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

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Statement of translational relevance:

Here we describe detailed biochemical, cellular and pharmacological properties of a highly potent and selective inhibitor (LGB321) of the three PIM kinases. When tested in over 500 cancer cell lines of diverse origins, we demonstrated almost exclusive anti-proliferative activity in cells from hematological lineages, including multiple myeloma (MM), ALL, AML and NHL. This finding correlated with higher levels of PIM kinases expression in these cancers, as compared to solid tumor cancers. To our knowledge, LGB321 is the first Pan-PIM inhibitor with activity on PIM2-dependent MM cells. LGB321 was effective in inhibiting tumor growth in an AML xenograft model, in addition to a previously reported MM model. Our findings suggest that single agent activity for Pan-PIM inhibitors is likely to be observed in hematological malignancies. Based on these findings we initiated early clinical testing of our development candidate in MM, emphasizing the translational relevance of the present work.
Abstract

Purpose: PIM kinases have been shown to act as oncogenes in mice, with each family member being able to drive progression of hematological cancers. Consistent with this, we found that PIMs are highly expressed in human hematological 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 hematological 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 (MM) cell lines, where 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 ALL, AML, MM and NHL. Furthermore, we demonstrate LGB321 activity in the KG-1 AML xenograft model, where 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 anti-proliferative 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 hematological 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 (MM), 16,060 cases of chronic lymphocytic leukemia (CLL) and 13,780 cases of acute myeloid 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 hematological 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 proto-oncogenes as a suitable target given that they are highly expressed in hematological malignancies. While 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 post-translational 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 auto-phosphorylated 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 tumorogenesis [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 knockout 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].

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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 hematological malignancies, including MM, AML, CML and B-Cell NHL. In vivo, the compound is orally available, demonstrates efficacy in tumor xenografts and is well-tolerated within the therapeutic exposure range in mice. Taken together, these findings establish PIM kinases as an attractive pharmacological target in cancer therapy.

**Material 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 prior to tissue resection were selected. After further removing redundant or poor-quality samples, 18960 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 [15] (HGU133Plus2_Hs_ENTREZG, version 14) 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. 1 and then visualized by boxplots in R (http://www.R-project.org).
PIM kinases biochemical assays and determination of LGB321 inhibition constants

PIM1 (Invitrogen PV3503), PIM2 (Invitrogen PV3649) and PIM3 (Novartis) enzyme reactions were run in 50 mM Hepes, pH 7.5, 5 mM MgCl₂, 0.05% BSA, 1 mM DTT buffer with a biotinylated BAD peptide (b-RSRHSSYPAGT_NH₂) and ATP substrates. Reaction products were measured using Perkin Elmer’s AlphaScreen IgG Detection Kit (Protein A) with anti-phospho-(Ser/Thr) AKT substrate antibody from Cell Signaling Technology. Apparent ATP Kₘ calculations were performed with Prism 5 (GraphPad Software Inc) using the Michaelis-Menten equation. Kᵢ measurements were performed at high ATP concentrations. For PIM1, the ATP concentration was 2800 μM, for PIM2 it was 500 μM and for PIM3 it was 2500 μM. The apparent Kᵢ from these measurements was calculated using the Morrison equation and converted to a true Kᵢ 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: \( \frac{V_i}{V_o} = 1 - \frac{(E + I + K_{i_{app}}) - \sqrt{(E + I + K_{i_{app}})^2 - 4EI}}{2E} \)

Cheng Prusoff equation: \( K_{i_{app}} = K_i(1 + S/K_m) \)

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, PI3Kβ, Vps34, PI4Kβ was determined by a luminescence assay based on ATP consumption (KinaseGlo; www.promega.com) with PI as the substrate, while PI3Kγ, PI3Kδ were 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 (ATCC, Manassas VA) supplemented with 10% FBS. AML cell lines KG-1 (ATCC) and MOLM-16 (DSMZ, Germany) were cultured in IMDM (ATCC) or RPMI-1640 (ATCC) respectively, supplemented with 20% FBS. The AML cell line P31/FUJ (HSRRB, 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 mM 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 Encyclopedia has been previously reported [18]. Prior to implantation in vivo, KMS-11.luc and KG-1 cells were cultured in DMEM (Corning, Manassas VA) plus 10% FBS and 1% L-Glutamine (Corning, Manassas VA).

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 μM to 2nM in 0.1% DMSO. After addition of LGB321 to cells, assay plates were returned to a humidified CO2 incubator (37°C; 5% CO2) for 3 days. To determine KMS-11.luc cell growth, 100μl per well of reconstituted CellTiter-Glo® reagent was added to the cell assay plates. Assay plates were then sealed and shaken on a DELFIA (Perkin Elmer) plateshaker for 10 minute at 400 – 600 RPM. Plates were then read on either a Microbeta Trilux (Perkin Elmer) 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 non-specific cytotoxic kinase inhibitor (defining 100% growth inhibition).

The activity of LGB321 was also tested in the Cancer Cell Line Encyclopedia screen [18] and further testing was performed on an expanded panel of hematological 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% RH and cultured in T75 flasks using standard culture techniques. They were expanded for at least 2 passages before being added in assay micro-titer 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 SNP-genotyping. Cell lines were dispensed into 384-well plates (Greiner Bio-One, #781098) with a final volume of 25µL and concentrations ranging from 250 to 4000 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 post-seeding compounds were transferred to the cells by delivering 5µL/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 µM 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 percent normalized on a plate by plate basis such that the median of the Neutral Controls wells (i.e. DMSO) are 100% growth and the D0 wells (optical density measured at seeding time) are 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; Rockville MD) 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 μM to 2nM 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 (Perkin Elmer, Waltham MA) plate shaker at 4°C and shaking the plates for 30 minutes at 600 RPM.

For *in vivo* assays, MDS lysis buffer (MSD, Rockville MD) was added to frozen pulverized tumor samples on ice and homogenates were prepared using the MagNA Lyser bead instrument (Roche Applied Science, Indianapolis, IN) by disrupting the samples with four cycles of 6000 RPM for 30 seconds at 4°C. Supernatants were created following centrifugation at 11000 RPM for 15 minutes at 4°C, and protein concentration determined using the BCA Protein Assay kit according to manufacturer’s instructions (Pierce Chemical Company, Rockford, IL). 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 (Perkin Elmer, Waltham MA) plate shaker. After overnight incubation, assay plates were processed according to manufacturer instructions.

**LGB321 in vivo studies**

All studies were done in an AAALAC-accredited animal facility and in compliance with the ILAR Guide for the Care and Use of Laboratory Animals. Scid/bg female mice (10-12 weeks old; Charles River, Hollister CA) were housed up to five animals per cage in clear polycarbonate micro-isolator cages with a 12-hour light, 12-hour dark cycle at temperatures between 70-80°F, and 30-70% relative humidity. Food (Purina rodent chow pellets) and water were provided *ad libitum.*

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For subcutaneous tumor models, cells were harvested at 80-90% confluency, washed and resuspended in cold Dulbecco’s Phosphate Buffered Saline (DPBS without Ca\(^{2+}\) or Mg\(^{2+}\), Cellgro, Manasas VA) at a concentration of 5 X 10\(^{7}\) cells/ml, mixed with an equal volume of Matrigel™ (Becton-Dickinson, Franklin Lakes NJ) and then 0.2 ml (5 X 10\(^{6}\) 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 \((\text{Length} \times \text{Width}^2) \times \pi/6\).

For pharmacodynamic and pharmacokinetic (PK/PD) studies, animals were enrolled on study when mean tumor volume reached 250-400mm\(^3\). For efficacy studies, animals were randomized into groups when tumor volume reached 200-250mm\(^3\). Tumor volume and body weights were captured and stored by StudyDirector software (StudyLog, South San Francisco, CA).

LGB321 was formulated for oral administration in 50mM Acetate buffer, pH4. The concentration of LGB321 in plasma was determined following extraction in acetonitrile using liquid chromatography and tandem mass spectroscopy (LC/MS/MS). Cytarabine (Hospira, Lake Forest IL) was diluted in bacteriostatic water and prepared fresh daily.

**Statistics**

For *in vivo* studies, statistical significance of differences in tumor volume was determined by a One-way Analysis of Variance (ANOVA), and pair-wise comparisons were made by the Uncorrected Fisher’s LSD post-test (GraphPad Prism software, La Jolla CA).
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 data sets derived from Affymetrix human genome U133 arrays from public depositories for PIM kinases expression. All primary data was 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. 1). Consistent with their known roles in cytokine signaling in hematopoiesis [2], all three PIMs are expressed at higher levels in hematological samples as compared to other tissues (Fig. 1 and Supp. Fig. 1). 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 hematological malignancies various PIM isoforms are expressed at higher levels in ALL, AML, MM or DLBCL-NHL samples, relative to samples of liver, lung, pancreas, prostate and stomach cancers (Fig. 1). PIM2 expression in MM appears to be significantly higher than all other tissues examined, including normal bone marrow, while PIM1 expression is higher in AML, ALL and DLBCL than in MM (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. Since we had observed that the MM KMS-11.luc cells are dependent on PIM2 for proliferation [14] and, since 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, since 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 $K_m$ (4 µM; Fig.2B) as compared to the $K_m$ for PIM1 or PIM3 (400 µM and 40 µM respectively; Fig.2B). Thus, the desired inhibitor needed to be potent enough to compete with the large excess of cellular ATP (in the range of 1-10mM) [19], about 250-2500 fold higher than the $K_m$ 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 ($K_i$) for LGB321 was determined to be in the single digit picomolar (pM) 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 to two other PIM inhibitors tested in clinical trials, it is clearly more potent (Supp. Fig.2; [20]).

The selectivity of LGB321 was first determined in biochemical assays of a panel of 7 lipid kinases and 68 diverse protein kinases that included PIM2 (Table 1). In this panel, only PIM2 was significantly inhibited by LGB321 with an $IC_{50}$ of <0.003 µM, the lowest sensitivity range for the assay. While biochemical potency in the range of 4-10 µM was demonstrated against eight other kinases in this assay, these $IC_{50}$ represent a greater than $10^6$ fold differential relative to the $K_i$ on all three PIM kinases. The biochemical $IC_{50}$ for all other kinases tested in this panel was >10 µM (Table 1). We further evaluated the selectivity of LGB321 using the KINOMESCAN™ binding displacement assay (Supp. Fig.3; [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 (Supp.Fig.3 and [20]).
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.4 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 (Supp. 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 EGF signaling in the cellular context (Supp. Fig. 5). 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 [22-24]) and two proteins downstream of the mTOR-C1 complex (S6K at Thr-389 and its substrate S6RP at Ser-235/6 [25]) 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, since 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]. Thus, PIM kinases likely act upstream of mTOR to regulate its activity, a finding which 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 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 3C). We obtained similar results in KMS-26, KMS-34 and H929 MM cells, in which LGB321 inhibition of proliferation and PIM signaling was observed [14]. Interestingly, the inhibition of pBAD by LGB321 in these MM 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 MM cell lines.

**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 hematological lineages where PIM kinases are most highly expressed. To do this, we took advantage of our high throughput compound screen of over 500 cell lines from the Cancer Cell Line Encyclopedia (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, CNS, 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 since LGB321 does not inhibit EGFR signaling in cells (Suppl. Fig. 5). 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 (Suppl. Table 1). 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 [26], which activates the MAP 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 hematological malignancies, we tested it in a screen that was adapted to evaluate the sensitivity of compounds on an extended panel of hematological cell lines (Fig. 4B, Supp. Table 2). In this screen cell lines from ALL, AML, MM and B-Cell NHL were represented with more than 18 cell lines each. MM cells appear to be the most broadly sensitive to LGB321 with 14 of the 18 cell lines tested having GI50 below 1 μM (Fig. 4B & Suppl. Table 2). Highly sensitive to LGB321 was also identified in ALL, AML, CML and NHL cell lines. However, the response was less broad than in the MM 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 μM. In addition, activity was also observed in Hodgkin’s lymphoma, CML and T-Cell NHL cell lines. However, the number of cell 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 hematological cell lines, including both lymphoid and myeloid lineages, suggests that PIM kinase inhibition may have a broad application on the treatment of these malignancies.

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Activity of LGB321 in vivo

We have reported elsewhere the antitumor activity of LGB321 in the MM KMS-11.luc xenograft model, together with a detailed characterization of its mechanism of action in MM 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 hematological cell line panel (Fig.4B and Supp. Table 2), KG-1 cells was 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 (GI$_{50}$ of 0.08±0.07 μM, N=7). We then tested if PIM inhibition in KG-1 cells resulted in modulation of pBAD and the mTOR signaling pathway and found that indeed LGB321 effectively inhibited both signaling pathways (Fig.5A). Having established in vitro the responses of KG-1 cells to PIM inhibition, we proceeded to evaluated in vivo the LGB321 pharmacokinetic-pharmacodynamic (PK/PD) 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, while 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 30mg/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 (QD) 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 QD regimen. This data establishes 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 MM models [14]. We have also recently reported similar *in vivo* activity of LGB321 in the AML EOL1 xenograft model [20]. In the current study, 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 which results in clinically relevant exposures [21]. When 30 mg/kg LGB321 QD dose was combined with 100mg/kg QD 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 two 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 which 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 sub-type of hematological cancer. This observation coupled with the fact that PIM family members can substitute for each other to generate lymphomas [6, 7] indicates that in order to effectively treat hematological 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]. Since 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 (Suppl. Fig. 3; [20]). Furthermore, cellular assays for the closest off-target kinase in each of these panels (Suppl. Fig. 4 & 5) 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 hematological cell lines amongst over 500 cell lines in the Cancer Cell Line Encyclopedia.

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. While 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 100mg/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 100mg/kg/day 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 co-administered 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 hematological tissues is consistent with a distinct role for PIM kinases in hematological cancers. We demonstrated that PIM kinase inhibition has an anti-proliferative effect primarily in hematological 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 hematological malignancies than in solid tumors. Interestingly, PIM kinases were identified as oncogenes by insertional 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 hematological lineage further strengthens the notion that PIM kinases play a lineage-related role in promoting proliferation of some hematological 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 hematological malignancies in general, and in MM and AML in particular. To test this hypothesis in humans, we have initiated the Phase 1 clinical testing of our development candidate LGH447 in relapsed and/or refractory multiple myeloma.

**Acknowledgements:** We would like to thank Dr. Suzanne Trudel at the University Health Network (UHN), Toronto, Ontario, Canada for generously providing the KMS-11.luc cell line and Dr. James D. Griffin at the Dana-Farber Cancer Institutes for his helpful advice. We
would like to thank Gary Vanasse, Chuck Voliva, Emma Lees and William Sellers for their support and critical reading of the manuscript.

**Conflict of Interest Statement:** All authors are employees at the Novartis Institutes for Biomedical Research. All authors declare no other competing financial interest.

Reference List


Garcia, PD et a (2013)


Garcia, PD et a (2013)
**Figure Legends**

**Figure 1: mRNA expression levels in cancer tissues.** PIM1, PIM2 and PIM3 expression in patient samples from the major hematological cancers sub-types (ALL N=350, AML N=2049, DLBCL-NHL N=640 and MM N=982) as compared to the expression in normal bone marrow (N=81). For comparison purpose, a few representative solid tumors are included: Breast (N=2422), Liver (N=132), Lung (N=973), Pancreas (N=128), prostate (N=193) and Stomach (N=101) cancers. Expression intensity was determined as described in Material 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 inter-quartile range (IQR) from the upper or lower quartile. Outliers that are at a distance of great than 1.5*IQR from the box are plotted individually as plus signs.

**Figure 2: LGB321 structure and biochemical properties.** A: Chemical structure of LGB321. B: Summary of the ATP $K_m$ and LGB321 inhibition constant ($K_i$) determined as described in Material and Methods.

**Figure 3: Effect of LGB321 on PIM2 Target Modulation & 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.

**Figure 4: LGB321 screen in Cancer Cell Line Encyclopedia.** A: Cell lines are grouped and color coded according to the primary site of the tumor from which they were derived. In
order to better demonstrate the differences in sensitivity across the cell lines, the
directionality of bars in the graph is based on a concentration of 1µM (log 0), with cell lines
in which 50% growth inhibition was achieved at lower concentrations than 1µM dropping
down, and those in which it was higher than 1µM rising up. Hematopoietic cancers are the
most sensitive to PIM inhibition. B: LGB321 screen in an expanded panel of cell lines from
hematological malignancies. LGB321 is most broadly effective in MM. In AML a subset of
cell lines are very sensitive to LGB321. Sensitivity in ALL, CML, Hodgkin lymphoma and
NHL cell lines was also observed.

**Figure 5: LGB321 activity in AML xenografts and demonstration of in vivo synergy
with cytarabine.** A: Modulation in vitro of pBAD and mTOR signaling in KG-1 cells. B:
Single does PK/PD of LGB321 in mice bearing subcutaneous KG-1 tumors. C: LGB321
efficacy as single agent and in combination with cytarabine. LGB321 was dosed by oral
gavage with vehicle, LGB321 at 30, 100 mg/kg once daily (QD), cytarabine at 100 mg/kg
QD by intraperitoneal injection, or with a combination of cytarabine and 30 or 100 mg/kg
LGB321 (n=9 per group). While 30 mg/kg QD of LGB321 resulted in significant tumor
growth inhibition (*, p<0.05), 100 mg/kg QD achieved the greater effect of partial
regression. In contrast, cytarabine has no effect in this model as a single agent. However,
when combined with 30 mg/kg QD of LGB321, a synergistic effect was achieved resulting in
tumor regression (p<0.05). When cytarabine was combined with 100 mg/kg LGB321, the
result was also increased tumor regression, though this result was not statistically
significant compared to the 100 mg/kg LGB321 group alone. The combination of LGB321
and cytarabine led to significant body weight loss after five successive days of dose, and the
combination groups were granted a two day dosing holiday before administration
continued.
FIGURE 1: PIM Expression in Representative tumor tissues

FIGURE 2

A. Structure of LGB321

B. Biochemical potency of LGB321 in PIM kinases

<table>
<thead>
<tr>
<th></th>
<th>ATP $K_m$</th>
<th>LGB321 $K_i$</th>
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<tbody>
<tr>
<td>PIM1</td>
<td>400 μM</td>
<td>1.0 pM</td>
</tr>
<tr>
<td>PIM2</td>
<td>4 μM</td>
<td>2.1 pM</td>
</tr>
<tr>
<td>PIM3</td>
<td>40 μM</td>
<td>0.8 pM</td>
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FIGURE 3: LGB321 activity in KMS11-luc MM Cells

A.

<table>
<thead>
<tr>
<th>LGB321 (μM)</th>
<th>pThr^{389}-S6K</th>
<th>Total-S6K</th>
<th>pSer^{235/6}-S6RP</th>
<th>Total-S6RP</th>
<th>pSer^{112}-BAD</th>
<th>Total-BAD</th>
<th>Actin</th>
<th>Pim2</th>
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</table>

B.

C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>EC_{50} (μM)</th>
<th>N</th>
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<tbody>
<tr>
<td>pBAD (S^{112})</td>
<td>0.010 ± 0.006</td>
<td>16</td>
</tr>
<tr>
<td>pS6RP (S^{235/6})</td>
<td>0.026 ± 0.013</td>
<td>4</td>
</tr>
<tr>
<td>pS6RP (S^{240/4})</td>
<td>0.038 ± 0.027</td>
<td>17</td>
</tr>
<tr>
<td>Proliferation</td>
<td>0.017 ± 0.011</td>
<td>26</td>
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</table>
FIGURE 4A:
Cancer Cell Line Encyclopedia Sensitivity to LGB321

FIGURE 4B:
Sensitivity to LGB321 of Hematopoietic cells in Cell Line Encyclopedia

Figure 5.

A) *in vitro* modulation of PD markers in KG-1 cells

<table>
<thead>
<tr>
<th>LGB321 (μM)</th>
<th>0</th>
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<th>0.2</th>
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<tr>
<td>pSer&lt;sup&gt;235/6&lt;/sup&gt;-S6RP</td>
<td>![Image]</td>
<td>![Image]</td>
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<tr>
<td>Total-S6RP</td>
<td>![Image]</td>
<td>![Image]</td>
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<tr>
<td>pSer&lt;sup&gt;112&lt;/sup&gt;-BAD</td>
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<td>Plm1</td>
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</tr>
</tbody>
</table>

B) Single-dose PK/PD in KG-1 tumor bearing mice

Figure 5.

C) Efficacy in KG-1 xenograft model

- **50mM Acetate buffer**
- **Cytarabine 100mg/kg qd**
- **30 mg/kg qd**
- **Cytarabine + 30 mg/kg qd**
- **100 mg/kg qd**
- **Cytarabine + 100mg/kg qd**

**Dosing duration for combination**

Tumor volume (mm³ ± SEM) vs. Days post implant.
Table 1: LGB321 Biochemical specificity Profile

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
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<tbody>
<tr>
<td>PIM2</td>
<td>&lt;0.003</td>
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<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>
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<tr>
<td>PKCα</td>
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<tr>
<td>PKN2</td>
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<tr>
<td>PKCτ</td>
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</tr>
<tr>
<td>ROCK2</td>
<td>9.4</td>
</tr>
</tbody>
</table>

**Kinases with IC<sub>50</sub> > 10 μM:**

cABL(T315I), cABL, ALK, AURORA-A, BTK, CDK1B, CDK2A, CDK4D1, CK1, COT1, CSK, CaMK2, ERK2, EPHA4, EPHB4, FAK, FGFR1, FGFR2, FGFR3, FGFR4, FGFR3(K650E), 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, PI4Kβ, PKBa, PLK1, RET, RON, cSRC, SYK, mTOR, TYK2, VPS34, WNK1, YES, ZAP70, p38α, p38γ
Hematological Cancers

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


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