not shown) glioblastoma cells, whereas PIK-75 treatment resulted in a dose-dependent loss of Mcl-1. Inhibition of PI3K signaling has also been shown to induce GSK3-mediated Mcl-1 degradation.25,26 However, GSK3 inhibition with AR-A014418 did not stabilize Mcl-1 nor did it prevent PIK-75-mediated apoptosis (supplemental Figure 2E). Thus, while in some cellular contexts PI3K inhibition may result in reduced Mcl-1, our findings suggest that targeting PI3K does not significantly impact on Mcl-1 levels, at least in the transformed cell types examined. Because the induction of apoptosis by PIK-75 observed was rapid and immediately preceded by the loss of Mcl-1 protein (Figure 2D), we further examined the mechanisms by which PIK-75 was able to downregulate Mcl-1.

**PIK-75 inhibits Cdk7 and Cdk9, resulting in a block of Mcl-1 gene transcription**

Mcl-1 is known to have a short half-life owing to its ubiquitination and targeting for degradation via the proteasome.27 However, because PIK-75 did not significantly alter Mcl-1 half-life in AML cells (data not shown), we examined its effect on MCL-1 gene expression. MCL-1 transcription is mediated by the transcriptional kinases, Cdk7 and Cdk9, which phosphorylate Ser2 and Ser5 within the C-terminal domain (CTD) of RNA polymerase II (RNPAP1).28 Using a luciferase reporter construct under the control of the Mcl-1 promoter, we observed a significant decrease in reporter activity in the presence of PIK-75 as well as 2 independent Cdk inhibitors, flavopiridol and SNS-032, but not the PI3K inhibitors LY294002, PI-103, IC87114, TGX-221, or AS252424 (Figure 3A). Furthermore, nuclear run-on experiments demonstrated that PIK-75 directly inhibited transcription of the endogenous MCL-1 gene (supplemental Figure 3A). These findings indicated that PIK-75 rapidly depleted Mcl-1 protein levels primarily through blockade of MCL-1 gene transcription.

In vitro kinase assays confirmed high-affinity binding of PIK-75 to the ATP binding pocket of both Cdk9 (Kd = 4.1 nM) and Cdk7 (Kd = 2.5 nM). Cheng et al reported that PIK-75 could block Cdk1 and Cdk2 and induce cell cycle arrest and apoptosis in glioblastoma cell lines.29 However, we and others have shown that the affinity of PIK-75 for Cdk2 is >100-fold lower (Kd = 540 nM) than Cdk9 (Kd = 4.1 nM; Figure 3B).30 In addition, our results indicate that PIK-75 is able to induce apoptosis in the CD34+CD38-CD123+ population enriched for quiescent leukemic stem/progenitor cells (Figure 1), suggesting that a cell cycle block is not a prerequisite for the induction of cell death. Consistent with its ability to inhibit Cdk7/9, PIK-75 treatment resulted in the inhibition of RNApol II CTD phosphorylation and the repression of Mcl-1, but not Bcl-2 or Bcl-XL expression, in MV4;11 cells (Figure 3C; supplemental Figure 3B), and primary human AML cells (Figure 3E) to levels comparable to that mediated by flavopiridol (Figure 3C-D) and SNS-032 (Figure 3C).

**Dual targeting of PI3K and Cdk9 induces apoptosis in AML cells**

To explore how PIK-75 might bind PI3K and Cdk9, which reside in evolutionarily diverse branches of the kinome tree, docking of PIK-75 into the crystal structures of p110α and Cdk9 was performed (supplemental Methods). Compared with its binding to PI3K p110α, PIK-75 adopted an entirely different binding mode to Cdk9, rotated 180° along its longitudinal axis (supplemental Figure 4). While PIK-75 represents a novel class of inhibitor with the ability to simultaneously inhibit PI3K and Cdk9, it was not clear whether the potent ability of PIK-75 to induce apoptosis in AML cells was due to the inhibition of PI3K alone, Cdk9 alone, or dual inhibition of PI3K and Cdk9. We therefore examined the survival of primary FACs-purified CD34+CD38-CD123+ leukemic stem and progenitor cells following inhibition of either Cdk9 alone (with either SNS-032 or flavopiridol) or in combination with PI3K (using LY294002). Under conditions where either SNS-032 or flavopiridol reduced cell survival by ~50%, addition of LY294002 resulted in a further significant reduction in cell viability (Figure 4A; supplemental Figure 5A). Furthermore, marked synergy was demonstrated (combination index < 1.0) in primary AML patient samples between SNS-032 and LY294002 over a variety of drug concentrations (supplemental Figure 5B).

To more specifically probe the importance of PI3K and Cdk9/ Mcl-1 for AML cell survival, the effect of the class I PI3K inhibitor, GDC-0941, and the p110α-selective inhibitor, A66, on cell viability was examined alone and in combination with siRNA-directed targeting of Cdk9 and Mcl-1 expression (supplemental Figure 5C-D). In primary human AML samples, only a minimal effect on cell viability was observed after exposure to GDC-0941 or A66 (Figure 4B-C). Similarly, siRNA directed against Mcl-1 and Cdk9 induced modest levels of apoptosis. In contrast, combining the PI3K inhibitors

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**Figure 2. PIK-75 induces apoptosis in a manner that is distinct from other inhibitors targeting the PI3K pathway.** (A) MV4;11 cells were cultured in the presence of the PI3K inhibitors, A66 (triangle), GDC-0941 (cross), YM-024 (open square), PI-103 (diamond), BEZ-235 (closed circle), and PIK-75 (open circle) for 24 hours and cell survival was determined by flow cytometric enumeration of PI-negative cells. (B) FACs-purified primary human CD34+CD38-CD123+ leukemic stem and progenitor cells were plated in DMEM, 100 nM PIK-75, or the p110α- (1 μM TGX-221), p110β- (5 μM IC87114), or p110γ- (100 nM AS252424) selective PI3K inhibitors and cell survival examined at 24 hours by annexin V staining. (C) Primary human AML cells were incubated in 1 μM PI-103 or 1 μM PIK-75 for 8 hours following which cell lysates were immunoblotted. (D) MV4;11 cells were treated with 100 nM PIK-75 following which cell lysates were immunoblotted with the indicated antibodies. Results are typical of at least 2 experiments.
(GDC-0941 or A66) with genetic or chemical inhibitors of Cd9 or Mcl-1 (siRNA or SNS-032) resulted in the significant loss of viability in primary AML cells (Figure 4B-C).

In addition to siRNA-mediated targeting of Mcl-1, we also employed a doxycycline-inducible BimS system that allows the selective targeting of Mcl-1. We used the BimS variant, Bim2A, in which the BH3 domain has been mutated so that it inhibits the prosurvival function of Mcl-1 but not Bcl-2, Bcl-xl, Bcl-w, or A1.31 Doxycycline induction of wt BimS, which targets all prosurvival proteins, resulted in significant apoptosis, whereas induction of an inert BimS mutant, Bim4E, had no impact on cell survival (Figure 4D). Importantly, induction of the Bim2A variant resulted in a partial reduction in cell survival, which was significantly enhanced by concomitant PI3K inhibition using GDC-0941 (Figure 4D). Furthermore, while PI3K inhibition using LY294002 (Figure 4E) or GDC-0941 (Figure 4F) in FACS-purified primary human CD34+CD38−CD123+ leukemic stem and progenitor cells had modest effects on cell survival, combined PI3K inhibition and siRNA-mediated Mcl-1 knockdown significantly increased apoptosis (Figure 4E-F).

PIK-75 disrupts Bak interaction with the prosurvival proteins, Mcl-1 and Bcl-xL, triggering Bak activation and apoptosis

To understand the mechanism by which PIK-75 induced cell death, we first examined whether any of the proapoptotic members of the Bcl-2 family were required for the induction of cell death. For these experiments, we examined the ability of PIK-75 to induce apoptosis in FDM cells derived from mice in which specific pro-death proteins had been knocked out. No resistance to PIK-75-mediated cell death was observed in FDM cells derived from BID−/−, PUMA−/−, BAD−/−, Bim−/−, PUMA−/−Bim−/−, or Noxa−/−Puma−/− mice when compared with wt FDM cells (Figure 5A). In fact, even FDM cells derived from Noxa−/−Puma−/−Bim−/− triple-knockout mice remained sensitive to PIK-75. Thus, while it is known that some kinase inhibitors induce apoptosis via the activation of BH3-only proteins such as Bim and Puma,32,33 our results show that such events are not essential for PIK-75-induced apoptosis. On the other hand, FDM cells from Bak−/− (and Bax−/−Bak−/−) knockout mice were completely resistant to PIK-75, whereas Bax−/− FDM cells were as sensitive to PIK-75 as wt FDM cells (Figure 5A). Furthermore, PIK-75 was able to induce cell death in p53−/− FDM cells; this is relevant for patients with complex karyotype AML in which almost one-third have defective p53.34 Together, these findings indicate that the mechanism by which PIK-75 induces apoptosis is via a Bak-dependent but p53-independent mechanism.

While the majority of AML patient samples in our cohort demonstrated PIK-75 IC50 values <500 nM, a subset of samples had IC50 values >900 nM (Figure 1D). As PIK-75–mediated apoptosis requires Bak (Figure 5A), we considered the possibility that Bak expression would represent a molecular determinant of PIK-75 sensitivity. Regression analysis of Bak expression in a panel of 8 AML samples demonstrated a good fit for a linear model (R2 = 0.74). Furthermore, patient samples with PIK-75 IC50 values greater than the median (>753 nM) demonstrated significantly lower Bak expression levels than samples below the median (supplemental

Figure 3. PIK-75 suppresses Mcl-1 through the inhibition of the transcriptional kinases, Cdk7 and Cdk9, and a block in MCL-1 gene transcription. (A) HEK293T cells were cotransfected with constructs expressing the Renilla luciferase gene (TK promoter-driven) and the Firefly luciferase gene (MCL-1 promoter-driven). Cells were treated with DMSO (vehicle), LY294002 (20 μM), PIK-75 (100 nM), PI-103 (100 nM), IC87114 (5 μM), TGX-221 (1 μM), SNS-032 (100 nM), SNS-032 (10 μM), or flavopiridol (10 μM) and the relative luciferase activity was determined after 20 hours. Error bars represent standard errors with asterisks indicating P < .05. (B) The binding affinity of PIK-75 to purified recombinant Cdk7 (data not shown), Cdk9, and Cdk2 was measured in the KINOMEscan competition assay (see supplemental Methods). (C) MV4;11 cells were treated with DMSO (vehicle), LY294002 (20 μM), flavopiridol, SNS-032, or PIK-75 for 8 hours following which cell lysates were immunoblotted with the indicated antibodies. (D) Bax−/−Bak−/− FDM cells were incubated with the indicated drugs following which cell lysates were immunoblotted. (E) Primary AML mononuclear cells were treated with DMSO or PIK-75 (100 nM) for 8 hours following which cell lysates were immunoblotted. Results are typical of at least 2 experiments.
tion is restrained solely by Mcl-1 and Bcl-xL. Although multiple members of the Bcl-2 prosurvival family, Bak activates a panel of 6 primary human AML samples (supplemental Figure 6D). No correlation between PIK-75 sensitivity and Mcl-1 expression in AML cells despite the significant overexpression of Mcl-1 achieved in MV4;11 cells, PIK-75 was still able to rapidly downregulate Mcl-1 to negligible levels within 2 hours, a rate that was similar to the observed rate of either GDC-0941 (GDC) or A66 (1 μM) and cell survival was assessed at 48 hours. (E-F) FACS-purified primary human CD34+CD38– leukemic stem and progenitor cells were transduced with lentiviruses for the inducible expression of wt Bim, Bim2A, or Bim4E and cell survival was assessed at 48 hours. (E-F) FACS-purified primary human CD34+CD38– leukemic stem and progenitor cells were transfected with 100 nM siRNA targeting Mcl-1 (siRNA-17 in E, siRNA-16, or siRNA-17 in F), occurring regardless of whether immunoprecipitations were performed using Bak antibodies (Figure 5F) or Bcl-xL antibodies (supplemental Figure 6E), occurred regardless of whether immunoprecipitations were performed using Bak antibodies (Figure 5F) or Bcl-xL antibodies (supplemental Figure 6E), and occurred in both MV4;11 cells (Figure 5F; supplemental Figure 6E) and primary human AML cells (Figure 5G). Importantly, PI3K inhibition did not affect intracellular levels of Mcl-1 in cell lysates that, not surprisingly, was accompanied by the loss of association between Mcl-1 and Bak (Figure 5E). On the other hand, while PI3K inhibition with GDC-0941 had no effect on Bcl-xL levels, we observed a disruption of the interaction between Bcl-xL and Bak following 6 hours of drug treatment (Figure 5F). Furthermore, the loss of Bcl-xL:Bak association was observed following treatment with either GDC-0941 or A66 (Figure 5F; supplemental Figure 6E), occurred regardless of whether immunoprecipitations were performed using Bak antibodies (Figure 5F) or Bcl-xL antibodies (supplemental Figure 6E), and occurred in both MV4;11 cells (Figure 5F; supplemental Figure 6E) and primary human AML cells (Figure 5G). Importantly, PI3K inhibition did not affect intracellular levels of Mcl-1 (Figures 2C and 3C-D; supplemental Figure 2C-D) nor did it disrupt the association of Mcl-1 with Bak (Figure 5F).

Thus, our findings indicate that Cdk9 inhibition is responsible for the loss of Bak association with Mcl-1 while PI3K inhibition results in the loss of Bak association with Bcl-xL and that dual targeting of PIK-75 and Cdk9 may have additive effects in the context of AML. Consequently, PIK-75 and Cdk9 may represent promising therapeutic targets for AML.
Figure 5. PIK-75 disrupts the interaction of Bak with the prosurvival proteins, Mcl-1 and Bcl-xL, to induce apoptosis via a Bak-dependent mechanism. (A) The ability of PIK-75 to promote cell death was assessed in hemopoietic FDM cell lines derived from wt mice or mice lacking proapoptotic members of the Bcl-2 family: Bid−/−, Puma−/−, Bad−/−, Bim−/−, Puma−/−Bim−/−, Noxa−/−, Puma−/−Bim−/−, Bak−/−, Bax−/−, Bak−/−Bax−/−, and p53−/−. FDM cell lines were plated in 0.25 ng/mL mIL-3 and cultured for 24 hours in the presence of PIK-75 and cell survival measured by flow cytometric enumeration of PI-negative cells. (B) The IC50 for PIK-75 for 8 primary human AML samples was determined as in Figure 1D and plotted against Bak protein expression levels as determined by western blotting and laser densitometry quantification. Regression analysis was performed (R² = 0.74) and a Pearson linear correlation coefficient was calculated (r = −0.86), indicating a strong inverse linear relationship between Bak expression and PIK-75 IC50 (P = .006 for a non-zero slope). (C) Primary AML blasts were transfected with 100 nM of the indicated siRNAs and/or treated with 1 μM of either GDC-0941 (GDC) or A66 and cell survival was measured at 48 hours. (D) MV4;11 cells were transfected with constructs for the expression of eGFP and either an empty vector or Mcl-1. After 24 hours, cells were treated with 50 nM PIK-75 and live GFP+ cells were counted using Flow-Count Fluorospheres by flow cytometry at 48 hours. (E-F) MV4;11 cells were treated with either (E) 100 nM SNS-032 or (F) 1 μM GDC-0941 for the indicated times following which cell lysates were subjected to immunoprecipitation (IP) using anti-Bak antibodies followed by immunoblotting with the indicated antibodies. Whole cell lysates (WCLs) were also immunoblotted as indicated. The asterisks indicate cross-reactivity of the IP IgG light chain with detection antibodies used in the western blots. (G) Primary human AML blasts were treated for 6 hours with either 100 nM SNS-032 or 1 μM GDC-0941 following which cell lysates were immunoprecipitated using anti-Bak antibodies and immunoblotted with the indicated antibodies. (H) Primary AML blasts were transfected with either a single targeting siRNA (50 nM targeting siRNA plus 50 nM control siRNA) or with 2 targeting siRNAs (50 nM of each targeting siRNA) and cell survival was measured at 72 hours. (I) Primary AML blasts were transfected with siRNAs as in (H) and Bak activation was measured by intracellular staining with anti-Bak antibodies (Ab-1) and flow cytometry (see supplemental Methods) at 48 hours. (J) MV4;11 cells were transfected with constructs for the expression of eGFP and either empty vector (Ctl) or human myr-Akt. After 24 hours, cells were either nontreated (-) or treated (+) with 50 nM of PIK-75 for 6 hours following which cell lysates were subjected to IP (bottom 2 panels) and immunoblotting with the indicated antibodies. WCLs (top 2 panels) were also immunoblotted as indicated. (K) MV4;11 cells transfected as in panel (J) were incubated in the absence (-) or presence (+) of PIK-75 and cell survival was examined after 48 hours. Results are typical of at least 2 experiments. Error bars represent standard deviations with asterisks indicating P < .05.
Mcl-1 and Bcl-xL appears critical for optimal apoptosis in human AML. Such a proposal is supported by our findings that targeting either Cdk9:Mcl-1 or PI3K:Bcl-xL alone is less effective at inducing apoptosis when compared with combined targeting in primary AML cells (Figures 4A-F and 5F-G; supplemental Figure 6E). Furthermore, combined siRNA-mediated knockdown of Mcl-1 and Bcl-xL (but not combined Mcl-1 and Bcl-2 knockdown; supplemental Figure 6F) was sufficient for the induction of apoptosis (Figure 5H) and Bak activation (Figure 5I). Together, these results support a mechanism in which inhibition of PI3K (which disrupts Bcl-xL:Bak association) combined with inhibition of Cdk9 (that depletes Mcl-1, leading to a loss in Mcl-1:Bak association) triggers Bak-dependent apoptosis.

To further examine the role of Bcl-xL in PIK-75–mediated cell death, we examined the possibility that enforced PI3K signaling could attenuate PIK-75–induced apoptosis. For these experiments, we overexpressed a constitutively active version of the downstream PI3K target, Akt1 (myr-Akt1), which results in increased 4EBP1 phosphorylation, even in the presence of PIK-75 (Figure 5J). While both GDC-0941 (supplemental Figure 6G) and PIK-75 (Figure 5J) treatment resulted in the loss of Bcl-xL:Bak association in vector control cells, overexpression of myr-Akt1 prevented the Bcl-xL:Bak disruption (Figure 5J; supplemental Figure 6G), leading to enhanced survival of MV4;11 (Figure 5K) and TF-1 erythroleukemia cells in the presence of PIK-75 (supplemental Figure 6H).

**PIK-75 selectively targets AML cells in vivo**

Next, we determined whether there was a therapeutic window separating the antileukemic activity of PIK-75 and the potential for toxicity. Doses of PIK-75 that significantly reduced growth of CFU-L from primary AML samples had no effect on the formation of CFU-GM from normal donors (Figure 6A–B). Additionally, PIK-75 treatment of AML blasts significantly reduced their engraftment in NOD-SCID mice, while not significantly affecting the engraftment of normal BM progenitors (Figure 6C–D). No adverse reactions were observed following daily administration of 10 mg/kg PIK-75 to mice for 14 days, with minimal changes to circulating blood hemoglobin, platelets, white cells, immunophenotypically defined hematopoietic BM subpopulations, clonogenic potential, or total mouse body weight (supplemental Figure 7A–F). The therapeutic tolerance of daily treatments with PIK-75 may relate to a limited duration of PIK-75 activity in mouse peripheral blood with >90% of drug activity (as measured by the ability of mouse plasma to induce apoptosis in MV4;11 cells) lost 2 hours after injection (Figure 6E).

HL-60 and MV4;11 human AML cells exhibit aggressive growth characteristics in NOD/SCID/IL2rgnull mice, leading to the rapid onset of disease, hemopoietic failure, and death (ethical endpoints). To determine if doses of PIK-75 found to be nontoxic to mice (supplemental Figure 7) would be efficacious in targeting AML, NOD/SCID/IL2rgnull mice with established leukemia after xenotransplantation of HL-60 or MV4;11 AML cells were examined. Administration of either 1 mg/kg PIK-75 or 10 mg/kg (both nontoxic; supplemental Figure 7) significantly reduced the leukemic burden in mice engrafted with HL-60 cells (Figure 6F). This was associated with detection of extensive apoptosis of leukemic cells in the BM (supplemental Figure 7G). Longer-term treatment with 1 mg/kg of PIK-75 significantly improved the survival of mice with established leukemia compared with vehicle-treated controls in both HL-60 and MV4;11 in vivo models (Figure 6G-H). Together, these results demonstrate that PIK-75 selectively targets AML cells in vivo, without inducing significant toxicity.

**Discussion**

In a screen for kinase inhibitors that suppress Mcl-1, we have identified PIK-75, which potently induces apoptosis in cytogenetically diverse primary human AML patient samples and cancer cell lines (Figure 1). While originally described as an inhibitor of the p110α isoform of PI3K,24 our findings show that PIK-75 also inhibits the transcriptional kinases, Cdk7 and Cdk9, and blocks RNA-Pri-mediated MCL-1 gene transcription (Figure 3), leading to the rapid loss of Mcl-1 protein (Figures 2 and 3). Importantly, concentrations of PIK-75 that induced apoptosis in primary human CD34+CD38-CD123+ leukemic stem and progenitor cells allowed the survival of normal BM progenitors (Figure 1). Furthermore, treatment of mice engrafted with human AML lines significantly reduced leukemic loads in the BM and extended mouse survival without significant toxicity (Figure 6).

PIK-75 simultaneously targets 2 independent and functionally distinct cell survival pathways: one via the inhibition of PI3K signaling and the other via the inhibition of Cdk7/9 that in turn blocks MCL-1 transcription. While Yu et al have shown that dual targeting of PI3K and Cdkks using LY294002 and flavopiridol induced apoptosis in AML cells,38 using selective siRNA knockdown approaches, we identified the p110α catalytic subunit of PI3K and the transcriptional kinase, Cdk9, as the critical targets for the induction of apoptosis in primary human AML cells (Figures 4 and 5). Others have shown that the inhibition of Bcl-2 and Bcl-xL when combined with PI3K inhibition induces apoptosis in human AML via a Bim-dependent mechanism that involves both Bax and Bak.7 Interestingly, the induction of apoptosis by PIK-75 in our studies did not require the activity of the proapoptotic BH3 proteins Bim, Bid, Bad, Noxa, or Puma and was strictly dependent on the multidomain proapoptotic protein Bak (Figure 5). Although Cdk9 inhibition frees Bax from prosurvival regulation by Mcl-1, optimal apoptosis of primary AML blasts appeared most effective when Bcl-xL was also targeted (Figures 4 and 5). This could be achieved by disruption of the binding interaction between Bcl-xL and Bak following inhibition of PI3K (Figure 5). Our findings suggest that dual Cdk9 and PI3K inhibition (as occurs with PIK-75) simultaneously liberates Bak from restraint by both Mcl-1 and Bcl-xL, triggering Bak activation (Figure 5).

A major therapeutic advantage of PIK-75 is that the proapoptotic activity of Bak might be elicited by a single agent that simultaneously targets 2 prosurvival regulators of Bak, namely, Mcl-1 and Bcl-xL.

While our studies show that Cdk9 inhibition blocks MCL-1 transcription resulting in the rapid depletion of Mcl-1 protein, others have shown that PI3K inhibition can also lead to a downregulation of Mcl-1 levels. For example, Chapuis et al and Park et al have shown that dual PI3K/mTOR inhibition (BEZ235 and PI-103) resulted in a decrease in Mcl-1 levels in both AML cell lines and primary AML blasts.22,23 Furthermore, dual PI3K/mTOR inhibition has also been shown to downregulate Mcl-1 levels in other tumors.39-42 It has been proposed that PI3K/mTOR inhibition leads to a downregulation of Mcl-1 due to a block in cap-dependent translation.23,43 Our studies show that PI3K inhibition using multiple pan-specific and isoform-selective PI3K inhibitors in either AML cell lines or primary AML blasts had very little impact on Mcl-1 levels (Figures 2-3 and 5). However, the kinetics of Mcl-1 downregulation following PI3K/mTOR inhibition observed by others appears slower (24 to 48 hours)23,24 when compared with the
Figure 6. In vivo therapeutic efficacy and selectivity of PIK-75 toward human AML in a mouse xenograft model. (A) AML blasts (n = 8 patient samples) or (B) BM cells from normal donors (n = 3) were plated in methyl cellulose in the indicated concentrations of vehicle (V), PIK-75, or PI-103 and CFU-L or CFU-GM colonies were counted using an inverted microscope. *P < .0001 with error bars representing standard errors. (C) Primary AML blasts or (D) nontransformed BM from normal donors were incubated in vehicle (V), 1 μM PIK-75 (Δ), or DMSO (■) for 3 hours, washed, and then engrafted into NOD-SCID mice. Engraftment was quantified after 4 to 6 weeks by assessing the percentage of hCD45+ cells in the BM of recipient mice. Each symbol represents the percentage hCD45+ cells observed in a separate mouse. (E) Mice were injected IP with 10 mg/kg of PIK-75 and then sacrificed at the indicated time points, with peripheral blood being collected by cardiac puncture. The peripheral blood was then diluted 1:2 with RPMI/1% FCS and incubated with MV4;11 cells for 24 hours following which apoptosis was quantified by annexin V staining. Each dot represents the observed apoptosis of MV4;11 cells following incubation with a peripheral blood sample collected from a separate mouse, with the horizontal line representing the mean. (F) NOD-SCID mice were engrafted with HL-60 cells and after disease was established (arrow), mice were injected IP with either vehicle (V) or PIK-75 for 3 days following which engraftment was quantified by assessing the percentage hCD45+ cells in the BM of recipient mice. Each symbol represents the percentage hCD45+ cells observed in a separate mouse (*P < .05; **P < .01). NOD-SCID/L26r/nu mice were engrafted with (G) HL-60 cells or (H) MV4;11 cells and after disease was established (arrow), mice were injected IP 5 times/week with 1 mg/kg PIK-75. P values were calculated using the log-rank Mantel-Cox test.

PI3K and Cdk7/9 regulate fundamental physiological processes in diverse cellular settings. In addition, gene knockout studies have shown that p110α expression is essential for mouse development, while Mcl-1 is essential for normal hematopoiesis. However, mice that are haploinsufficient for either gene are viable, suggesting that transient or partial blockade of either both survival nodes might represent a clinically achievable and tolerable therapeutic approach. While clinical studies have demonstrated the safety of using transcriptional repressors such as flavopiridol and SNS-032 that inhibit Cdk family members and block Mcl-1 expression, the limited clinical activity observed in patients with leukemias suggests that targeting Mcl-1 alone may only be effective in a subset of AML cases. Our findings show that dual targeting of both PI3K and transcriptional kinases by PIK-75 demonstrates antileukemic activity in primary human AML cells without impacting on the survival, colony-forming potential, or engraftment capacity of nontransformed hematopoietic BM progenitors (Figure 6). Thus, while PIK-75 targets 2 fundamental cell survival pathways, our data indicate that there is a therapeutic window that permits selective targeting of AML cells.

Small molecule inhibitors of Bcl-2, Bcl-xL, and Bcl-w, such as ABT-737, have been shown to induce apoptosis in primary follicular lymphomas, chronic lymphocytic leukemias, and AML. However, it is becoming increasingly clear that a variety of malignancies including chronic and acute leukemias are resistant to ABT-737 in part due to deregulated Mcl-1 expression. In fact, downregulation of Mcl-1 may potentiate the apoptosis induced by ABT-737. Furthermore, Mcl-1 overexpression in some solid and hemopoietic malignancies is a prognostic marker of poor patient outcome. Our findings indicate that PIK-75 is able to induce apoptosis in AML cells irrespective of the level of Mcl-1 expression (supplemental Figure 6). Thus, Mcl-1 represents an important therapeutic target and small molecule inhibitors, such as PIK-75, that block Mcl-1 expression may improve clinical responses in the treatment of cancer. PIK-75 activity strongly correlated with Bak expression in patient samples tested (Figure 5), suggesting Bak might be an informative biomarker that predicts resistance to drugs that target Mcl-1 and Bcl-xL.

The characteristically complex nature of the AML genome, as revealed by next-generation sequencing, will likely present major obstacles to the development of targeted therapies against specific oncogenes. Furthermore, the increased frequency of mutations at relapse highlights the substantial genomic instability in AML, which will likely further thwart target-selective approaches. For example, while FLT3-activating mutations (eg, FLT-ITD) are clearly oncogenic, clinical studies using FLT3 inhibitors have reported rapid evolution of “escape” mutations or activation of parallel pathways despite encouraging initial responses. Our studies showing that PIK-75 is broadly effective in ~90% of primary AML patient samples (n = 46) as well as in cancer cell lines (n = 16), representing leukemia, multiple myeloma, and brain and breast cancer (Figure 1; supplemental Table 3), indicate that PIK-75 likely targets “convergent” cell survival pathways common to a wide range of transforming lesions. To date, although the clinical efficacy of PI3K or Cdk inhibitors as monotherapies has been limited, our findings would indicate that combining their targeted actions for the treatment of cancer may be more effective and could overcome the molecular complexity inherent to multigenic diseases such as AML.

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References


Targeting acute myeloid leukemia by dual inhibition of PI3K signaling and Cdk9-mediated Mcl-1 transcription