CAL-101, a p110δ selective phosphatidylinositol-3-kinase (PI3K) inhibitor for the treatment of B cell malignancies inhibits PI3K signaling and cellular viability

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Running title: CAL-101 displays potent antitumor activity in B cells

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Abstract

PI3K p110δ serves as a central integration point for signaling from cell surface receptors known to promote malignant B cell proliferation and survival. This provides a rationale for the development of small molecule inhibitors that selectively target p110δ as a treatment approach for patients with B cell malignancies. We thus identified CAL-101, a highly selective and potent p110δ small molecule inhibitor (EC_{50} 8 nM). Using tumor cell lines and primary patient samples representing multiple B cell malignancies, we have demonstrated that constitutive PI3K pathway activation is p110δ-dependent. CAL-101 blocked constitutive PI3K signaling resulting in decreased phosphorylation of Akt and other downstream effectors, an increase in PARP and caspase cleavage and an induction of apoptosis. These effects have been observed across a broad range of immature and mature B cell malignancies thereby providing a rationale for the ongoing clinical evaluation of CAL-101.
Introduction

Dysregulation of the PI3K pathway plays an important role in the etiology of human malignancies including those of hematologic origin\textsuperscript{1,2}. In B-cell malignancies, aberrant PI3K signaling may be the result of constitutive B-cell receptor (BCR) activation and/or the response to proliferation and survival factors present in bone marrow and lymph node microenvironments\textsuperscript{3-5}. Activation of the PI3K pathway by cell surface receptors is directly mediated by Class I isoforms p110\textalpha, p110\textbeta, p110\textdelta and p110\textgamma. The p110\textdelta isoform is highly expressed in cells of hematopoietic origin being predominantly detected in leukocytes\textsuperscript{6}. Genetic and pharmacological approaches that specifically inactivate the p110\textdelta isoform have demonstrated its important role in B-cell signaling\textsuperscript{7-9}. We identified CAL-101, a potent and selective inhibitor of p110\textdelta, in a kinome-wide screen using purified enzymes and in cell-based PI3K isoform-specific assays. To investigate the therapeutic potential of p110\textdelta inhibition by CAL-101 in B-cell tumors we utilized malignant B-cell tumor lines and primary tumor cells.

Methods

Cell culture conditions

Cell lines were cultured in RPMI 1640 medium (ATCC) supplemented with 10% fetal bovine serum, FBS, 100 U/L penicillin-streptomycin and incubated at 37° C/5% CO\textsubscript{2}. Blood samples were obtained after written informed consent following the Declaration of Helsinki according to local institutionally approved protocols by the Calistoga Pharmaceuticals institutional review board. Mononuclear cells were isolated from peripheral blood or bone marrow by density centrifugation over Ficoll. Cells were analyzed on a FACSARia flow cytometer using lineage and
blast-specific cell surface markers, confirming that neoplastic cells accounted for greater than 90% of the total cell population in all patient samples analyzed.

**In Vitro kinase profiling**

All biochemical *in vitro* protein kinase assays were analyzed using the SelectScreen kinase inhibitor assay service (Invitrogen, Carlsbad, CA).

**Western and ELISA analysis**

Whole cell lysates were analyzed on 10% polyacrylamide gels. Transfer to nitrocellulose, blocking, probing with antibodies, and chemiluminescence were performed as previously described. Total Akt1, total S6, phospho-Akt1 (Ser473), phospho-S6 (Ser 235/236), cleaved caspase-3 and cleaved PARP were measured using the PathScan® Sandwich ELISA kit (Cell Signaling Technology) following manufacture’s protocol.

**Cell viability assays**

Cell viability was assessed using Cell Titer Aqueous One Solution Cell Proliferation Assay reagent (Promega, Madison, WI) following manufacture’s protocol. Fluorescence was measured with the Spectramax M5 plate reader (Molecular Devices, Sunnyvale, CA).

**Flow cytometry**

Apoptosis was measured by Annexin-V-FITC/7-AAD labeling followed by fluorescence-flow cytometry (FC) as previously described. Intracellular FC, cells were labeled with either anti-CD19-FITC or anti-CD5-FITC and either anti-phospho-Akt T308 (Alexa Fluor 488), anti-
phospho-Akt Ser473 (Alexa Fluor 488), or an isotype-matched control antibody (mouse IgG1-Alexa Fluor 488 conjugate). Cells were assayed by FC using the Cytomics FC 500MPL cytometer and data was collected and analyzed using CXP software (Beckman Coulter).

Results and Discussion

CAL-101 is a potent selective inhibitor of PI3Kδ

CAL-101 (5-Fluoro-3-phenyl-2-[(S)-1-(9H-purin-6-ylamino)-propyl]-3H-quinazolin-4-one; Figure 1A) is an oral p110δ inhibitor currently under clinical evaluation in patients with B cell malignancies. CAL-101 was 40-300-fold more selective for p110δ relative to other PI3K class I enzymes (IC\text{50} for p110δ=2.5 nM; p110α, p110β, and p110γ IC\text{50} were 820, 565, and 89 nM respectively; Figure 1B). Greater selectivity (400-4000-fold) was seen against related kinases C2β, hVPS34, DNA-PK and mTOR (Figure 1B), while no activity was observed against a panel of 402 diverse kinases at 10 μM (Ambit KinomeScan, data not shown).

In fibroblasts, the receptor tyrosine kinase PDGFR signals through p110α and the GPCR receptor for LPA signals through p110β\textsuperscript{11}. We stimulated murine embryonic fibroblasts with PDGF or LPA and monitored phosphorylation of Akt to measure pathway activation. CAL-101 reduced PDGF-induced pAkt by only 25% at 10 μM while the positive control, PI-10312, had an EC\text{50} of 90 nM (Figure 1C & Supplemental Figure 1A). CAL-101 inhibited LPA-induced pAkt with an EC\text{50} of 1.9 μM (Figure 1C & Supplemental Figure 1B). Expression of p110δ and p110γ is normally restricted to leukocytes. In basophils, FcεRI signals through p110δ while fMLP signals through GPCR-p110γ\textsuperscript{13,14}, and activation through either stimulus results in surface expression of CD63 that can be monitored by flow cytometry. CAL-101 blocked FcεRI p110δ-mediated CD63 expression with an EC\text{50} of 8 nM while fMLP activation of p110γ was inhibited
with an \( \text{EC}_{50} \) of 3.0 \( \mu \text{M} \) (Figure 1C & Supplemental Figure 1C). Thus in cell-based assays CAL-101 had 240-2500-fold selectivity for p110\( \delta \) over the other Class I PI3K isoforms.

**Sensitivity of primary cells from patients with lymphoid and myeloid leukemias to CAL-101**

Primary leukemia cells from patients with a variety of malignancies were tested for sensitivity to CAL-101 *in vitro*. In total, 134 leukemia patient and 5 control PBMC samples were tested. We observed significant sensitivity (defined as an \( \text{EC}_{50} \) less than 1 \( \mu \text{M} \)) to CAL-101 in 23% of B-ALL samples (5/22), 26% of CLL samples (11/42), 3% of AML samples (1/31), and 0% of MPN samples (0/39). Normal PBMCs showed no sensitivity to CAL-101 (Figure 2A). Thus, CAL-101 exhibited far greater activity in B-ALL and CLL cells compared with AML and MPD cells, suggesting a greater therapeutic potential for patients with B-cell malignancies.

**CAL-101 inhibits constitutive PI3K signaling and induces apoptosis**

DLBCL (SU-DHL-5), FL (KARPAS-422) and B-ALL (CCRF-SB) cell lines showed pAkt\(^{S473}\) expression in an initial assay. In these cell lines, CAL-101 produced a concentration-dependent reduction in pAkt\(^{S473}\), pAkt\(^{T308}\) and the downstream target S6 with an \( \text{EC}_{50} \) of 0.1-1.0 \( \mu \text{M} \), demonstrating a central role for p110\( \delta \) in constitutive PI3K signaling (Figure 2B). Patient-derived malignant B-cells (5/5 CLL and 5/5 MCL samples) displayed constitutive levels of pAkt\(^{T308}\) which was significantly reduced by CAL-101 (\( \text{EC}_{50} < 100 \text{nM} \); Figure 2C). In these cells, phosphorylation of Akt was low or undetectable at S473. Since Akt phosphorylation at both sites is required for full kinase activity, we investigated if tumor microenvironment signals could cause further p110\( \delta \)-dependent PI3K pathway activation through S473 phosphorylation.
Treatment of patient CLL or MCL cells with sCD40L or BCR-crosslinking caused rapid 
induction of pAktS473 that was completely inhibited by CAL-101 at 0.1-1.0 μM (Figure 2D). 
Comparable observations were made using MCL cell lines (supplementary Figure 2). Similarly, 
knockdown of p110δ in CLL cells by siRNA resulted in inhibition of CD40-induced Akt 
phosphorylation and a decrease in the prosurvival factor Mcl-1 (Supplementary Figure 3), 
supporting the role of the p110δ isoform.

The functional role of p110δ was evaluated by measuring apoptosis in SU-DHL-5, WSU-
NHL (FL) and CCRF-SB tumor cell lines. CAL-101 treatment resulted in a 3-5-fold increase in 
Annexin-V staining indicating a significant level of apoptosis induction (Figure 2E). We also 
observed a dose-dependent 2-8-fold increase in both caspase 3 and PARP cleavage at 24 hours 
with CAL-101 treatment compared to vehicle controls (Figure 2F).

We have thus described the biochemical and cellular activity of CAL-101, a selective and 
potent inhibitor of p110δ; such targeted inhibition has the potential to avoid adverse effects that 
may result from non-selective PI3K inhibition. Further, we have demonstrated an essential role 
for PI3K p110δ in constitutive PI3K signaling that is required for the survival of malignant B-
cells. Oncogenic mutations in components of the PI3K pathway are infrequent in B-cell 
malignancies, and much less is known about the importance of constitutive PI3K signaling in 
these tumors. A potential mechanism for PI3K pathway activation in this setting is tonic 
antigen-independent BCR signaling that requires p110δ for the transduction of proliferation and 
survival signals8. In this regard, CAL-101 blocks constitutive oncogenic signaling, resulting in 
apoptosis, and inhibits survival signals provided by the microenvironment. Our studies have 
identified a novel targeted approach for the treatment of patients with B cell malignancies and 
provided the rational for ongoing clinical studies.
Acknowledgments

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Authorship

Contribution: B.J.L. planned the research, performed experiments, analyzed data, drafted the first and subsequent drafts of the paper and approved the final version of the paper; S.A.M, A.K., and B.S. were involved in planning components of the research, performing experiments, and approved the final version of the paper; S.E.H.H performed experiments; A.J.J Provide primary cells and reviewed drafts; J.C.B. was involved in planning components of the research, reviewed drafts and approved the final version of the paper; J.W.T. and M.M.L. obtained patient samples, performed experiments, reviewed drafts of the paper and approved the final version of the paper; M.D. and B.J.D. were involved in planning components of the research, and approved the final version of the paper; K.D.P. was involved in planning components of the research; R.G.U. and N.A.G, planned research, supervised the research, analyzed data, reviewed drafts, and approved the final version of the paper.

Conflict-of-interest disclosure: Brian J. Lannutti, Sarah A. Meadows, Adam Kashishian, Bart Steiner, Kamal D. Puri, Roger G. Ulrich, and Neill A. Giese are employees of Calistoga Pharmaceuticals.

References


**Figure 1.** CAL-101 inhibits PI3Kδ with high selectivity. **A)** Chemical structure of CAL-101. **B)** CAL-101 *in vitro* activity profiles (IC₅₀ values) against recombinant enzymes of Class I, II, III, and IV PI3Ks. CAL-101 was diluted in DMSO at a concentration of 10 mM and ten-point kinase inhibitory activities were measured over a concentration range (5.0 – 10⁴ nM) with ATP at a concentration consistent with each enzymes Kₘ. **C)** Potency of CAL-101 in PI3K Class I isoform-specific cell based assays. For the analysis of p110α and p110β signaling murine embryo fibroblast were stimulation with PDGF or LPA, soluble protein was analyzed by Western blotting for Akt and pAkt⁴⁷³ levels. For the analysis of p110δ and p110γ signaling, basophil activation was measured in isolated PBMC or whole blood using the Flow2 CAST kit according to the manufacture’s standardized methods (Buhlman Laboratories AG, Switzerland). p110δ was activated with anti-FCεRI and p110γ was activated with fMLP. To monitor the basophil cell population and cellular activation, anti-CD63-FITC and anti-CCR3-PE antibodies was added to each sample. Cells were fixed and analyzed on a FC500MPL flow cytometer (Beckman Coulter, Brea, CA).

**Figure 2.** **A)** CAL-101 screening of primary cells from patients with ALL, CLL, AML, or MPN or from normal healthy volunteers (NHV). Cell viability was determined using an MTS assay, and the sensitivity of each sample relative to untreated cells was calculated for estimation of the EC₅₀. The heat map readout shown was generated using GenePattern software (Broad Institute) and indicates the percent EC₅₀ for each sample relative to the maximum drug concentration tested (10 μM). **B)** CAL-101 inhibition of p110δ blocks PI3K signaling in malignant B cells lines and primary patient tumor cells. Serum starved cells were incubated with 1 μM CAL-101
and total cell lysates were subjected to Western blot analysis using anti-phospho AktS473, anti-Akt, anti-phospho S6S235/236 and anti-S6 antibodies (see supplemental Methods). Starved cells were incubated with vehicle or serial dilution of CAL-101 for 1 hr and pAktT308, pS6S235/236, total Akt, and total S6 was detected by PathScan® sandwich ELISA (see supplemental Methods).

C & D) CLL (N=5) and MCL (N=5) patient whole blood samples were subjected to ficoll-hypaque separation. Isolated cells were incubated in RPMI with vehicle or serial dilutions of CAL-101 prior to fixation and staining with anti-phospho-AktT308 Alexa Fluor 488 or isotype matched Alexa Fluor 488 antibody. Cells pretreated with CAL-101 or vehicle for one hr and then stimulated with 10µg/mL anti-IgM (BCR activation), or 50 ng/ml sCD40L (CD40 stimulation) for 10 min prior to fixation and staining with anti-phospho-AktS473 Alexa Fluor 488 or isotype matched Alexa Fluor 488 antibody. FITC-CD5+ cells were gated and analyzed by two color flow cytometry to quantify intracellular p-AktT308 levels using the Beckman Coulter Cytomics FC 500MPL employing CXP software. Bar graphs represent the percent difference in mean fluorescence intensity (MFI) values between isotype-matched control Ig and phospho-AktT308.

E) CAL-101 induces apoptosis in DLBCL, FL, and B-ALL cell lines. Cells were treated with vehicle, 0.5 µM or 1.0 µM CAL-101 for 24 hr. The percentage of apoptotic cells were determined by Annexin V-FITC/7AAD staining followed by two-color flow cytometric analysis. Percentages represent both Annexin V-FITC/7AAD negative and Annexin V-FITC/7AAD double-positive.

F) Cells were cultured in RPMI/10% FBS with CAL-101 or vehicle alone for 24 hr, cells were lysed and analyzed by PathScan® Sandwich 96-well ELISA for the detection of cleaved caspase-3 and cleaved PARP as indicated (see supplemental Methods). Results are expressed as mean ± SD. Statistically significant differences between means were determined.
using a one-way ANOVA. Data is expressed as the fold change, and is representative of three separate experiments.
### Figure 1

#### A) Molecular Structure

#### B) Table of PI3K Inhibitor Activities

| Class I PI3Ks  | Class II PI3Ks  | Class III PI3Ks  | Class IV PI3Ks  | Other Phosphoinositide Kinas
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#### C) Table of PI3K Activity in Different Cell Types

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<td>3,000 (n=5)</td>
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Figure 2
CAL-101, a p110δ selective phosphatidylinositol-3-kinase inhibitor (PI3K) for the treatment of B cell malignancies inhibits PI3K signaling and cellular viability

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