PAK1 Mediates Resistance to PI3K Inhibition in Lymphomas

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Abstract

Purpose: The phosphoinositide 3-kinase (PI3K) pathway is known to play an active role in many malignancies. The role of PI3K inhibition in the treatment of lymphomas has not been fully delineated. We sought to identify a role for therapeutic PI3K inhibition across a range of B-cell lymphomas.

Experimental Design: We selected three small molecule inhibitors to test in a panel of 60 cell lines that comprised diverse lymphoma types. We tested the selective PI3K inhibitor BKM120 and the dual PI3K/mTOR inhibitors BEZ235 and BGT226 in these cell lines. We applied gene expression profiling to better understand the molecular mechanisms associated with responsiveness to these drugs.

Results: We found that higher expression of the PAK1 gene was significantly associated with resistance to all three PI3K inhibitors. Through RNA-interference-mediated knockdown of the PAK1 gene, we showed a dramatic increase in the sensitivity to PI3K inhibition. We further tested a small-molecule inhibitor of PAK1 and found significant synergy between PI3K and PAK1 inhibition.

Conclusion: Thus, we show that PI3K inhibition is broadly effective in lymphomas and PAK1 is a key modulator of resistance to PI3K inhibition.

Introduction

Lymphomas are a common group of malignancies that collectively affect more than 100,000 new patients in the United States alone (1). These tumors are molecularly and clinically heterogeneous, with dramatically different responses and outcomes with standard therapies. While some patients can be cured of their disease with standard regimens, the majority of these patients relapse and eventually succumb to their tumors. There is an urgent need for new therapies in this disease.

Over the past decades, lymphomas have been classified extensively into more than 30 individual diagnoses (2), with new categories continuing to emerge. These classifications have been useful in understanding the clinical behavior of these tumors. However, many of these classifications can be difficult to render clinically (3, 4), and do not always capture the diversity of clinical behavior and molecular heterogeneity of these tumors. Gene expression profiling has elucidated the molecular diversity of these tumors even further (5, 6) and showed that the activation of different oncogenic pathways frequently overlap among different lymphoma types (3, 7).

Activation of the phosphoinositide 3-kinase (PI3K) pathway is known to be a key oncogenic event in a number of malignancies (8) and results in increased proliferation and cell survival (9). Multiple PI3K inhibitors that either target PI3K selectively or in combination with the mTOR, which lies downstream of PI3K (10), are under clinical investigation in several malignancies (11). The clinical role of PI3K inhibition in lymphomas has not been fully explored. Early preclinical data in lymphomas as well as other hematologic malignancies and solid tumors show potential use of PI3K inhibition in these diseases (12–15). However, the overall patterns of responsiveness to PI3K inhibition in different lymphomas and the molecular patterns underlying their response remain unknown.

Most preclinical studies to date in lymphomas have generally focused on relatively small numbers of cell lines within a single lymphoma subtype. The role of PI3K inhibition has not been systematically studied in most lymphoma histologies. We hypothesized that testing the role of PI3K inhibition in a broad set of lymphoma histologies in conjunction with gene expression profiling would reveal the broad applicability of PI3K inhibition in different lymphomas and would allow us to identify the molecular underpinnings of resistance to therapy.

Recent work has shown that gene expression patterns associated with response to targeted therapy in cell lines are similarly associated with a clinical response in patients (16). We hypothesized that gene expression patterns associated with response to PI3K inhibition might provide a useful tool to understand the underlying biology associated with responsiveness to drugs that inhibit this pathway. We selected 3 small molecule-inhibitors that targeted PI3K either singly or in combination with its downstream target...
mTOR, and tested these drugs broadly in 60 different cell lines that predominantly represented different lymphoma types. Through gene expression profiling in the same cell lines, we show that signaling mediated by the PAK1 gene is a significant contributor to resistance to PI3K inhibition in lymphomas. We experimentally show that knockdown of the PAK1 gene through RNA interference and pharmacologic approaches overcomes the resistance to PI3K inhibition and may represent a rational combination for the treatment of patients with lymphoma.

Materials and Methods

Cell culture
A total of 60 cell lines that were representative of different lymphoma types were cultured under standard conditions. The cell lines were obtained from the American Type Culture Collection and maintained in accordance with provided guidelines. The cell lines were tested within 6 months after resuscitation. Supplementary Table S1 provides the full list of cell lines tested and their growth conditions.

Small-molecule inhibitors
The PI3K p110 inhibitor BKM120, dual PI3K/mTOR inhibitor BEZ235, and dual PI3K/mTOR inhibitor BG1226 were obtained from Novartis. The PAK1 inhibitor IPA-3 was purchased from Tocris Bioscience.

Cell viability
We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay to determine cell viability in response to drug treatment. We carried out 5 replicates per concentration tested in serial dilutions with a total of 10 concentrations with a 50% reduction in drug concentration per dilution for each plate. A total of 15 μL of MTT reagent was added to each drug-treated well and incubated for 3 hours at 37°C. Detergent was added to each drug-treated well and incubated overnight at room temperature without exposure to light. Absorbance was measured at 570 nm using a plate reader (Tecan Group). The IC50 concentration was determined using the publicly available ED50 plus v1.0 software program.

Western blot
RIPA Lysis buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 10 mmol/L phenylmethylsulfonfly fluoride, 1 μg/mL aprotinin, and 100 mmol/L sodium orthovanadate) was added to 750,000 cells and incubated on ice for 30 minutes. The mixture was spun down, and the supernatant was transferred to a new tube as the whole-cell extract. A total of 20 μg cell lysate was separated on a 4% to 18% Tris-Bis NuPAGE gel (Invitrogen) and transferred using the iBlot transfer device (Invitrogen) program 3 for 7 minutes. The blots were probed with a 1:100 p-AKT, 1:1,000 total AKT, 1:100 p-GSK3alpha, 1:100 p-GSK3beta, or 1:100 p-s6 (Cell Signaling), or 1:5,000 goat-anti–β-actin (SC-47 778; Santa Cruz Biotechnology) for 1 hour at room temperature. The antibodies were detected by the use of 1:10,000 goat-anti–mouse horseradish peroxidase (HRP)–conjugated antibodies (Santa Cruz Biotechnology). Western Blotting Luminol Reagent (Santa Cruz Biotechnology) was used to visualize the bands corresponding to each antibody.

Confirmation of pathway downregulation
We used the Western blot technique and a time-course experiment to confirm PI3K pathway downregulation with BEZ235. We sought to confirm pathway downregulation in both a dose- and time-dependent manner using Western blot analysis. We treated 1 million BJAB DLBL cells with increasing doses of BEZ235 and, after 6 hours, we looked for a dose-dependent effect at 15, 30, 60, 125, 250, and 500 nmol/L concentrations. We measured cell count and viability for each concentration. For the time-course experiment, we treated BJAB cells with BEZ235 at 30 nmol/L (selected on the basis of its IC50 concentration of 0.0269 μmol/L) and measured cell count at 0, 3, and 6 hours after treatment. We looked for downregulation of phosphorylation of the downstream targets of PI3K inhibition: p-AKT, p-GSK3 alpha and beta, and p-S6 through Western blot analysis.

Gene expression profiling and statistical analysis
Gene expression profiling using Affymetrix Gene 1.0 ST arrays were carried out, scanned, and normalized as previously described (17). These data are available through the Gene Expression Omnibus (GEO). The microarray data will be available through accession number GSE22898.

The gene expression data for 60 cell lines were divided into independent training (n = 29) and validation sets (n = 31). In the cell lines from the training set, we applied the Cox proportional hazards regression model to identify...
genes that were associated with a response to BEZ235. BEZ235 was the first drug that we tested in all the cell lines. The associations between BEZ235 and gene expression served as the starting point for the exploration of the molecular predictors of response. In all cases, the experimentally identified IC_{50}s for each of 3 drugs for the cell lines were considered the response variable.

To identify overlap between genes associated with a response to each of the 3 drugs, the Cox model was applied to the entire drug-response data for all the available cell lines. Genes associated with a response to BEZ235 (n = 60) and BKM120 (n = 60) were deemed significant at P < 0.01 and those associated with BGT226 (n = 31) were deemed significant at P < 0.05. Two-tailed probabilities were used in all analyses involving BEZ235 and 1-tailed probabilities were used with BKM120 and BGT226, with the direction of the effect being indicated by the association with response to BEZ235 in the same cell lines.

The Cox model identified genes that were associated with a response to BEZ235. We identified 881 genes as being associated with a response to the drug (P < 0.05) in the training set of 29 cell lines. We applied hierarchical clustering (18) to these genes in the training set and noted 2 predominant clusters of cases in the training set. These clusters served as the base model for identifying additional cell lines that were sensitive or resistant to BEZ235. We then constructed a Bayesian model using these clusters to predict the sensitivity or resistance to the independent validation set cases treated with BGT226 and BKM120. A posterior probability value of 0.9 or higher was used to signify predicted sensitive cell lines and posterior probability value of 0.1 or lower was used to signify predicted resistant cell lines. These class distinctions were rendered similar to those that we have used to distinguish gene expression-based subgroups previously.

The Wilcoxon-rank test was used to identify differences between IC_{50}s of the predicted sensitive and resistant groups.

**RNA interference**

Lentiviral pGIPZ constructs containing either a scrambled sequence (non-silencing; cat. no. RHS4346; Open Biosystems) as control, or a hairpin sequence targeting the PAK1 gene (cat. no. RHS4430; Open Biosystems) were mixed with Lentiviral packaging mix (cat. no. TLP4691; Open Biosystems) and transfected into TLA-HEK-293T cells using Arrest-in as a transfection reagent. Virus was harvested 72 hours posttransfection and frozen at -80°C until use. DLBCL cell lines were infected with virus in the presence of 8 μg/mL of polybrene, using 1 × 10^6 cells per well in a 24-well plate. The plates containing cells and virus were spun at 700g for 90 minutes at room temperature. Cells were selected by puromycin at 72 hours postinfection and observed to express GFP. Successful knockdown of PAK1 was confirmed by Western blot and quantitative PCR (qPCR). We selected the DLBCL Ly8 cell line for its transfectability, relatively high PAK1 expression, and relatively high (resistant) IC_{50} for BEZ235.

**Synergy testing between small-molecule inhibitors**

We tested the PI3K inhibitor BEZ235 in combination with the PAK1 inhibitor IPA-3 (19) using the previously described CalcuSyn software by applying the Chou–Talalay method (20). We did not anticipate significant single-agent activity, but were interested in assessing whether combination therapy was synergistic and a potential method to overcome resistance to PI3K inhibition. We first explored our hypothesis with the BJAB cell line and subsequently tested 5 cell lines (HBL1, HBL2, HT, Ly8, and Riva) that were predicted to be resistant to PI3K inhibition. We also tested the addition of IPA-3 to BEZ235 in normal tonsil lymphocytes. Ly8 and tonsillar lymphocytes were cultured in 96-well plates (~500,000 cells/mL) for 36 hours in Iscove’s modified Dulbecco’s medium (IMDM) with 20% human AB serum. Viability was assessed with alamar blue dye and normalized to the lowest concentration of BEZ235 in each group.

**Results**

**PI3K inhibition is an effective strategy in a broad range of lymphomas**

We first addressed the question of whether PI3K inhibition is a potentially effective therapeutic approach in lymphomas. We selected 60 cell lines that were predominantly derived from lymphomas and represented the following distinct histologies: Burkitt lymphoma (n = 11), germinal center B–cell-derived diffuse large B-cell lymphoma (n = 19), primary mediastinal B-cell lymphoma (n = 3), activated B-cell-derived diffuse large B-cell lymphoma (n = 6), mantle cell lymphoma (n = 5), Hodgkin lymphoma (n = 3), multiple myeloma (n = 6), acute leukemias (n = 4), and T-cell lymphoma (n = 3).

We selected 3 separate small-molecule inhibitors that targeted the PI3K pathway: BKM120, a selective PI3K inhibitor, and 2 separate dual PI3K/mTOR inhibitors, BEZ235 and BGT226. We began by examining whether these 3 small molecule inhibitors were effective in these diverse cell lines at physiologically achievable concentrations. The complete list of cell lines and their IC_{50}s are shown in Supplementary Table S1.

We tested the PI3K inhibitor (BKM120) and a dual PI3K/mTOR inhibitor (BEZ235) in each of the 60 cell lines chosen in this study. We also tested the effects of a second PI3K/mTOR inhibitor (BGT226) in 31 cell lines. The drug concentrations needed to achieve 50% growth inhibition (IC_{50}) in these cell lines were calculated for each cell line. We observed that the IC_{50} of these drugs ranged from 0.01 to 0.003 to 9 μmol/L for the dual inhibitor BZ235, 0.003 to 0.2 μmol/L for the dual inhibitor BGT226 (n = 31), and 0.2 to 7.8 μmol/L for the selective PI3K inhibitor BKM120. The drug-response for each cell line to BEZ235, BKM120, and BGT226 are shown in log-scale in Fig. 1A–C, respectively. All these drugs were nearly 100% lethal at 48 hours.

We, thus, concluded that these drugs were effective at physiologically achievable concentrations. We noted that the dual PI3K and mTOR inhibitors BEZ235 and BGT226 were more potent than the selective PI3K inhibitor.
BKM120. Within nearly every lymphoma histology, we found that the respective cell lines varied by as much as 10-fold in their sensitivity to these drugs. Generally, the histology of the cell line was not associated with response to the drug; the observed heterogeneity in drug response within a particular histology was generally comparable with that observed between different histologies.

We further examined the role of histology and response to the PI3K inhibitors. We carried out pair-wise cross-histology comparisons between all lymphoma groups using the Wilcoxon-rank test, adjusted for multiple comparisons using the Bonferroni method. We found that the association between histology and response to the inhibitors was similar to that expected by chance alone (adjusted p > 0.05 in all cases).

Next, we confirmed the inhibition of the PI3K pathway by these drugs through separate time-course and dose-escalation experiments and by measuring the expression of key downstream members of the pathway. We tested the drugs at the IC50 concentration in the BJAB lymphoma cell line and tested the effects on phosphorylated AKT (p-AKT), phosphorylated S6 (p-S6), and the phosphorylated forms of the α- and β-isofoms of GSK3 (P-GSK3-α and -β, respectively). As shown in the Western blot in Fig. 1D (left), we observed a dose-dependent downregulation of the known targets of PI3K at 6 hours. We also found a time-dependent downregulation of these targets at 3 and 6 hours, respectively (Fig. 1D, right). Total AKT and actin controls were unchanged. We ensured that there was no associated decrease in cell viability at these early time points by

Figure 1. PI3K inhibition is broadly effective as a therapeutic strategy in lymphomas. A, the dual PI3K/mTOR inhibitor BEZ235 was lethal in all histologies tested with a range of IC50 concentrations of 0.01 to 9 µmol/L. The individual IC50 (in nmol/L) are displayed on Log10 scale for each of the 60 cell lines tested with the color corresponding to the lineage. The cell lines are depicted in the same order as they appear in Supplementary Table S1. The color legend is common to Fig. 1A–C. B, the selective PI3K p110 inhibitor BKM120 was lethal within all the histologies tested in a total of 60 cell lines, with IC50 ranging from 0.2 to 7.8 µmol/L. C, the dual PI3K/mTOR inhibitor BGT226 was lethal within all histologies tested in a total of 31 cell lines, with IC50 ranging from 0.003 to 0.2 µmol/L. D, PI3K inhibition showed a time-dependent and dose-dependent downregulation of the PI3K pathway. Data shown are for the GCB DLBCL cell line BJAB. In the left panel, BJAB was treated with increasing concentrations (in nmol/L) of BEZ235. Western blots were carried out 6 hours after treatment with drug. We observed increasing downregulation of PI3K downstream targets with increasing concentration of drug. In the right panel, BJAB was treated with BEZ235 at 30 nmol/L (based on its IC50 concentration of 26.9 nmol/L) at 3 and 6 hours. Western blots were carried out and we observed increasing downregulation of PI3K downstream targets, including the phosphorylated forms of AKT, GSK3α, GSK3β, and S6 at each successive time point, compared to beta-actin controls.
Figure 2. Gene expression profiles are associated with response to PI3K inhibition. A, hierarchical clustering of the 881 genes associated with a response to BEZ235 in the training set cell lines (n = 29) are shown here. The relative expression of these differentially expressed genes is depicted in a heat map over a 2-fold range, with higher expression shown in red and lower expression in green. This analysis was carried out using the IC50 value for each cell line in response to incubation in BEZ235 for 48 hours. B, left, the Bayesian probabilities of 29 cell lines in the BEZ235 training set are shown for the cell lines predicted to be sensitive or resistant to PI3K inhibition. The cases with a probability of 90% or higher were deemed as "predicted sensitive", while those with a probability of 10% or lower were deemed as "predicted resistant". Right, the IC50s for the predicted resistant and predicted sensitive groups for the 29 cell lines in the BEZ235 training set were significantly different at P < 0.05 (Wilcoxon-rank test). C, left, the Bayesian probabilities of 31 cell lines in the BGT226 validation set are shown for the cell lines predicted to be sensitive or resistant to BEZ235 inhibition. The cases with a probability of 0.9 or higher were deemed as "predicted sensitive", while those with a probability of 0.1 or lower were deemed as "predicted resistant". The analysis was carried out using the IC50 value for each cell line in response to incubation with BGT226 for 48 hours. Right, the IC50s for the predicted resistant and predicted sensitive groups using a 0.9/0.1 cut-off to identify the predicted sensitive and resistant cell lines among the 31 cell lines in the BGT226 validation set were significantly different at P < 0.05 (Wilcoxon-rank test). D, we also separately tested the 0.5 probability cut-off to identify predicted sensitive (probability > 0.5) or predicted resistant cell lines (probability < 0.5). The IC50s for the predicted resistant and predicted sensitive groups among the 31 cell lines in the BGT226 validation set were significantly different at P < 0.05 (Wilcoxon-rank test).
quantifying the proportion of viable cells at these time points using trypan blue staining (data not shown). These data are similar to those reported previously and confirm that these drugs directly inhibit the PI3K pathway.

Gene expression profiles associated with a response to PI3K/mTOR inhibition

Given the lack of correlation between histology and response to PI3K inhibition, we examined the potential association of gene expression profiles across all the different histologies that comprise these cell lines and response to these small-molecule inhibitors.

We carried out gene expression profiling on all 60 cell lines and divided these cell lines randomly into a training set (n = 29) and a validation set (N = 31). All the cell lines tested with the BGT226 drug (n = 31) were assigned to the validation set. Similar to that described previously (21), we applied the Cox proportional hazards model to identify genes associated with drug IC_{50}s (i.e., the response variable). We identified 881 genes that were associated with a response to BEZ235 in the training set (P < 0.05), which were associated with either relative sensitivity (i.e., lower IC_{50}s) or resistance (i.e., higher IC_{50}s).

We carried out hierarchical clustering on these 881 genes for all the cases in the training set to group these cell lines on the basis of shared patterns of gene expression. The cell lines clustered into 2 main clusters (Fig. 2A) that were predicted to be sensitive or resistant to BEZ235, the combined PI3K/mTOR inhibitor. We then generated a Bayesian predictor using these 881 genes to identify cases in the training and validation sets that most closely resembled these clusters. For each case, the Bayesian model generated a probability that estimated the similarity of these cases with that of the original clusters.

In the training set, we deemed those cases with a Bayesian probability of 0.9 or higher as those predicted to be sensitive and those with a Bayesian probability of 0.1 or lower as those predicted to be resistant to PI3K inhibition (Fig. 2B, C).

Figure 3. PAK1 is associated with resistance to PI3K inhibition. A, the PAK1 gene was well expressed, but variable among different cell lines and histologies. The cell lines and histologies are shown in the same order as Fig. 1A. B, left, higher PAK1 expression was associated with poorer response (i.e., higher IC_{50}) to all 3 drugs tested. The scatter-plot depicts the association of PAK1 expression with response to BEZ235, with a fitted regression line (r^2 = 0.5041, P < 0.001). Similar trends were noted with the association of PAK1 and response to BKM120 (P = 0.009) and BGT226 (P = 0.03). Right, gene set enrichment analysis showed that the PAK pathway was significantly upregulated in the predicted resistant group of cell lines. The core group of enriched genes is depicted from the output of the program, and the genes in the pathway were noted to be highly significant (P < 0.001). C, PAK1 expression was tested in primary human DLBCL cases using gene expression and Western blotting. Gene expression is shown in the top image with red representing relatively high PAK1 expression and green representing relatively low PAK1 expression. The Western blot in the lower image shows protein expression for the same samples. There was excellent correlation between gene and protein expression.
Figure 4. Resistance to PI3K inhibition can be overcome by inhibiting PAK1. A, left, RNA interference using 3 separate PAK1 constructs in the Ly8 DLBCL cell line led to successful knockdown of PAK1 protein expression in all 3 shRNA constructs by Western blot analysis. β-actin loading controls were carried out in each experiment. Middle, an example of the MTT assay is shown for the cell line expressing shRNA construct #3 directed against PAK1 that was treated with 3 µmol/L of BEZ235. The proportion of viable cells at each concentration of the drug is shown for both the wild-type cells (shown as the black line) and the cells with reduced PAK1 expression (shown as the red line). Right, the bar graph summarizes the results from MTT assays from each of the 3 PAK1 knockdown constructs that were treated with BEZ235. In all 3 PAK1 knockdown constructs, there was restoration of sensitivity to PI3K inhibition with at least a 3-fold reduction in IC50.
left). We found that, in the training set cases that were treated with BEZ235 \( (n = 29) \), the cases that were predicted to be either sensitive or resistant to PI3K inhibition differed in their IC\(_{50}\) by more than 2-fold (Fig. 2B, right, \( P < 0.01 \)). We then noted similar results \( (P = 0.03) \) in the independent validation set cell lines \( (n = 31) \) that were treated with BGT226, another drug from the same class (Fig. 2C). We also found a similar trend in the validation set cell lines treated with BEZ235 with a more than 2-fold difference between the IC\(_{50}\) between the predicted sensitive and resistant groups \( (P = 0.14) \). We further examined the effect of altering the Bayesian probability for identifying the predicted sensitive and resistant cases. We alternatively identified those cases with a Bayesian probability of greater or less than 0.5 as predicted sensitive and resistant cases respectively. We found that the difference between the 2 groups remained significant \( (P = 0.01) \), with a more than 2-fold difference of IC\(_{50}\) (Fig. 2D) in the validation set cases \( (n = 31) \) treated with BGT226. We found that the genes associated with a response to the dual PI3K/mTOR inhibitors were not significantly associated with a response to the selective PI3K inhibitor, suggesting that the additional inhibition of mTOR might affect different mechanisms in these cell lines.

We also examined the potential role of PTEN mutations in the response to PI3K inhibition in 21 DLBCL cell lines and found all of those cell lines to be wild type. These data suggest that PTEN mutations are not a significant mediator of resistance to PI3K inhibition in these lymphomas.

These results suggest that the association of gene expression and drug response produced robust results for the combined PI3K/mTOR inhibitors.

**PAK1 expression is associated with resistance to PI3K inhibition**

To better understand the genes associated with resistance to PI3K inhibition, we identified genes associated with resistance to treatment with all 3 drugs. We identified all genes that were associated with resistance to BEZ235 \( (n = 60, P < 0.01) \), BKM120 \( (n = 60, P < 0.01) \), and BGT226 \( (n = 31, P < 0.05) \). We found that PAK1 was 1 of only 2 genes to meet these stringent criteria, and the only gene previously associated with the PI3K pathway, suggesting that the additional inhibition of mTOR might affect different mechanisms in these cell lines.

We then tested these 3 engineered cell lines with lower PAK1 expression and compared their sensitivity to PI3K inhibition with the wild-type cell line. We found that reduced PAK1 expression resulted in increased sensitivity to PI3K inhibition with a significantly increased sensitivity to the effects of the drug \( (P < 0.01, \text{Fig. 4A, middle}) \). These results were repeated with all drugs alone. Bottom, the same experiment was conducted in Ly8 (the cell line used to create the PAK1 knockdown constructs) and showed that the combination of PI3K and PAK1 inhibition (shown in red on the left) was more effective than either agent alone (BEZ235 and IPA-3). The combination of PI3K and PAK1 inhibition (shown in red on the left) was more effective than either agent alone (BEZ235 and IPA-3) at a fixed concentration of 1:10 BEZ235:IPA-3 diluted in log\(_{2}\), fashion are shown. The combination of the drugs is much more effective than either drug alone. Bottom, the same experiment was conducted in Ly8 (the cell line used to create the PAK1 knockdown constructs) and showed that the combination of PI3K and PAK1 inhibition (shown in red on the left) was more effective than either agent alone (BEZ235 and IPA-3). The combination of PI3K and PAK1 inhibition was tested at fixed ratio dilutions of 3:5 BEZ235:IPA-3 in Ly8. C, 5 DLBCL cell lines that were predicted to be resistant to PI3K inhibition were treated with a combination of PI3K inhibition and PAK1 inhibition. In all of the 5 cell lines, we noted synergy indicated by synergy combination index less than 1 using the Chou-Talalay method. This enrichment was highly significant \( (P < 0.001) \). However, when PAK1 was knocked-down using shRNAs, the observed synergism between PI3K inhibitors and IPA-3 either diminished or disappeared for 2 of the 3 inhibitors tested (C, right). These data suggest that IPA-3 effectively inhibits PAK1 and that the observed synergism is dependent on PAK1 expression.
3 cell lines expressing the PAK1 shRNA and lower levels of PAK1. We found that these cell lines were, on average, 3.5-fold more sensitive to the effects of PI3K inhibition (Fig. 4A, right), a difference that was statistically significant ($P < 0.01$).

We further tested the hypothesis that PAK1 is a major mediator of resistance to PI3K inhibition using small-molecule inhibition of PAK1. We selected a small-molecule inhibitor, IPA-3, that has previously been shown to selectively inhibit PAK1 (19). We tested the effects of this PAK1 inhibitor in 9 different lymphoma cell lines, both individually and in combination with BEZ235.

As a single agent, the PAK1 inhibitor had limited effectiveness with observed IC$_{50}$ ranging from 0.697 $\mu$mol/L to no cell death at 10 $\mu$mol/L. However, even in cell lines that were relatively resistant to the effects of the PAK1 inhibitor as a single agent, we observed that PAK1 inhibition conferred increased sensitivity to BEZ235. We first explored the potential role of combined PI3K and PAK1 inhibition in the BJAB cell line. Fig. 4B (top) shows the combination of both drugs (red curve) is more effective than either drug alone (black curve = BEZ235, blue curve = IPA-3). Identical effects were observed in the cell line Ly8, with the combination of the 2 drugs (red curve) found to be much more effective than either drug alone (Fig. 4B, bottom). In both sets of experiments, IPA-3 had minimal activity as a single agent at therapeutically achievable concentrations.

We tested the combination of BEZ235 and the PAK1 inhibitor in 5 different cell lines that were predicted to be resistant to BEZ235 (HBL1, HBL2, HT, Ly8, and Riva). We found that the inhibition of PAK1 greatly potentiated the effects of PI3K inhibition. We used the Chou–Faltyt method to identify synergy between the 2 agents in these 5 cell lines. We found that all 5 of the cell lines showed synergy in cell killing between the PI3K and the PAK1 inhibitors ($P < 0.01$, Fisher exact test), with a synergy combination index ranging from 0.3 to 0.86 (Fig. 4C, left). We further tested the combination of the 3 different PI3K inhibitors and IPA-3 in Ly8 cells with and without PAK1 knockdown. We found that, in the setting of shRNA-mediated PAK1 knockdown, there was substantial decrease in synergy between each of the three drugs which were all synergistic with IPA-3 in the cells with preserved (wild type) PAK1 expression (Fig. 4C, right). These data suggest that the observed synergism between the 2 drugs depends on higher PAK1 expression and that a combination of PI3K inhibition and PAK1 inhibition might provide a means to overcome resistance to PI3K inhibition.

Potential therapeutic window between malignant and normal cells

We further tested the effects of BEZ235, IPA-3, and the combination of the 2 drugs in normal B cells to determine whether a therapeutic window between malignant and normal cells existed. We found that both drugs individually and in combination were selectively toxic in the B-cell lymphoma cell line Ly8 compared with normal B cells (Supplementary Fig. S1).

Discussion

In this study, we showed that the PI3K pathway inhibition is broadly lethal in lymphomas of all histologies at physiologically achievable concentrations of 3 different novel small-molecule inhibitors (BEZ235, BKM120, and BGT226). However, we observed considerable variability in response to the same drug even within individual lymphoma histologies, suggesting that histology, in itself, is not the primary determinant of response to PI3K inhibition. These observations led us to further explore the molecular mechanisms underlying the variability in response using gene expression profiling across all these histologies.

In correlating gene expression profiles with response to these inhibitors, we found that the gene expression profiles associated with responsiveness to the 2 dual PI3K/mTOR inhibitors overlapped and response to 1 drug generally predicted response to the other. We also found that the dual PI3K/mTOR inhibitors were more potent than the selective PI3K inhibitor. The genes associated with a response to PI3K inhibition alone had a lower degree of overlap with the dual inhibitors. These observations could arise due to differing affinities for different PI3K targets for these drugs or from the additional effects of mTOR inhibition in the 2 dual inhibitors.

The common thread that united the response to these 3 different inhibitors was the association of PAK1 gene expression with relative resistance to PI3K inhibition. $PAK1$ is a gene that regulates diverse cellular functions related to signaling and promotion of cell survival (25, 27). Early data suggest that the substrate of PI3K,PIP$_2$, can directly activate PAK1 and, thus, potentially bypass PI3K inhibitors (28) and provide a potential mechanism of resistance. PAK1 is associated with poor prognosis in a number of different solid tumors, with higher PAK1 expression linked to poor outcome including shortened progression free and overall survival (24, 29). In bladder and breast cancers, high PAK1 expression has been associated with lymph node metastases and tamoxifen resistance, respectively (24, 30).

The importance of PAK1 in resistance to PI3K inhibition was confirmed by our RNA-interference experiments, which showed that knock-down of PAK1 led to a significant improvement in responsiveness to PI3K inhibition. These experiments produced congruent results with those obtained by small-molecule inhibition of PAK1. Our work shows significant synergy between PI3K and the PAK1 inhibition, suggesting a role for PAK1 inhibition in combination with PI3K inhibition as a way to overcome resistance to PI3K.

There are currently few known markers of response to PI3K inhibition. Our data suggest that PAK1 gene expression could serve as a potential marker for response. This association serves as a testable hypothesis as PI3K inhibitors enter clinical trials. Taken together, our data provide a rationale for studying the clinical role of PI3K inhibition in a wide range of lymphomas.
PI3K Inhibition in Lymphoma

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

Authors’ Contributions
Conception and design: K.J. Walsh, S.S. Dave
Development of methodology: Q. Liu, S.S. Dave
Acquisition of data: provided animals and managed patients, provided facilities, etc.: K.J. Walsh, M.S. McKinney, C. Love, A. Fan, A. Patel, J.L. Smith
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