Cancer Therapy: Preclinical

Pan-PIM Kinase Inhibition Provides a Novel Therapy for Treating Hematologic Cancers

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Abstract

Purpose: PIM kinases have been shown to act as oncogenes in mice, with each family member being able to drive progression of hematologic cancers. Consistent with this, we found that PIMs are highly expressed in human hematologic cancers and show that each isoform has a distinct expression pattern among disease subtypes. This suggests that inhibitors of all three PIMs would be effective in treating multiple hematologic malignancies.

Experimental Design: Pan-PIM inhibitors have proven difficult to develop because PIM2 has a low 
\( K_m \) for ATP and, thus, requires a very potent inhibitor to effectively block the kinase activity at the ATP levels in cells. We developed a potent and specific pan-PIM inhibitor, LGB321, which is active on PIM2 in the cellular context.

Results: LGB321 is active on PIM2-dependent multiple myeloma cell lines, where it inhibits proliferation, mTOR-C1 signaling and phosphorylation of BAD. Broad cancer cell line profiling of LGB321 demonstrates limited activity in cell lines derived from solid tumors. In contrast, significant activity in cell lines derived from diverse hematological lineages was observed, including acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), multiple myeloma and non-Hodgkin lymphoma (NHL). Furthermore, we demonstrate LGB321 activity in the KG-1 AML xenograft model, in which modulation of pharmacodynamics markers is predictive of efficacy. Finally, we demonstrate that LGB321 synergizes with cytarabine in this model.

Conclusions: We have developed a potent and selective pan-PIM inhibitor with single-agent antiproliferative activity and show that it synergizes with cytarabine in an AML xenograft model. Our results strongly support the development of Pan-PIM inhibitors to treat hematologic malignancies.

Introduction

As a group, hematologic cancers are the fourth most common cancer type in the United States (1). In 2012, an estimated 70,130 cases of non-Hodgkin lymphoma (NHL), 21,700 cases of multiple myeloma, 16,060 cases of chronic lymphocytic leukemia (CLL), and 13,780 cases of acute myelogenous leukemia (AML) were diagnosed (1). In spite of considerable advances with novel therapeutics, including monoclonal antibodies, stem cell transplantation, and targeted therapies, the number of patients succumbing to these diseases remains high, with more than 45,000 deaths estimated in 2012 (1).

In both hematologic cancers and solid tumors, therapies that target cancer drivers have been shown to be clinically beneficial. With this in mind, we identified the PIM family of protooncogenes as a suitable target given that they are highly expressed in hematologic malignancies. Although gene expression alone is not always a fair predictor of cancer relevance, PIM kinases display a few unique features. Once properly folded, PIM kinases require no further posttranslational modifications for their activity (2); indeed, the...
crystal structure of PIM1 supports a model in which the unphosphorylated protein adopts an active conformation (3). In addition to transcriptional regulation, PIM kinases are phosphorylated and dephosphorylated to regulate their stability (2). Thus, the cellular activity of each PIM kinase is primarily regulated by the balance of synthesis and degradation of the protein itself (2), suggesting that expression alone may be a useful predictor of where PIM kinases are active.

The PIM kinases have roles in cell-cycle progression, cell survival, and tumorigenesis (4). PIM1 was originally discovered as an oncogene by frequent proximal proviral insertions of murine leukemia virus in lymphomas of infected mice (5, 6). Similar unbiased insertional mutagenesis screens were used to demonstrate the oncogenic potential of PIM2 (7) and PIM3 (8) in PIM1 and PIM1/2 knock-out mice, respectively, suggesting that PIMs may have functional similarities in oncogenesis. These studies also demonstrated that PIM kinases in conjunction with c-Myc result in synergistic induction of cancer (9).

Here, we describe LGB321, a potent and selective ATP-competitive small-molecule inhibitor of all three PIM kinases (Pan-PIM kinase inhibitor). LGB321 is unique relative to previously described PIM inhibitors (10–13), in that it is active in PIM2-dependent cell lines (14), a kinase that has proven difficult to inhibit in the cellular context. Consistent with its activity on all three PIM kinases, LGB321 inhibits proliferation of a number of cell lines derived from diverse hematologic malignancies, including multiple myeloma, acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), and non-Hodgkin lymphoma (NHL). This finding correlated with higher levels of PIM kinases expression in these cancers, as compared with solid tumor cancers. To our knowledge, LGB321 is the first Pan-PIM inhibitor with activity on PIM2-dependent multiple myeloma cells. LGB321 was effective in inhibiting tumor growth in an AML xenograft model, in addition to a previously reported multiple myeloma model. Our findings suggest that single-agent activity for Pan-PIM inhibitors is likely to be observed in hematologic malignancies. On the basis of these findings, we initiated early clinical testing of our development candidate in multiple myeloma, emphasizing the translational relevance of the present work.

Translational Relevance
Here, we described detailed biochemical, cellular, and pharmacologic properties of a highly potent and selective inhibitor (LGB321) of the three PIM kinases. When tested in more than 500 cancer cell lines of diverse origins, we demonstrated almost exclusive antiproliferative activity in cells from hematologic lineages, including multiple myeloma, acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), and non-Hodgkin lymphoma (NHL). This finding correlated with higher levels of PIM kinases expression in these cancers, as compared with solid tumor cancers. To our knowledge, LGB321 is the first Pan-PIM inhibitor with activity on PIM2-dependent multiple myeloma cells. LGB321 was effective in inhibiting tumor growth in an AML xenograft model, in addition to a previously reported multiple myeloma model. Our findings suggest that single-agent activity for Pan-PIM inhibitors is likely to be observed in hematologic malignancies. On the basis of these findings, we initiated early clinical testing of our development candidate in multiple myeloma, emphasizing the translational relevance of the present work.

in mice. Taken together, these findings establish PIM kinases as an attractive pharmacologic target in cancer therapy.

Materials and Methods
Sources of gene expression data
All gene expression microarray datasets were retrieved from public depositories, including Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov GEO/) and ArrayExpress (http://www.ebi.ac.uk/arrayexpress/). Only human primary tumor or normal tissue samples that were hybridized on Affymetrix human genome U133 Plus 2.0 arrays and were obtained from patients that did not receive any treatment before tissue resection were selected. After further removing redundant or poor-quality samples, 18,960 samples across 40 tissue types were processed for data analysis.

Microarray data processing and analysis
To estimate expression level of each gene, microarray raw CEL files were processed using the Michigan custom CDF (HGU133Plus2_Hs_ENTREZC, version 14; ref. 15) with an extended reference-based RMA summarization method (16). Fourteen-hundred samples from the entire data set were randomly selected to estimate parameters of the RMA fit, which were then applied directly to the remaining microarray samples to produce gene-level expressions. After data processing, expression levels of PIM1, PIM2, and PIM3 were extracted for the samples described in Fig. 1 and Supplementary Fig. S1 and then visualized by boxplots in R (http://www.R-project.org).

Biochemical assays for PIM kinases and determination of LGB32I inhibition constants
PIM1 (Invitrogen; PV3503), PIM2 (Invitrogen; PV3649), and PIM3 (Novartis) enzyme reactions were run in 50 mmol/L Hepes, pH 7.5, 5 mmol/L MgCl2, 0.05% bovine serum albumin (BSA), 1 mmol/L dithiothreitol buffer with a biotinylated BAD peptide (b-RSRHSSYPAGT_NH2) and ATP substrates. Reaction products were measured using the PerkinElmer AlphaScreen IgG Detection Kit (protein A) with anti-phospho-(Ser/Thr) AKT substrate antibody from Cell Signaling Technology. Apparent ATP \( K_m \) calculations were performed with Prism 5 (GraphPad Software Inc.) using the Michaelis–Menten equation. \( K_i \) measurements were performed at high ATP concentrations. For PIM1, the ATP concentration was 2,800 \( \mu \)mol/L, for PIM2 it was 50 \( \mu \)mol/L, and for PIM3 it was 2,500 \( \mu \)mol/L. The apparent \( K_i \) from these measurements was calculated using the Morrison equation and converted to a true \( K_i \) using the Cheng–Prusoff relationship for an ATP site–competitive mechanism. Active site enzyme titrations were performed on all three enzyme isoforms for use in the Morrison equation.

Morrison equation:
\[
V_a/V_o = 1 - \left( \frac{E + I + K_{app}}{\sqrt{\text{sqr}(E + I + K_{app})}} \right) (4 \times E \times I)/(2 \times E)
\]
Cheng–Prusoff equation:
\[
K_{app} = K_i \times (1 + S/K_m)
\]
Figure 1. mRNA expression levels in cancer tissues. PIM1, PIM2, and PIM3 expression in patient samples from the major hematologic cancers subtypes [ALL, N = 350; AML, N = 2,049; DLBCL-NHL, N = 640; and multiple myeloma (MM), N = 982] as compared with the expression in normal bone marrow (N = 81). For comparison purpose, a few representative solid tumors are included: breast (N = 2,422), liver (N = 132), lung (N = 973), pancreas (N = 128), prostate (N = 193), and stomach (N = 101) cancers. Expression intensity was determined as described in Materials and Methods. The median expression of each gene is represented by the black center line within each box, and the first and third quartiles are depicted by the edges of the box. The whiskers extending from each box indicate expression values that are within 1.5 times the interquartile range (IQR) from the upper or lower quartile. Outliers that are at a distance of greater than 1.5 × IQR from the box are plotted individually as plus signs.
Biochemical kinase specificity profile

The kinase specificity profile for LGB321 was determined as previously described (17). Briefly, protein kinase activity was assayed using either the LanthaScreen (www.invitrogen.com) or the Caliper (www.caliperls.com) technologies. The biochemical activity for lipid kinases PI3Kα (phosphoinositide 3-kinase α), PI3Kβ, Vps34, and PI4Kβ was determined by a luminescence assay based on ATP consumption (KinaseGlo; www.promega.com) with phosphoinositol as the substrate, whereas PI3Kγ, PI3Kδ was determined by the Adapta TR-FRET technology (www.invitrogen.com). For determination of the biochemical activity of recombinant mTOR an antibody-dependent TR-FRET assay was used with 4EBP1 as substrate as well as an "ATP-binding assay," which measures the occupancy of compounds in the ATP site of mTOR.

Cell lines and reagents

The KMS-11.luc human multiple myeloma tumor cell line, a KMS-11 clone expressing firefly luciferase, was obtained from the University Health Network (UHN; Toronto, Ontario, Canada). KMS-11.luc and OPM-2 (DSMZ, Germany) were cultured in RPMI-1640 (American Type Culture Collection, ATCC), supplemented with 10% FBS. AML cell lines KG-1 (ATCC) and MOLM-16 (DSMZ, Germany) were cultured in Iscove’s modified Dulbecco’s medium (IMDM; ATCC) or RPMI-1640 (ATCC), respectively, supplemented with 20% FBS. The AML cell line P31/FUJ (Health Sciences Research Resources Bank, Japan) was cultured in RPMI-1640 (ATCC) media supplemented with 10% FBS. All cell culture media for in vitro work was additionally supplemented with 20 mmol/L glutamine, 1,000 IU/mL penicillin and 1,000 μg/mL streptomycin. The origin and in vitro methods for the 947 independent cancer cell lines in Cancer Cell Line Encyclopaedia (CCLE) has been previously reported (18). Before implantation in vivo, KMS-11.luc and KG-1 cells were cultured in Dulbecco’s Modified Eagle Medium (Corning) plus 10% FBS and 1% t-glutamine (Corning).

Cellular proliferation assays

To assess the effect of LGB321 on proliferation, cells were seeded in 96-well tissue culture plates followed by addition of compound that had been serially diluted to achieve a final concentration range of 10 μmol/L to 2 nmol/L in 0.1% dimethyl sulfoxide (DMSO). After addition of LGB321 to cell cultures, assay plates were returned to a humidified CO2 incubator (37°C; 5% CO2) for 3 days. To determine KMS-11.luc cell growth, 100 μL per well of reconstituted CellTiter-Glo (PerkinElmer) plate-shaker for 10 minutes at 400 to 600 rpm. Plates were then read on either a Microbeta Trilux (PerkinElmer) or SpectroMax L (Molecular Devices) luminometer. Cell growth was determined by comparing assay signals of LGB321 treated cells with the control conditions of untreated cells (defining 0% growth inhibition) or cells treated with Ki-1, a potent nonspecific cytotoxic kinase inhibitor (defining 100% growth inhibition). The activity of LGB321 was also tested in the CCLE screen (18) and further testing was performed on an expanded panel of hematologic cell lines. Cell lines were obtained from commercial sources (ATCC or DSMZ) and were cultured in RPMI or IMDM plus 10% to 20% FBS (Invitrogen) as supplier recommended. All cell lines were thawed from frozen stock, grown at 37°C, 5% CO2, 95% relative humidity and cultured in 175 flasks using standard culture techniques. They were expanded for at least two passages before being added in assay microtiter plates. Cell count was measured using a CASY Model TT counter (Roche Applied Science). All cell lines were tested for and shown to be free of mycoplasma using PCR detection. In addition, cell line identity was verified by single-nucleotide polymorphism genotyping. Cell lines were dispensed into 384-well plates (Greiner Bio-One; #781098) with a final volume of 25 μL and concentrations ranging from 250 to 4,000 cells per well in duplicate plates. Cell viability was assessed 3 hours after seeding in the first plate (start value) and 120 hours after seeding in the second plate by adding 25 μL Cell Titer-Glo (Promega; #G7571) per well. The doubling time was calculated for each condition and the optimal seeding density leading to the shortest doubling time was selected for further profiling. Three hours after seeding, compounds were transferred to the cells by delivering 5 μL per well of the intermediate dilution using the Velocity-Bravo Automated Liquid Handling Platform (Agilent). This resulted in a final compound concentration range of 10 to 0.01 μmol/L in a final volume of 25 μL and a final DMSO concentration of 1%. The cell–compound mixture was incubated for 120 hours. Cell Titer-Glo was added and luminescence was read on a Magellan plate reader (TECAN). On all plates, wells containing vehicle only were included. Cell lines were seeded in parallel into a 384-well plate (Corning; #732-5528) in the same conditions and Cell Titer-Glo was added 3 hours after seeding to evaluate cell viability before treatment (start value). Start values were subtracted and raw values were percentage normalized on a plate-by-plate basis such that the median of the neutral control wells (i.e., DMSO) is 100% growth and the D0 wells (optical density measured at seeding time) is the 0% growth (100% inhibition). The crossing point defines the concentration at which growth is inhibited by 50% and is reported as GI50 in this report.

Phosphoprotein assays

Commercial electrochemiluminescence (ECL) assay kits from Meso Scale Discovery (MSD) were used to quantify the effects of LGB321 on the levels of phosphorylated S6RP and BAD in KMS-11.luc cells.

For in vitro assays, KMS-11.luc cells were seeded in 96-well tissue culture plates, and then LGB321 was serially diluted and added to cell plates to achieve a final concentration range of 10 μmol/L to 2 nmol/L in 0.1% DMSO and incubated with cells for 1 hour at 37°C. Cells were pelleted by centrifugation for 7 minutes at 1,500 rpm, media was gently aspirated, and MSD lysis buffer added. Cells were lysed by placing plates on a DELFIA (PerkinElmer) plate
shaker at 4°C and shaking the plates for 30 minutes at 600 rpm.

For in vivo assays, MDS lysis buffer (MSD) was added to frozen pulverized tumor samples on ice and homogenates were prepared using the Magna Lyser bead instrument (Roche Applied Science) by disrupting the samples with four cycles of 6,000 rpm for 30 seconds at 4°C. Supernatants were created following centrifugation at 11,000 rpm for 15 minutes at 4°C, and protein concentration determined using the BCA Protein Assay Kit according to the manufacturer’s instructions (Pierce Chemical Company). For both assays, samples were transferred to ECL assay plates previously blocked with 3% BSA, sealed, and incubated at 4°C overnight while undergoing gentle shaking on a DELFIA (PerkinElmer) plate shaker. After overnight incubation, assay plates were processed according to the manufacturer’s instructions.

LGB321 in vivo studies

All studies were done in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited animal facility and in compliance with the ILAR Guide for the Care and Use of Laboratory Animals. Scid/bg female mice (10–12-week-old mice weighing about 20 g each; Charles River Laboratories) were housed up to 5 animals per cage in clear polycarbonate microisolator cages with a 12-hour light, 12-hour dark cycle at temperatures between 70°F to 80°F, and 30% to 70% relative humidity. Food (Purina rodent chow pellets) and water were provided ad libitum.

For subcutaneous tumor models, cells were harvested at 80% to 90% confluency, washed, and resuspended in cold Dulbecco’s Phosphate-Buffered Saline (without Ca²⁺ or Mg²⁺; CellGro) at a concentration of 5 × 10⁶ cells/mL, mixed with an equal volume of Matrigel (Becton-Dickinson) and then 0.2 mL (5 × 10⁶ cells) was implanted subcutaneously into the right flank of female Scid/bg mice. Tumor volume was measured in two dimensions using digital calipers and calculated as (length × width²) × π/6.

For pharmacodynamic and pharmacokinetic studies, animals were enrolled on study when mean tumor volume reached 250 to 400 mm³. For efficacy studies, animals were randomized into groups when tumor volume reached 200 to 250 mm³. Tumor volume and body weights were captured and stored by StudyDirector software (StudyLog).

LGB321 was formulated for oral administration in 50 mmol/L acetate buffer, pH4. The concentration of LGB321 in plasma was determined following extraction in acetonitrile using liquid chromatography and tandem mass spectroscopy. Cytarabine (Hospira) was diluted in bacteriostatic water and prepared fresh daily.

Statistical analysis
For in vivo studies, statistical significance of differences in tumor volume was determined by a one-way ANOVA, and pair-wise comparisons were made by the uncorrected Fisher LSD posttest (GraphPad Prism software).

Results

Comprehensive analysis of PIM kinases mRNA Expression

Elevated expression of PIM kinases and their role in cancer progression have been extensively reported in the literature (4). However, a comprehensive evaluation of their expression in a large number of samples across normal and cancer tissue types has not been reported. Given that PIMs are constitutively active kinases (3), we reasoned that high mRNA expression could be used to identify cancers in which PIM kinases are active. We evaluated datasets derived from Affymetrix human genome U133 arrays from public repositories for PIM kinases expression. All primary data were normalized to allow direct comparison of expression across all tissues (Materials and Methods). Expression of PIM1, PIM2, and PIM3 was examined in normal and malignant samples derived from 17 different tissue types (Supplementary Fig. S1). Consistent with their known roles in cytokine signaling in hematopoiesis (2), all three PIMs are expressed at higher levels in hematologic samples as compared with other tissues (Fig. 1 and Supplementary. Fig. S1). Increased expression of PIMs in most cancer types over their normal tissues counterpart is rather modest on average, suggesting that PIM kinases expression is primarily hematologic lineage-specific.

Interestingly, across hematologic malignancies various PIM isoforms are expressed at higher levels in acute lymphoblastic leukemia (ALL), AML, multiple myeloma, or DLBCL-NHL samples, relative to samples of liver, lung, pancreas, prostate, and stomach cancers (Fig. 1). PIM2 expression in multiple myeloma seems to be significantly higher than all other tissues examined, including normal bone marrow, whereas PIM1 expression is higher in AML, ALL, and DLBCL than in multiple myeloma (Fig. 1). In addition to the functional similarities as oncogenes (7, 8), this observation suggested that in order for a small-molecule inhibitor to have broad clinical utility the effective inhibition of all three PIM kinases (Pan-PIM) would be required. Because we had observed that the multiple myeloma KMS-11.luc cells are dependent on PIM2 for proliferation (14) and because PIM2 inhibition is the most challenging in the cellular context (as described in the next section), we chose this cell line to drive the development of potent Pan-PIM inhibitors in cells.

Biochemical activity and selectivity of the Pan-PIM inhibitor LGB321

We reasoned that an ATP-competitive inhibitor was most likely to inhibit all three PIM kinases, because the ATP-binding pocket is highly conserved among the three PIM kinases and has some unique features relative to the ATP pocket for other kinases (3). The development of a cell-active ATP-competitive inhibitor for PIM2 was particularly challenging, given its low ATP Kᵦ (4 μmol/L; Fig. 2B) as compared with the Kᵦ for PIM1 or PIM3 (400 and 40 μmol/L, respectively; Fig. 2B). Thus, the desired inhibitor needed to be potent enough to compete with the large
Pan-PIM Kinase Inhibition in Hematologic Cancers

Figure 2. LGB321 structure and biochemical properties. A, chemical structure of LGB321. B, summary of the ATP Kᵢ and LGB321 inhibition constant (Kᵢ) determined as described in Materials and Methods.

excess of cellular ATP (in the range of 1–10 mmol/L; ref. 19), about 250- to 2,500-fold higher than the Kᵢ for ATP of PIM2. We developed a series of highly potent and selective inhibitors of all three PIM kinases (20) and selected LGB321 (Fig. 2A) as a tool compound with such characteristics. The inhibition constant (Kᵢ) for LGB321 was determined to be in the single-digit picomolar (pmol/L) range for each of the three PIM kinases (Fig. 2B). Several groups have developed inhibitors to the PIM kinases in both academic and industry settings (10–13); however, to our knowledge LGB321 is the most potent compound so far described. When directly compared with two other PIM inhibitors tested in clinical trials, it is clearly more potent (Supplementary Fig. S2; ref. 20).

The selectivity of LGB321 was first determined in biochemical assays of a panel of seven lipid kinases and 68 diverse protein kinases that included PIM2 (Table 1). In this panel, only PIM2 was significantly inhibited by LGB321 with an IC₅₀ of <0.003 μmol/L, the lowest sensitivity range for the assay. Although biochemical potency in the range of 4 to 10 μmol/L was demonstrated against eight other kinases in this assay, these IC₅₀ represent a greater than 10⁷-fold differential relative to the Kᵢ for all three PIM kinases. The biochemical IC₅₀ for all other kinases tested in this panel was >10 μmol/L (Table 1). We further evaluated the selectivity of LGB321 using the KINOMESCAN-binding displacement assay (Supplementary. Fig. S3; ref. 20). The results demonstrate that LGB321 has a high selectivity score with activity against only two other kinases (EGFR and ERK8) in this assay, in addition to the PIM kinases (Supplementary Fig. S3; ref. 20).

Table 1. LGB321 Biochemical specificity profile

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC₅₀ (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIM2</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>GSK3β</td>
<td>4.4</td>
</tr>
<tr>
<td>PKN1</td>
<td>4.4</td>
</tr>
<tr>
<td>PKA</td>
<td>6.7</td>
</tr>
<tr>
<td>S6K</td>
<td>6.8</td>
</tr>
<tr>
<td>PKCγ</td>
<td>7.0</td>
</tr>
<tr>
<td>PKN2</td>
<td>7.2</td>
</tr>
<tr>
<td>PKCγ</td>
<td>7.7</td>
</tr>
<tr>
<td>ROCK2</td>
<td>9.4</td>
</tr>
</tbody>
</table>

NOTE: Kinases with IC₅₀ > 10 μmol/L: cABL(T315I), cABL, ALK, AURORA-A, BTK, CDK1B, CDK2A, CDK4D1, CK1, COT1, CSK, CalM2, ERK2, EPHA4, EPHA4, FAK, FGFR1, FGFR2, FGFR3, FGFR4, FGFR2(650E), FLT3, FYN, HCK, HER1, HER2, HER4, IGF1R, INS1R, IRAK4, JAK1, JAK2, JAK3, JNK2, JNK3, KDR, cKIT, LCK, LYN, cMET, MK2, MK5, MNK1, MNK2, PAK2, PDGFRα, PDK1, PI3Kα, PI3Kβ, PI3Kδ, PI3Kγ, PI4Kα, PI4Kβ, PI4Kδ, PKBa, PLK1, RET, RON, cSRC, SYK, mTOR, TYK2, VPS34, WNK1, YES, ZAP70, p38α, p38γ.

We further evaluated the activity of LGB321 at the cellular level on two of the potential off-targets. First, we monitored the activity of LGB321 on GSK3β, the most potent off-target kinase identified in the biochemical assay (Table 1). As seen in Supplemental Fig. S4 early compounds of the LGB321 series showed cellular inhibition of GSK3β, as demonstrated by the stabilization of β-catenin. However, compound optimization increased the biochemical selectivity and resulted in complete lack of activity of LGB321 on GSK3β in cells (Supplementary. Fig. 4). We next evaluated the activity of LGB321 on EGFR, as it was identified as the most potent off-target in the KINOMESCAN-binding displacement assay. Despite this result, we found no evidence of inhibitory activity in EGFR signaling in the cellular context (Supplementary. Fig. S5). Together these results reinforce that LGB321 is a highly potent and selective Pan-PIM inhibitor.

Cellular activity of LGB321

The cellular activity of LGB321 was evaluated in KMS-11. luc cells, a KMS-11 clone expressing firefly luciferase that we have demonstrated previously to be dependent on PIM2 kinase activity (14). Western blotting (Fig. 3A) was used to demonstrate that LGB321 inhibited the phosphorylation of BAD at Ser-112 (a direct PIM kinase substrate; refs. 21–23) and two proteins downstream of the mTOR-C1 complex (S6K at Thr-389 and its substrate S6RP at Ser-235/6; ref. 24) in a concentration-dependent manner. The effect of LGB321 in phosphorylation of S6K or S6RP is not due to an off-target effect on mTOR, because the compound was inactive in the mTOR biochemical assay shown in Table 1 and did not inhibit the phosphorylation of S6K or S6RP in 293A/TSC2-null cells with constitutively active mTOR (14).
Activity of LGB321 in a large panel of cancer cell lines

Having established the potency, selectivity, and cellular activity of LGB321, we proceeded to use it to test the hypothesis that PIM inhibition would have the greatest impact on cell lines derived from hematologic lineages in which PIM kinases are most highly expressed. To do this, we took advantage of our high-throughput compound screen of more than 500 cell lines from the CCLE (18). It should be noted that in addition to test compounds of interest, many other well-characterized inhibitors and cancer drugs have been included in the CCLE screens, including panobinostat, erlotinib, and PLX4720 (18). These inhibitors serve as controls for specificity by showing differential activity in cell lines of diverse cancer lineages or genetic backgrounds (18). LGB321 has been tested in this screen on three different occasions, and in each case with similar results (data not shown). For simplicity, the data from a single screen are presented here to demonstrate the pattern of LGB321 activity. As is apparent in Fig. 4A, LGB321 displays very limited activity in most cell lines derived from solid tumors, including breast, central nervous system, kidney, large intestine, liver, lung, ovarian, pancreas, prostate, and stomach cancer cell lines (Fig. 4A). The limited activity observed in some lung cancer cell lines was not due to potential activity against EGFR because LGB321 does not inhibit EGFR signaling in cells (Supplementary Fig. S5). This was further supported by the fact that the lung cell lines tested showed differential sensitivity to LGB321 and erlotinib, a potent and specific EGFR inhibitor (Supplementary Table S1). In general, the lack of activity of LGB321 in cell lines derived from solid tumors, including cancer indications for which evidence of roles for PIM kinases have been reported, was somewhat surprising. A possible explanation for this observation is suggested by the recent report that PIM kinase inhibition may result in increased expression of several tyrosine kinase receptors in prostate cancer cells (25), which activates the mitogen-activated protein kinase and PI3K/AKT pathway as the main drivers of proliferation in these cells. Nevertheless, our results with a highly potent and selective inhibitor suggest that in solid tumors PIM kinases are not the primary driver of proliferation.

To further explore the LGB321 activity in hematologic malignancies, we tested it in a screen that was adapted to evaluate the sensitivity of compounds on an extended panel of hematologic cell lines (Fig. 4B; Supplementary Table S2). In this screen, cell lines from ALL, AML, multiple myeloma, and B-cell NHL were represented with more than 18 cell lines each. Multiple myeloma cells seem to be the most broadly sensitive to LGB321 with 14 of the 18 cell lines tested having GI50 below 1 μmol/L (Fig. 4B, Supplementary Table S2). Highly sensitive to LGB321 was also identified in ALL, AML, CML, and NHL cell lines. However, the response was less broad than in the multiple myeloma panel, with 4 of 21 ALL, 15 of 26 AML, and 10 of 27 B-cell NHL cell lines showing sensitivity to LGB321 with GI50 below 1 μmol/L. In addition, activity was also observed in Hodgkin lymphoma, CML, and T-cell NHL cell lines. However, the number of cell

Thus, PIM kinases likely act upstream of mTOR to regulate its activity, a finding that agrees with our recent data demonstrating that PIM2 regulates mTOR through phosphorylation of TSC2 in the tuberous sclerosis complex (14).

To better understand the relationship between the biochemical and cellular potency of LGB321, we used quantitative Meso Scale assays designed to measure levels of phospho-BAD (pBAD) and phospho-S6RP (pS6RP). Similar to the Western blot analysis results, these assays confirm that LGB321 inhibits phosphorylation of both BAD and S6RP (at both the Ser-235/6 and Ser-240/6 sites, respectively) in a concentration-dependent manner in KMS-11.luc cells (Fig. 3B). In parallel experiments, we also determined the effect of LGB321 on the proliferation of KMS-11.luc cells using CellTiter-GLO assays. The potency of LGB321 in modulating PIM signaling correlated well with the inhibitory effects on KMS-11.luc cell proliferation (Fig. 3B and C). We obtained similar results in KMS-26, KMS-34, and H929 multiple myeloma cells, in which LGB321 inhibition of proliferation and PIM signaling was observed (14). Interestingly, the inhibition of pBAD by LGB321 in these multiple myeloma cell lines did not correlate with significant induction of PARP cleavage (14). Taken together, our results demonstrate that LGB321 is a potent and selective Pan-PIM inhibitor capable of inhibiting PIM kinase signaling and proliferation in PIM2-dependent multiple myeloma cell lines.

**Figure 3.** Effect of LGB321 on PIM2 target modulation and proliferation in KMS-11.luc cells. A, Western blot analysis of KMS-11.luc cells treated with a dose-response of LGB321 for 3 hours. B, quantitative analysis of the effects of LGB321 on KMS-11.luc proliferation and phosphorylation of S6RP and BAD; cells treated with a dose–response of LGB321 were tested in 72-hour proliferation assays as well as quantitative ECL assays as described in Materials and Methods. C, summary of the quantitative cellular activities on LGB321 in KMS-11.luc cells.
lines representing these diseases is rather low. Furthermore, the genetic alterations frequently observed in these diseases are represented in only a few cell lines. Thus, further exploration will be required to understand the role of PIM kinases in these malignancies and their diverse genetic backgrounds. The broad distribution of LGB321 sensitivity among hematologic cell lines, including both lymphoid and myeloid lineages, suggests that PIM kinase inhibition may have a broad application on the treatment of these malignancies.

**Activity of LGB321 in vivo**

We have reported elsewhere the antitumor activity of LGB321 in the multiple myeloma KMS-11.luc xenograft model, together with a detailed characterization of its mechanism of action in multiple myeloma cells (14). Here, we focus on the characterization of the LGB321 activity in an AML xenograft model. Among the sensitive AML cell lines tested in the expanded hematologic cell line panel (Fig. 4B; Supplementary Table S2), KG-1 cells were chosen as a model that readily grows in vivo. We first verified that...
KG-1 cells were indeed sensitive to LGB321 in several independent CellTiter-GLO proliferation assays (GI50 of 0.08 ± 0.07 μmol/L; N = 7). We then tested whether PIM inhibition in KG-1 cells resulted in modulation of pBAD and mTOR signaling in vitro (Fig. 5A). Having established in vitro the responses of KG-1 cells to PIM inhibition, we proceeded to evaluated in vivo the pharmacokinetic–pharmacodynamic relationship in KG-1 subcutaneous tumors (Fig. 5B). Following a single oral dose of LGB321 (30 or 100 mg/kg), at the indicated time points plasma and tumor
samples were collected for pharmacokinetics and pharmacodynamics analysis, respectively. PIM kinase inhibition was determined by assessing the modulation of pS6RP and pBAD in tumor lysate using the quantitative Meso Scale assay and the results were expressed as a ratio of their phosphorylated to unphosphorylated forms (Fig. 5B). A dose-dependent increase in the plasma concentration of LGB321 was observed (Fig. 5B). At the 100 mg/kg dose, the plasma concentration was maintained at higher levels through 24 hours, whereas at the 30 mg/kg dose a gradual reduction in the plasma concentration occurred. The modulation of pS6RP in tumors was also dose-dependent with more sustained inhibition at 100 mg/kg but only transient inhibition at 30 mg/kg. In contrast, both doses achieved equally significant and sustained pBAD inhibition through 24 hours. This observation raises the question of which target modulation marker would better predict the in vivo efficacy of LGB321.

To address this question, we tested the same 30 and 100 mg/kg doses of LGB321 in a daily regimen of oral dosing in an 11-day efficacy study. At the 30 mg/kg daily regimen of LGB321, we observed near stasis with only minor increase in tumor volume relative to the vehicle control (Fig. 5B). In contrast, slight tumor regression was observed with the 100 mg/kg daily regimen. These data establish that the extent and duration of pS6RP, and not pBAD, modulation correlates with efficacy in this AML model, a finding in agreement with our previous data in multiple myeloma models (14). We have also recently reported similar in vivo activity of LGB321 in the AML EOL1 xenograft model (20). Here, we tested the combination of LGB321 with the nucleoside analog cytarabine, a standard-of-care in the clinical treatment of AML. KG-1 tumors did not exhibit a significant response to cytarabine alone when delivered at 100 mg/kg daily, a dose that results in clinically relevant exposures (26). When 30 mg/kg LGB321 daily dose was combined with 100 mg/kg daily dose of cytarabine, a synergistic effect resulting in slight regression was achieved (Fig. 5B). When combined with the higher dose of LGB321, the synergistic effect was not statistically significant, given that regression was achieved with 100 mg/kg LGB321 alone. However, the combination of cytarabine with LGB321 induced significant body weight loss following the fifth day of administration (not shown), which required dosing to be halted only in the combination arms for 2 days before resuming treatment. These results demonstrate the efficacy of LGB321 both as a single agent as well as in combination with cytarabine, even in a model that is refractory to this standard of care as a single agent.

**Discussion**

Here, we demonstrated that each of the three PIM kinase family members is most highly expressed in at least one subtype of hematologic cancer. This observation coupled with the fact that PIM family members can substitute for each other to generate lymphomas (6, 7) indicates that to effectively treat hematologic cancers, an inhibitor of all three isoforms is required. Furthermore, we describe LGB321, a potent and selective inhibitor of the PIM kinase family members capable of inhibiting PIM2 at the high cellular concentrations of ATP (20). Because LGB321 is a picomolar inhibitor of all three PIM family members, we carefully evaluated its selectivity against other kinases in both biochemical and cellular contexts. The selectivity was tested biochemically in a panel of 75 kinases (Table 1) and by binding displacement assays in a panel of 386 kinases (Supplementary Fig. S3; ref. 20). Furthermore, cellular assays for the closest off-target kinase in each of these panels (Supplementary Figs. S4 and S5) were used to demonstrate that LGB321 was inactive on these kinases at concentrations well above the on-target activities on PIM2-dependent cells (Fig. 3). Selectivity was further evident by the almost exclusive activity of LGB321 in hematologic cell lines among more than 500 cell lines in the CCLE.

LGB321 as single agent was well-tolerated in vivo after multiple doses, a finding predicted by the viability and fertility of mice with knockout of the three PIM kinase genes (27). LGB321 can be administered orally to reach plasma exposures that allowed us to evaluate the relationship between pharmacokinetics, pharmacodynamics, and efficacy of PIM inhibition in xenograft models. Although oral administration with LGB321 leads to a dose-dependent increase in plasma exposure, the increased exposure was achieved through extended plasma drug concentrations, rather than successive increases in peak plasma concentration, as exhibited by differences between the 30 and 100 mg/kg doses (Fig. 5B). The pharmacokinetic properties of LGB321 at higher doses are somewhat advantageous, as the extended exposure is concomitant with sustained target inhibition as evidenced by pS6RP inhibition. The maximal in vivo effect was slight tumor regression at the 100 mg/kg/d regimen in the AML KG-1 model (Fig. 5C) and tumor stasis in the KMS-11.luc (14) and the EOL-1 models (20). As this dose is near the maximal tolerated dose, we could not assess whether LGB321 would achieve tumor regression at higher exposures. Nevertheless, stasis or slight regression are consistent with our results that LGB321 leads to inhibition of cell proliferation and not to apoptosis as assessed by PARP cleavage in the KMS-11.luc model (14). In the KG-1 model, tumor regression of approximately 50% was observed when LGB321 was coadministered with cytarabine, a nucleoside analog that is integral to the current standards of care in AML (28). Interestingly, in this model cytarabine alone had no effect, highlighting the suggestion that the combined use of this agent and PIM inhibitors could enhance the clinical response (29).

The higher level of PIM mRNA expression in hematologic tissues is consistent with a distinct role for PIM kinases in hematologic cancers. We demonstrated that PIM kinase inhibition has an antiproliferative effect primarily in hematologic cell lines, with very limited activity in cell lines from solid tumors. Although the role of PIM kinase in some solid tumors has been extensively described in the literature (2, 4), our data suggest that PIM kinases play a more significant role in promoting proliferation in hematologic malignancies than in solid tumors. Interestingly, PIM
kinases were identified as oncogenes by insertion mutagenesis screens in lymphomas from murine leukemia virus–infected mice (8, 30), but not in tumors induced by the mouse mammary tumor virus (31). These observations suggest that PIM kinases, in addition to being specifically expressed in the hematologic lineage, are hematologic lineage-specific oncogenes. Our results that the inhibitory activity of LGB321 is observed primarily in cell lines of hematologic lineage further strengthen the notion that PIM kinases play a lineage-related role in promoting proliferation of some hematologic cancers and suggest that potentially single-agent activity may be observed in these cancers.

In aggregate, our results strongly suggest that the use of potent and selective pan-PIM inhibitors, either as single agent or in combination with other agents, will be very useful for the treatment of hematologic malignancies in general, and in multiple myeloma and AML in particular. To test this hypothesis in humans, we have initiated the phase I clinical testing of our development candidate LGH447 in relapsed and/or refractory multiple myeloma.

Disclosure of Potential Conflicts of Interest

P.D. Garcia has ownership interest (including patents) in Novartis Shares. J.L. Langowski is an investigator for Novartis. J. Lan is a research investigator for Novartis. M. Lindvall has ownership interest (including patents) in Novartis. J. Holash has ownership interest (including patents) in Novartis. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

The authors thank Dr. Suzanne Trudel at the UHN for generously providing the KMS-11 luc cell line and Dr. James D. Griffin at the Dana–Farber Cancer Institutes (Boston, MA) for his helpful advice. The authors also thank Gary Vanasse, Chuck Voliva, Emma Lees, and William Sellers for their support and critical reading of the article.

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Received July 29, 2013; revised December 6, 2013; accepted January 7, 2014; published OnlineFirst January 28, 2014.
Pan-PIM Kinase Inhibition Provides a Novel Therapy for Treating Hematologic Cancers


Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-13-2062

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