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Original Article
PIK-75 overcomes venetoclax resistance via blocking PI3K-AKT signaling and MCL-1 expression in mantle cell lymphoma

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Abstract: Therapeutic resistance is the major challenge in clinic for patients with mantle cell lymphoma (MCL), an aggressive subtype of B-cell lymphoma. In addition to the FDA-approved Bruton’s tyrosine kinase (BTK) inhibitors, multiple clinical trials have demonstrated clinical benefits in targeting BCL-2 by venetoclax and reported to greatly improve clinical outcome for refractory/relapsed patients with MCL alone or in combination with BTK inhibitors. However, resistance to venetoclax is no exception and marks as a new clinic challenge. To decode the underlying mechanisms driving venetoclax resistance, we established two MCL cell lines, Mino-Re and Rec1-Re, with acquired resistance to venetoclax from sensitive Mino and Rec-1. Using reverse phase protein assay (RPPA), an agnostic proteomic approach, we identified targetable signaling pathways that are associated with acquired venetoclax resistance in Mino-Re and Rec1-Re cells. A panel of pro-survival signals was identified to correlate well with venetoclax-resistance, including increased expression of MCL-1, BCL-xL and AKT phosphorylation, and decreased expression of BIM, BAX and PTEN. Based on a high throughput drug screening of over 320 FDA-approved/investigational drugs in the paired venetoclax-sensitive and -resistant cell lines Mino-Re and Rec1-Re, we identified the top candidates that are capable to overcome acquired venetoclax resistance in these cells. The best candidate is PIK-75, a dual inhibitor targeting both PI3K and CDK9. Its action to overcome venetoclax resistance was further confirmed in additional cell lines with primary venetoclax resistance (n=4) and primary patient samples (n=21). Mechanistically, PIK75 treatment potently diminished the elevated MCL-1 expression and AKT activation in cells with acquired or primary venetoclax resistance and resulted in potent anti-MCL activity to overcome these resistances. In addition, PIK75 is also potent in overcoming tumor microenvironment (TME)-associated venetoclax resistance. Furthermore, PIK-75 treatment is efficacious in overcoming primary and acquired venetoclax resistance in xenograft models and inhibited tumor cell dissemination to spleen in mice. Altogether, our data demonstrated that PIK-75 is highly potent in overcoming primary, acquired, or stromal cells-induced venetoclax resistances in MCL cells and revealed a new tumor vulnerability that can be exploited clinically in difficult to treat MCL cases, especially those with venetoclax resistance.

Keywords: PIK75, venetoclax resistance, PI3K, MCL-1, AKT, mantle cell lymphoma

Introduction

Targeting the B-cell leukemia/lymphoma-2 (BCL-2) protein has shown potent therapeutic effect in hematologic malignancies [1-3]. BCL-2 specific inhibitor venetoclax has been approved for treating chronic lymphocytic leukemia (CLL) and has been utilized alone or in combination with BTK inhibitors in numerous clinical trials and demonstrates clinical benefits in other hematologic cancers [4], including mantle cell lymphoma (MCL) [5, 6]. However, drug resistance develops frequently and is one of the major challenges for relapsed/refractory (R/R) patients. Although resistance to venetoclax has been studied in several hematologic cancers, its mechanism has not been well defined in MCL yet. Discovering the potential mechanisms of venetoclax resistance lays a foundation for preventing its occurrence or developing new therapeutic strategies to overcome it. Three main mechanisms have been reported to con-
tribute to venetoclax resistance: 1) acquired mutations in BCL-2 family proteins with proliferation-enhancing effects under the selection pressure or aberrant expression of antiapoptotic genes, such as MCL-1 and BCL-xL; 2) dysregulated signaling pathways, such as the PI3K-AKT pathway; and 3) interaction with TME such as stromal cells, which protect cancer cells through cell-cell interaction and cytokine secretion [7-9].

Importantly, many of these dysregulated proteins that play crucial roles in the acquired resistance, including PI3K-AKT/mTOR and B-cell receptor signaling, are also targetable and thus offer potential strategies for overcoming venetoclax resistance. Particularly, novel compounds that inhibit MCL-1 and BCL-xL have shown great efficacy in targeting venetoclax resistance [10-12]. Additionally, TME-associated venetoclax resistance can be counteracted by CD20-directed antibodies or by PI3K-AKT kinase dependent inhibitors.

To uncover the mechanisms of venetoclax resistance and develop a strategy that is tailored to the MCL characteristics, we first generated two venetoclax-resistant MCL cell lines and patient-derived primary cells. RPPA analysis as an agnostic approach was used to investigate the mechanism of acquired venetoclax resistance in these cells, and high throughput drug screening was applied to find candidates to potentially overcome this resistance. The potency of the best candidates was further confirmed in MCL cell lines with primary resistance and primary patient samples with primary or TME-associated resistance. We found that PIK-75, a PI3K/CDK9 dual inhibitor, had great efficacy in inhibiting cell growth in vitro and in vivo not only in MCL cells with acquired resistance, but also in those with primary or TME-associated resistance in vitro and in vivo. Mechanistically, we demonstrated that PIK-75 actions through inhibiting PI3K-AKT signaling and blocking MCL-1 expression to induce cell apoptosis and thus overcomes venetoclax resistance in MCL cells.

Materials and methods

**MCL primary tumor samples and cancer cell lines**

Twenty-one MCL patient samples were collected after obtaining informed consent and approval from the Institutional Review Board at The University of Texas MD Anderson Cancer Center (MDACC). The patient samples were processed and cryopreserved before use.

MCL cell lines Mino, Rec-1, Maver-1, and Z-138 and human stromal cell line HS-5 were purchased from ATCC (Manassas, VA, USA). Granta-519 cells were kindly provided by Dr. Felipe Samaniego from MDACC (Houston, TX, USA). JeKo-R cells with acquired ibrutinib resistance were generated from ibrutinib-sensitive JeKo-1 cells and provided by Dr. Lan Pham at MDACC. SP-49 cells were kindly provided by Dr. Jianguo Tao at Moffitt Cancer Center (Tampa, FL, USA). JeKo-luc cells were derived from JeKo-1 cells to stably express the luciferase protein for bioluminescence in vivo studies. All MCL cell lines were cultured in RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin. HS-5 cells were maintained in DMEM medium with 10% FBS and 1% penicillin/streptomycin.

**Drugs**

PIK-75 (S1205), GSK1059615 (S1360), venetoclax (S8048), and a drug library of 320 drugs (FDA-approved/investigational) were purchased from Selleckchem (Houston, TX, USA).

**Induction of venetoclax resistance in cell lines and primary samples in vitro**

Two venetoclax-sensitive MCL cell lines Mino and Rec-1 were subjected to long-term exposure to venetoclax using a stepwise dose-increase approach from 1 nM to 1000 nM (Figure 1A). The stable resistant cells Mino-Re and Rec1-Re were cryopreserved for future use.

To establish venetoclax-resistant primary samples, primary cells from two venetoclax-sensitive patients were co-cultured with the stromal cell line HS-5 and cytokine cocktail (50 ng/ml of IL-10, 50 ng/ml of BAFF, 1 ng/ml of IGF-1, 1 ng/ml of IL-6, and 50 ng/ml CD40L). The fibroblast-like HS-5 cells secrete significant levels of cytokines to promote the survival and growth of macrophages, including granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF), macrophage-CSF (M-CSF) and macrophage-inhibitory protein-1 alpha [13]. IL-10, BAFF and CD40L can activate macrophages [14]. Tumor associated macro-
phages (TAMs) have been shown to correlate with poor prognosis in various types of cancer including solid cancer and hematologic malignancies [15]. Together with the supplemental cytokines, TAMs can be activated to promote the survival and growth of primary MCL cells. HS-5 cells were pre-treated with 10 μg/ml mitomycin-C for 2 h before the co-culture system set-up. The ratio of HS-5: primary MCL cells was 1:10. Primary cells were isolated from the stromal cells once a week and diluted in fresh medium with HS-5 cells and a cytokine cocktail.

**High throughput drug screening**

A library of 320 FDA-approved/investigational drugs from Selleckchem (Houston, TX) was
PIK-75 overcomes venetoclax resistance in MCL

Table 1. \( \text{IC}_{50} \) levels of venetoclax, GSK1059615 and PIK-75 treatments in MCL cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Venetoclax (nM)*</th>
<th>GSK1059615 (nM)*</th>
<th>PIK-75 (nM)*</th>
</tr>
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<tr>
<td>Mino</td>
<td>1.50</td>
<td>3.35</td>
<td>3.82</td>
</tr>
<tr>
<td>Mino-Re</td>
<td>&gt;100</td>
<td>5.71</td>
<td>1.50</td>
</tr>
<tr>
<td>Rec-1</td>
<td>10.94</td>
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</tr>
<tr>
<td>Rec1-Re</td>
<td>&gt;100</td>
<td>6.59</td>
<td>10.90</td>
</tr>
<tr>
<td>Maver-1</td>
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<td>7.15</td>
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<td>Granta519</td>
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<td>17.19</td>
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<td>147.50</td>
<td>7.03</td>
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<td>8.09</td>
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<td>5.27</td>
<td>5.14</td>
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</table>

*IC\(_{50}\) values were calculated 72 h post-treatment.

used for a high throughput drug screening. Four cell lines (Mino, Rec-1, Mino-Re and Rec1-Re) were treated with each drug at 5 \( \mu \)M for 72 h, and cell viability was determined as described previously.

Cell viability and cell apoptosis assay

These assays were performed as described previously [16].

RPPA analysis

5\( \times \)10\(^6\) cells of each cell line with/without treatment with vehicle or 50 nM PIK-75 in triplicate for 24 h were subjected to RPPA analysis as described previously [16]. In total, 425 antibodies and four secondary antibodies (as negative controls) were included in the analysis. NormalLog2_MedianCentered values were selected for heatmap generation. We set up the parental cell lines or untreated cell lines as “1” (upregulated as compared with resistant or treated cell lines) or “-1” (downregulated as compared with resistant or treated cell lines). The differences in proteins values between parental (or untreated) samples and resistant (or treated) samples \(< \) and \(>0.5\) were selected for the heatmap. The heatmap was generated in Cluster 3.0, visualized in Treeview, and presented as a high-resolution Bmp format. Each treatment was set up in triplicate.

Western blotting

Western blotting was performed as described previously [16]. All primary antibodies against PARP, cleaved PARP, Caspase-3, cleaved Caspase-3, BIM, BAX, BAK, BCL-2, BCL-xl, MCL-1, p-S6, S6, p-AKT, AKT, p-p38, p38, PDCD4, PTEN, PDCD4, and GAPDH and secondary antibodies (anti-mouse or anti-rabbit) were purchased from Cell Signaling Technology (Danvers, MA, USA) and used at the manufacturer’s suggested concentration.

Animal studies

All the animal studies were performed after obtaining approval from the Institutional Animal Care and Use Committee, at MDACC.

In vivo long-term drug efficacy evaluation

Approximately 5\( \times \)10\(^6\) cells of Rec1-Re or luciferase-engineered JeKo-luc were injected into each 6-8-week-old female NSG mice. Once the tumors became palpable, the mice (n=5 per group) were treated with vehicle, venetoclax (10 mg/kg, oral, daily), or PIK-75 (10 mg/kg, IP, daily). Mouse blood was collected weekly. Human beta-2-microglobulin (B2M) level was detected by ELISA (Thermo Fisher Scientific) according to the manufacturer’s instructions. Tumor burden was measured weekly by caliper or by IVIS Luminescence In Vivo Imaging System (IVIS100, PerkinElmer, USA). Tumor volume was calculated using the following formula: tumor volume (mm\(^3\)) = length (mm) × width (mm\(^2\))/2. Total flux was calculated based on live imaging data. Tumor weights were measured at the end of experiments.

Statistical analysis

The IC\(_{50}\) values were calculated from dose-dependent drug inhibition values using GraphPad Prism-8. Statistically significant differences among treatments were determined by 2-tailed Student t test. Experiments were repeated three times and representative results were presented, as appropriate.

Results

Establishment of MCL cell lines with acquired resistance to venetoclax

To understand the mechanism of venetoclax resistance, we first established two venetoclax-resistant cell lines Mino-Re and Rec1-Re to have acquired resistance to venetoclax at 1000 nM in culture from parental venetoclax-sensitive cell lines Mino (IC\(_{50}\)=1.5 nM) and Rec-1 (IC\(_{50}\)=10.9 nM), respectively (Figure 1A, 1B and Table 1). Venetoclax treatment at 2 nM
PIK-75 overcomes venetoclax resistance in MCL

(P=0.003) or 5 nM (P=0.003) induced robust cell apoptosis (Figure 1C), PARP cleavage and caspase-3 cleavage in Mino and Rec-1 cells, respectively, but not in Mino-Re and Rec1-Re cells with acquired resistance (Figure 1D). The resistant phenotype was stable even in the absence of venetoclax for >20 passages indicating that the developed venetoclax-resistant phenotype was stable and acquired survival and growth advantage changes without the need for venetoclax for cell re-charging. Therefore, these paired cell lines were ideal cellular models to study the mechanism underlying venetoclax resistance.

**Dysregulated apoptosis pathway and PI3K-AKT signaling confer venetoclax resistance in MCL**

The mechanism of venetoclax resistance may vary from one cancer type to another, but factors disturbing the balance between pro- and anti-apoptotic proteins always play a major role in driving venetoclax resistance. RPPA is an unbiased proteomics method that can simultaneously characterize hundreds of proteins by their expression and phosphorylation status, and therefore emerged as our preferred method for interrogating the status of protein profiles and signaling networks. We found that both the pro-apoptotic and anti-apoptotic family members were significantly off balance in resistant cell lines Mino-Re and Rec1-Re compared to parental cell lines Mino and Rec-1 (Figure 1E). For example, anti-apoptotic proteins such as MCL-1 and BCL-xL were significantly up-regulated, while pro-apoptotic proteins such as BIM, BAX, and BAK were down-regulated in the venetoclax-resistant cell lines. The BCL-2 itself was slightly decreased in the resistant cells. Of interest, pro-survival and stress-related proteins such as AKT, S6, and p38 were also activated in venetoclax-resistant cell lines. These findings were further validated by western blotting in these paired venetoclax-sensitive and -resistant cell lines (Figure 1F).

**High throughput drug screening identified PIK-75 and GSK1059615 as the top candidates that overcome venetoclax resistance**

To find candidate compounds that can overcome venetoclax resistance, we performed a high throughput drug screening over a library consisting of 320 drugs FDA-approved or under clinical investigation in the paired venetoclax-sensitive and -resistant cell line models (Mino vs Mino-Re and Rec-1 vs Rec1-Re) (Figure 2A). Forty-four drugs (at 5 µM) were identified to inhibit at least 80% cell viability compared to the DMSO control (Figure 2A, 2B). These drugs were further grouped based on their targeted signaling pathways, and the five most effective groups targeted PI3K-AKT, DNA topoisomerase, Aurora, VEGFR, and mTOR signaling pathways. The top six drugs (4 targeting PI3K and 2 targeting Aurora kinase) were chosen for validation via a four-dose-dependent cell viability assay (Figure 2C). As a result, PIK-75 and GSK1059615 showed the best efficacy in the paired venetoclax-sensitive and -resistant cell lines. PIK-75 is a dual inhibitor targeting PI3K and CDK9, and has been shown to be selectively potent in targeting AML cells but with significantly less sensitivity to CD34+ normal bone marrow progenitor cells [17]. GSK1059615 is a dual inhibitor targeting PI3K and mTOR pathways [18].

**PIK-75 is highly potent in overcoming a variety of venetoclax resistances**

To further evaluate the potency and potential of PIK-75 and GSK1059615 in overcoming venetoclax resistance, we performed an eight-dose cell viability assay. Interestingly, both PIK-75 and GSK1059615 were potent inhibitors with IC_{50}=1.5-10.9 nM and 3.4-6.6 nM at 72 hours upon treatment, respectively, in the paired venetoclax-sensitive and -resistant cell lines (Figure 3A and Table 1). This demonstrated that both compounds are potent in overcoming acquired venetoclax resistance.

To test whether PIK-75 is also capable to overcome primary venetoclax resistance, in addition to acquired venetoclax resistance, we screened an additional panel of eight MCL cell lines for cell viability to venetoclax and PIK-75. Two cell lines, Maver-1 and Granta-519, were identified as venetoclax-sensitive (IC_{50}=2.9 and 5.8 nM, respectively), in addition to Mino and Rec1. Four cell lines JeKo-1, JeKo R, Z138 and SP-49 were venetoclax-resistant (IC_{50}>100 nM). Consistently, both PIK-75 (IC_{50}=1.5-17.2 nM) and GSK1059615 (IC_{50}=3.4-15.2 nM) were potent in targeting all these MCL cell lines (Figure 3B, 3E and Table 1).

To ascertain the clinical potential of PIK-75 and GSK1059615, we next asked if this also applies...
PIK-75 overcomes venetoclax resistance in MCL

Figure 2. High throughput drug screening to identify candidates for overcoming venetoclax resistance. (A) Schematic illustration of the high-throughput drug screen. The drug screen was performed over a library consisting of 320 drugs in two parental and venetoclax-resistant paired cell lines by a 72 h cell viability test with one dose (5 μM) as first round screen followed by 2nd round drug validation via 4-dose viability assay in the same cell lines to identify the top candidates in overcoming venetoclax resistance. (B) 1st round drug screen at 5 μM by a cell viability inhibition assay in Mino, Mino-Re, Rec-1 and Rec1-Re cells. Each treatment for the cell viability assay was set up in triplicate. Relative cell viability was normalized to DMSO control. Cell viability less than 20% was considered as positive (red box). (C) 2nd round drug validation for the top candidates (six drugs) was performed by a four-dose (three-fold dilution) cell viability assay at 72 h in the same cell lines. The heatmap shown represents the IC_{50} values (µM). Each treatment for the cell viability assay was set up in triplicate and the experiment was repeated three times.

to primary patient samples. Among a panel of 21 primary MCL patient samples, PT 1-10 were determined to be venetoclax sensitive (IC_{50}=5.2-125.1 nM) and PT 11-21 were resistant (IC_{50}>244.8 nM), based on IC_{50} values at 24 hours upon treatment (Figure 3C, 3E and Table 2). Intriguingly, PIK-75 showed higher potency in targeting primary patient samples ex vivo (IC_{50}=6.3-425.2 nM) while only a partial efficacy was observed for GSK1059615 (IC_{50}>110.7 nM) (Figure 3C, 3E and Table 2). These data suggested that PIK-75 may have more clinical potential than GSK1059615 in targeting MCL cells and in overcoming venetoclax resistance in primary MCL patient samples.

It is well known that TME plays a critical role in drug resistance in many cancer types [19]. To mimic TME-associated venetoclax resistance, we co-cultured two primary patient samples PT2 (from a clinically venetoclax resistant patient) and PT3 (from a venetoclax-naïve patient), with human stromal cell line (HS-5) and a panel of supplemental cytokine cocktail (see Materials and Methods). Both PT2 and PT3 were sensitive to venetoclax ex vivo (IC_{50} = 3.1 and 3.4 nM, respectively) in the absence of co-culture and became highly resistant to venetoclax (IC_{50}>2000 nM) after two weeks of co-culture (Figure 3D, 3E, left panel). Interestingly, PIK-75 showed high potency at similar levels in targeting both parental venetoclax-sensitive patient samples and their derived samples with TME-associated venetoclax resistance (IC_{50} = 5.8-9.0 nM) (Figure 3D, 3E, right panel). In contrast, GSK1059615 was only slightly effective in the parental samples (IC_{50}=110.7 and 458.4 nM, respectively) and much less potent in those with TME-associated venetoclax resistance (IC_{50}=1112 and 2566 nM, respectively) (Figure 3D, 3E, middle panel). Together, these
PIK-75 overcomes venetoclax resistance in MCL

Figure 3. PIK-75 is potent in overcoming a variety of venetoclax resistances. (A, B) In vitro efficacy of BCL2i venetoclax, PI3Ki PIK-75 and GSK1059615 in paired venetoclax-sensitive (Mino and Rec-1) and -resistant (Mino-Re and Rec1-Re) cell lines (A), and additional 8 MCL cell line panel (B) by eight-dose cell viability assay at 72 hours upon treatment. (C) In vitro efficacy of venetoclax, PIK-75 and GSK1059615 in 21 primary MCL patient samples at 24 hours upon treatment. (D) Two venetoclax-sensitive (ex vivo) patient samples (PT2 and PT3) were co-cultured with HS-5 stromal cells and supplemental cytokines for two weeks. An eight-dose 24-hour cell viability assay was conducted. Each treatment for the cell viability assay was set up in triplicate. (E) Heatmaps of IC\textsubscript{50} of venetoclax, GSK1059615 and PIK-75 in MCL cell lines and primary patient samples with/without coculture with HS-5 cells based on the data (A-D). Sensitivity to venetoclax was indicated in the right for each cell line or patient sample.

data support the notion that PIK-75 is more potent than GSK1059615 in overcoming primary, acquired, and TME-associated venetoclax resistance in primary patient samples and therefore, PIK-75 was chosen for further investigation.

**PIK-75 overcomes venetoclax resistance by blocking inhibiting PI3K-AKT signaling and MCL-1 expression**

We showed by RPPA and subsequent western blot that up-regulation of pro-survival molecules MCL-1 and BCL-xL and activation of PI3K-AKT signaling were the major events in driving venetoclax-acquired resistance (Figure 1E, 1F), indicating that targeting these pathways has the potential to overcome the resistance. To understand the mechanism of PIK-75 action, we treated both Rec-1 and Rec1-Re cells with 50 nM PIK-75 for 24 h and performed RPPA analysis again as an unbiased proteomics approach. Multiple proteins were dysregulated in both cell lines upon PIK-75 treatment. In particular, PIK-75 induced loss of MCL-1 protein expression in both MCL-1 high-expressing Rec1-Re cells (venetoclax-resistant) and MCL-1 low-expressing Rec-1 cells (venetoclax-sensitive) (Figure 4A). AKT phosphorylation was also
PIK-75 overcomes venetoclax resistance in MCL

Table 2. IC_{50} levels of venetoclax, GSK1059615 and PIK-75 treatments in patient samples

<table>
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<tr>
<th>Patient ID</th>
<th>Venetoclax (nM)</th>
<th>GSK1059615 (nM)</th>
<th>PIK-75 (nM)*</th>
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*IC_{50} values were calculated 24 h post-treatment.

PIK-75 overcomes venetoclax resistance in MCL

PIK-75 overcomes venetoclax resistance in MCL and Rec1-Re cells (Figure 4A). To verify this, we treated both Rec-1 and Rec1-Re cells with designated concentrations of venetoclax and PIK-75 for 24 h and then tested the expression of MCL-1 and AKT phosphorylation by western blotting. As expected, PIK-75 potently blocked MCL-1 and AKT phosphorylation in both cell lines (Figure 4B). Of note, expression of another pro-apoptosis protein BAK was not dramatically affected by PIK-75 treatment. Based on RPPA analysis, caspase-3 and -7 cleavage were highly induced upon PIK-75 treatment. Consistently, PIK-75 at 10 and 50 nM induced dose-dependent cell apoptosis in both Rec-1 (P=0.0030 and 0.0002, respectively) and Rec1-Re cells (P=0.0014 and 0.0002, respectively). PIK-75 at either dose was more potent in targeting Rec1-Re cells than venetoclax at 100 nM (P=0.031 and 0.0003, respectively) (Figure 4C). Consistently, higher levels of cleaved PARP and caspase-3 were observed in Rec1-Re cells treated with PIK-75 compared to venetoclax (Figure 4B). Together, these data demonstrated that PIK-75 induces cell apoptosis and overcomes venetoclax resistance via inhibiting PI3K-AKT signaling and blocking MCL-1 expression.

PIK-75 blocks in vivo homing of MCL cells to the mouse spleen

PI3K-AKT signaling plays a crucial role in regulating the tumor-TME interplay and targeting PI3K-AKT signaling has been shown to block malignant B-cell homing [20, 21]. Therefore, we hypothesized that PIK-75 may also interfere with the malignant B-cell homing to different sites via targeting PI3K. To address this, we performed a short-term in vivo B-cell homing assay using a clinically-assessed venetoclax-resistant primary patient sample (PT2) (Figure 3D). The MCL cells homed predominantly to the spleen instead of the bone marrow site (Figure 5A, 5B). A 30 min pretreatment with PIK-75 at 100 nM inhibited MCL homing to mouse spleen at 16 h post-treatment (P=0.011) (Figure 5A, 5B). We also observed a concomitant increase of increased retention of circulating tumor cells in the blood of mice injected with PIK-75-pretreated MCL cells, compared to vehicle-pretreated cells (P=0.025). As expected, PIK-75 pretreatment did not induce cell apoptosis in ex vivo culture (data not shown). Therefore, these data may suggest that ex vivo short-term PIK-75 pretreatment blocks MCL homing to mouse spleen, likely via targeting PI3K-AKT mediated tumor-TME interplay.

PIK-75 overcomes acquired and primary venetoclax resistance in mouse xenograft models

To test the in vivo potency of PIK-75 in overcoming venetoclax resistance, we generated cell line-derived xenograft (CDX) models by subcutaneous injection of Rec1-Re cells. The mice bearing xenografts were treated with vehicle, venetoclax (10 mg/kg, oral, daily), or PIK-75 (10 mg/kg, IP, daily). As expected, venetoclax failed to inhibit tumor growth in Rec1-Re CDXs (P=0.21) confirming the in vivo venetoclax-resistant phenotype (Figure 4D-F). In contrast, PIK-75 reduced tumor burden (P=0.0002) and production of B2M significantly (P=0.000002) as compared to the vehicle-treated group.
PIK-75 overcomes venetoclax resistance in MCL

Figure 4. PIK-75 inhibits PI3K-AKT activity and overcomes acquired venetoclax resistance in vitro and in vivo. (A) Rec-1 and Rec1-Re cells were treated with 50 nM PIK-75 for 24 h and subjected to RPPA analysis. A heatmap was made with NormLog2_MedianCentered values that were < and >0.5 times between untreated and treated samples. (B, C) Rec-1 and Rec1-Re cells were treated with designated venetoclax or PIK-75 concentrations for 24 h. Cells were harvested and analyzed by western blotting (B) and cell apoptosis assay (C) to confirm the RPPA data and biological effects. (D-F) 5×10^6 Rec1-Re cells were injected subcutaneously into each NSG mouse and treated with vehicle, 50 mg/kg venetoclax, or 10 mg/kg PIK-75, daily. Tumor volumes were measured weekly (D), and mouse blood was collected weekly; human B2M in mouse serum was measured by ELISA assay (F). Tumor weight was measured at the end of experiment (E). Student t test was used for statistical analysis, n=5.
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(Figure 4D-F). In addition, no gross abnormality was detected in mice treated with PIK-75. This demonstrated that PIK-75 is safe and highly potent in overcoming acquired venetoclax resistance in vivo. Next, we tested if this also applies to primary resistance. JeKo-1, Z138, JeKo R and SP-49 were intrinsically resistant to venetoclax while highly sensitive to PIK-75 treatment (IC_{50} =5.1-9.6 nM) (Figure 3B, 3E and Table 1). Similar to the Rec1-Re venetoclax resistance model system, PIK-75 at 10 nM induced robust cell apoptosis in JeKo-1 as evident by PARP cleavage and caspase-3 cleavage, and diminished expression of MCL-1 and AKT phosphorylation (Figures 4B and 6A). Furthermore, PIK-75 (P<0.05), but not venetoclax (P=0.64), effectively inhibited tumor growth of luciferase-expressing JeKo-luc derived xenografts in vivo, compared to vehicle-treated group (Figure 6B-D). Again, no gross abnormality was detected in mice treated with PIK-75. Altogether, these data demonstrated that PIK-75 is a safe and potent candidate for overcoming primary and acquired venetoclax resistance in MCL.

Discussion

In this study, we demonstrated that PIK75 targeting both PI3K and CDK9, is potent for over-
PIK-75 overcomes venetoclax resistance in MCL

PIK-75 overcomes venetoclax resistance in MCL by suppressing PI3K-AKT signaling and blocking MCL-1 expression (Supplementary Figure 1). PIK-75 therapeutic efficacy has been reported in acute myeloid leukemia (AML); the mechanism involves primarily dual inhibition of the PI3K pathway and CDK9-mediated MCL-1 transcription [22]. PIK-75 targets PI3Kα isoform and dissociates the interaction between BCL-xL and BAK [23]. In this study, PIK-75 was one of the top hits from the high throughput drug screening, for the first time, it was shown to overcome venetoclax resistance. AKT phosphorylation was significantly diminished in both venetoclax-sensitive and -resistant cells, and MCL-1 expression elevated in venetoclax-resistant cells was effectively blocked upon PIK75 treatment. This demonstrates its potency in overcoming venetoclax resistance. The tumor microenvironment plays an important role in drug resistance and subsequently, drugs that are capable of targeting tissue homing, such as the BTK inhibitor ibrutinib, have an increased therapeutic efficacy [24, 25]. Interestingly, we showed that PIK-75 is also able to inhibit B-cell homing to the spleen in a short-term in vivo homing assay. This indicates that PIK-75 may overcome tissue niche-specific drug resistance by also targeting the tumor-microenvironment crosstalk. Taken together, PIK-75 has potential to overcome venetoclax resistance in MCL by dually targeting PI3K and MCL-1 and by interfering with the tumor microenvironment.

Cell apoptosis is tightly regulated by a balance between pro-apoptotic and anti-apoptotic proteins. Sensitivity to venetoclax relies not only on BCL-2 expression, but also on the expression of other BCL2 family proteins, as they form complexes to coordinate cell apoptosis [3, 26]. In CLL, phosphorylation of BCL-2 indicates the status of venetoclax resistance, as BCL-2 phosphorylation inhibits interaction between venetoclax and the BCL-2/BIM/BAX complex [27]. In multiple myeloma (MM), sensitivity to venetoclax is correlated with high BCL-2 and low BCL-xL or MCL-1 expression [28]. We have previously reported that sensitivity of MCL or DLBCL to venetoclax correlated strongly with the BCL-2 protein expression level, although other BCL2 family members also modulate the therapeutic response [29]. For example, BCL-xL and MCL-1 expression correlated with venetoclax resistance, while BIM and BAK levels were negatively correlated with the resistance. Taken together, aberrant expression of BCL-2 family proteins may contribute to primary resistance and acquired resistance to venetoclax.
Gene mutations are also considered an important factor for venetoclax resistance. Acquisition of mutations within BCL-2 or BAX results in venetoclax resistance in MCL-like murine lymphoma cells and in a human MCL cell line (HBL-2), respectively [30]. Mutations (F101C or F101L) within the BCL-2 BH3 domain impair venetoclax binding, and BAK mutation interferes with its anchoring to the mitochondrial membrane [31]. However, these mutations were not identified in Rec1-Re and Mino-Re cells with acquired venetoclax resistance (data not shown). Instead, we revealed non-genetic aberration of PI3K signaling and elevated expression of MCL-1 and BCL-xL, which likely drive the venetoclax resistance in our system.

Acquired drug resistance models can be generated by chronic drug exposure or interaction with the microenvironment [7, 9, 30, 32, 33]. In MCL, chronic exposure of HBL-2 cells to venetoclax led to an increase in MCL-1 expression and moderate decrease in BIM expression [30]. Similar results were observed in a murine MCL model developed to be resistant to venetoclax. Upon stimulation by CD40 antibody or CD40L-expressed stromal cells, MCL cell lines and patient samples can acquire venetoclax-resistant properties [32]. This phenotype included dramatic up-regulation of BCL-xL and a slight up-regulation of MCL-1. For BCL-2 itself, changes in protein expression were not unanimously shared by all malignant phenotypes. Consistently, our results showed that a dysregulated balance between anti-apoptotic proteins such as MCL-1 and BCL-xL and pro-apoptotic proteins such as BIM, BAX, and BAK is the hallmark of venetoclax-resistant cells in MCL. Additionally, pro-survival and stress-related signaling pathways were elevated in the venetoclax-resistant cells. These included up-regulation of p-S6, p-AKT, and p-p38, also reported previously to contribute to venetoclax resistance [29, 34-36]. Of interest, two tumor suppressors, PTEN and PDCD4, showed decreased expression in venetoclax-resistant MCL cells. We speculated that suppression of PTEN could lead to activation of AKT and S6. PDCD4 can alter PI3K-AKT by regulating miR-184 expression [37]. Whether down-regulation of these survival pathway-related tumor suppressors plays a role in venetoclax resistance requires further investigation.

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Disclosure of conflict of interest

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**Supplementary Figure 1.** Proposed model for the mechanism of action of PIK75 in overcoming venetoclax resistance in MCL. Aberrant PI3K-AKT signaling and MCL-1 expression contribute to venetoclax resistance in MCL cells. PIK75 targets PI3K to inhibit cell survival mediated by PI3K-AKT-signaling and targets CDK9 to block CDK9-mediated gene transcription, leading to diminished expression of MCL-1.