**PI3Kδ Inhibitors in Cancer: Rationale and Serendipity Merge in the Clinic**

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**ABSTRACT**

Several phosphoinositide 3-kinase (PI3K) inhibitors are in the clinic and many more are in preclinical development. CAL-101, a selective inhibitor of the PI3Kδ isoform, has shown remarkable success in certain hematologic malignancies. Although PI3Kδ signaling plays a central role in lymphocyte biology, the degree of single-agent therapeutic activity of CAL-101 during early-phase development has been somewhat unexpected. CAL-101 works in part by blocking signals from the microenvironment that normally sustain leukemia and lymphoma cells in a protective niche. As PI3Ks enter the arena of molecular-targeted therapies, CAL-101 provides proof of principle that isoform-selective compounds can be effective in selected cancer types and patient populations.

**SIGNIFICANCE:** A key question is whether compounds targeting a single PI3K catalytic isoform can provide meaningful single agent efficacy in cancer cells that express multiple isoforms. Clinical studies of the drug CAL-101 have provided a significant advance by showing that selective targeting of PI3Kδ achieves efficacy in chronic lymphocytic leukemia, in part through targeting the tumor microenvironment. Cancer Discovery; 1(7), 562–72. ©2011 AACR.

**PHOSPHOINOSITIDE 3-KINASE AS A DRUG TARGET**

Phosphoinositide 3-kinase (PI3K) was first discovered as an enzymatic activity coprecipitating with oncoproteins and activated growth factor receptors (1, 2). This spurred research in PI3K as an interesting new signaling molecule and a potential drug target. Subsequent cloning efforts identified 8 distinct genes encoding PI3K catalytic subunits (3–5). Four of these PI3K isoforms (PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ) are categorized as class I enzymes because they can use phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P2) as a substrate to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3). Elevated PIP3 in cellular membranes drives several hallmarks of the cancer phenotype: cell proliferation, survival, metabolic reprogramming, and migration (6, 7).

Diverse mechanisms can contribute to increased PIP3 levels. Some of these are cell-intrinsic, such as oncogenic tyrosine kinases (e.g., EGFR, BCR-ABL) or loss of the lipid phosphatase tumor suppressor PTEN. Cell-extrinsic cues, including growth factors, cytokines, chemokines, and adhesion molecules, also promote PI3K activation and PIP3 production. One reason that PI3K has received such attention as a cancer target is that inhibiting PI3K should block growth and survival signals emanating from both the cell-intrinsic mutations and the microenvironment. Supporting this prediction, first-generation PI3K inhibitors wortmannin and LY294002 cause cell-cycle arrest and death in a broad array of tumor cell lines and stromal coculture models.

These studies led to the concept of PI3K as a convergence point for diverse upstream oncogenic inputs such that PI3K inhibition could provide a general strategy for cancer therapeutics (8). The fact that PI3K enzymes are primarily lipid kinases with distinct structure from classic protein kinase targets afforded the opportunity for drug selectivity. However, it soon became apparent that LY294002 (and, to a lesser extent, wortmannin) are not selective; they inhibit not only class I PI3Ks, but many other lipid and protein kinases in cells, including mTOR, a central regulator of cell growth and proliferation (9–11). Consequently, LY294002 has been instrumental in establishing the importance of class I PI3K and mTOR signaling in tumor cell biology but is a blunt instrument for understanding whether a specific PI3K isoform or subgroup has a required function (Fig. 1).

These considerations, along with the poor pharmacologic properties of wortmannin and LY294002, prompted development of second-generation, more selective “pan-PI3K” inhibitors. Compounds that target all class I PI3Ks are effective in mouse models of cancer with acceptable toxicity, and several pan-PI3K inhibitors have finally entered the stage of clinical
Success of PI3Kδ Inhibitors in Leukemia and Lymphoma

Figure 1. Targeting PI3K and mTOR has broad applicability in cancer. A first-generation PI3K inhibitor (LY294002, center) inhibits all class I PI3K isoforms and also inhibits mTOR directly. The concentric circles indicate the PI3K isoform-selective inhibitors or mTOR inhibitors and their most likely targets with respect to tumor type and genetics. Bold print refers to topics of focus in this review. GBM, glioblastoma multiforme; MCL, mantle cell lymphoma; NET, neuroendocrine tumor; RCC, renal cell carcinoma; TBD, to be determined; TME, tumor microenvironment.

Development in oncology (12–19). On the other hand, targeting the entire group of class I PI3Ks is a “one-size-fits-all” strategy that might not achieve the selectivity for cancer cells that is a primary goal of molecular medicine: to target a selected patient population based on a defined molecular defect such that inhibiting the molecule has a greater impact in cancer cells than normal tissue. It is generally assumed that targeting a single or subgroup of isoforms will achieve the goal of limiting systemic toxicities (20). However, a central question has been whether anticancer efficacy can be achieved by just targeting a single isoform alone. Cell line studies suggest considerable redundancy among class I PI3Ks for sustaining proliferation and survival (21). Another challenge has been for medicinal chemists to design compounds that selectively target PI3K isoforms that are closely related in sequence and structure. Progress in this area has been advanced by x-ray crystal structures of each class I isoform (22–26) and by a landmark survey of diverse PI3K inhibitor chemotypes (11).

PI3Kα has received the most attention as a target in oncology. This is because gain-of-function mutations in the gene encoding PI3Kα (PIK3CA) occur very frequently in human tumors (27–29) (Fig. 1). Genetic experiments using cell lines and mouse models have supported the idea that activating PIK3CA mutations are both necessary and sufficient for tumor formation and maintenance (30–32). In addition, pharmacologic interrogations of cancer cell line panels have shown a good correlation between PIK3CA mutation status and sensitivity to pan-PI3K inhibitors or to compounds targeting both PI3K and mTOR (33–35). Early results from clinical studies support this correlation between PIK3CA mutation status and response to PI3K/mTOR inhibitors (36). PI3Kα is also expressed in lymphocytes and one study found that compounds with some selectivity for PI3Kα inhibit chemotaxis and survival of chronic lymphocytic leukemia (CLL) cells in vitro (37). Compounds with high selectivity for PI3Kβ are in early-stage clinical development (INK1117 and BYL-719), and one preclinical compound (A66) showed at least equivalent efficacy to a pan-PI3K/mTOR inhibitor in mouse models of PIK3CA mutant tumors (38).

The role of PI3Kβ in cancer is less clear. Overexpression of wild-type PI3Kβ is sufficient to transform chicken embryo fibroblasts (39), and a constitutively active mutant can cause prostate cancer in mice (40). However, activating mutations in the PIK3CB gene are not observed in human tumors. A recent crystal structure of PI3Kβ (26) provided a potential explanation by showing that the catalytic subunit (p110β) is constrained by an additional inhibitory contact with the regulatory subunit, an interaction that is not present in PI3Kα. Despite the multiple inhibitory contacts between p110β and the regulatory subunit, PI3Kβ seems to havebasal activity in cells (11). Indeed, evidence has emerged that PI3Kβ is required for PIP2 accumulation in epithelial tumor cells lacking PTEN phosphatase activity (Fig. 1). In a mouse model of prostate cancer driven by PTEN loss, deletion of PI3Kβ but not PI3Kα reduced tumorigenesis (41). PI3Kβ activation downstream of tyrosine kinases is difficult to detect (42); nevertheless, inactivating PI3Kβ impaired breast cancer development in a model driven by an activated tyrosine kinase, ERBB2 (43) (Fig. 1). PI3Kβ activity might also provide a resistance mechanism in tumors treated with selective PI3Kα inhibitors.

Published OnlineFirst November 29, 2011; DOI: 10.1158/2159-8290.CD-11-0249

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inhibitors (44). These findings have raised interest in clinical testing of PI3Kβ inhibitors or combined PI3Kα/PI3Kβ inhibitors in cancer.

PI3Kγ is expressed at highest levels in leukocytes but is also detectable in some other cell types. There have been sporadic reports of PI3Kγ activity in certain tumor types, yet overall the role of PI3Kγ as a cell-intrinsic oncoprotein seems limited. To date, much more effort has been devoted to PI3Kγ as a target in inflammatory diseases driven by leukocytes (16, 20). Intriguingly, PI3Kγ might have a very important role in the development of solid tumors through its role in innate immune cells. Inflammation driven by tumor-associated myeloid cells is now considered a hallmark of cancer, contributing to both cancer cell expansion and angiogenesis (7). A recent study showed that PI3Kγ is essential for myeloid cell adhesion to endothelium and extravasation into tumor sites in mice (45) (Fig. 1). Blockade of PI3Kγ specifically in myeloid cells is sufficient to diminish tumor growth with a concomitant reduction in vascular endothelial growth factor production and angiogenesis.

This discussion highlights the prominent role of PI3Kα and the potential contributions of PI3Kβ and PI3Kγ in cancer. What about PI3Kδ, the focus of this review? Like PI3Kγ, the PI3Kδ isoform is mainly expressed in leukocytes and has been widely viewed as a target for immune-related diseases (46). A large amount of effort has been devoted to understanding the biology of PI3Kδ in the immune system and to develop and characterize PI3Kδ-selective inhibitors. PI3Kδ is expressed in most lymphoid tumors but so are other class I isoforms, and activating mutations have not been found for PI3Kδ. Selective inhibitors of PI3Kδ are not strongly cytotoxic in vitro (47–49). It has therefore been surprising and exciting that the PI3Kδ-targeted compound CAL-101 has achieved remarkable clinical responses in certain B-cell malignancies (Fig. 1). As discussed in this review, the efficacy and tolerability of CAL-101 make sense in light of the somewhat restricted role for PI3Kδ in leukocyte biology and the specific diseases where CAL-101 has been effective. From the perspective of pharmaceutical development, the success of CAL-101 emphasizes the importance of (i) knowing your target; (ii) selecting the right disease/patient population; and (iii) treating the disease with an isoform-selective PI3K drug.

Target Validation of PI3Kδ

The B-cell receptor (BCR) for antigen and the coreceptor CD19 strongly activate PI3K, as first reported in the mid-1990s (50–52). Subsequent work showed that inhibitory receptors limit B-cell activation by reducing PIP3 (53, 54). The first genetic evidence for PI3K function in B cells came from studies of mice lacking the class I regulatory isoform p85α (55, 56). Deletion of p85α diminished the numbers of mature B cells and caused profound defects in B-cell proliferation and antibody production. In 2002, three groups reported similar B-cell defects in mice with targeted inactivation of Plik3δ, the gene encoding the PI3Kδ catalytic subunit (often referred to as p110δ) (57–59). One of these studies, from Okkenhaug and colleagues (59), used a knock-in approach in which a point mutation (D910A) produced a p110δ protein lacking lipid kinase activity but expressed at normal levels. The development of a kinase-dead knock-in model was a historic event in the PI3K field, because this approach prevents compensatory upregulation of other isoforms and retains potential noncatalytic functions of the protein (60). Hence, the kinase-dead knock-in strains are more faithful models of pharmacologic kinase inhibitors. Indeed, the p110δD910A strain has been the mouse model of choice for most subsequent studies of PI3Kδ function. As a case in point, p110δD910A B cells were used as a reference in a study of IC87114, the first PI3Kδ-selective inhibitor to be published (47). Comparison of IC87114-treated and p110δD910A B cells revealed similar impairments in BCR-mediated signaling and proliferation and equivalent defects in survival mediated by the cytokine interleukin-4 (IL-4).

IC87114 was originally discovered by ICOS Pharmaceuticals and subsequently developed further by Calistoga Pharmaceuticals. The high degree of selectivity of IC87114 towards PI3Kδ was achieved well before the p110δ crystal
structure was reported, yet IC87114 remains one of the most selective PI3Kδ inhibitors known. We now understand that IC87114 binding is favored by the conformational flexibility of the catalytic domain of p110δ and by the ability of IC87114 to interact with amino acid residues outside the ATP-binding pocket (Fig. 2) (22). The high selectivity of IC87114, together with the clear-cut B-cell deficits and overall benign phenotype of p110δ<sup>+/−</sup> mice, validated this compound class as potential therapeutics. Medicinal chemistry efforts using the IC87114 lead compound yielded the clinical candidate CAL-101 (Fig. 2). Before discussing CAL-101 efficacy and mechanisms, we first review the known functions of PI3Kδ in B cells.

**PI3Kδ in B Cells**

In mice, the naïve mature B-cell pool is divided into three major subsets: follicular (FO) B cells that recirculate, marginal zone (MZ) B cells that reside in the spleen, and B-1 cells that are abundant in body cavities. Genetic inactivation of PI3Kδ function greatly reduces development of MZ and B-1 cells (57–59). Mice treated with IC87114 also display an altered MZ B-cell compartment (48); indeed, loss of MZ B cells is a useful biomarker of PI3Kδ inhibition in mice. Although selective loss of PI3Kδ has little impact on numbers of mature FO B cells, the function of these cells is severely compromised (57–59). In vitro, B cells fail to enter the cell cycle after BCR stimulation and display reduced proliferation in response to Toll-like receptor ligands (e.g., LPS) (Fig. 3). Survival mediated by the cytokines IL-4 or BAFF is also diminished (Fig. 3). These defects are not a consequence of altered development as wild-type B cells treated with IC87114 show equivalent changes (47). In vivo, the antibody response to T-cell–independent antigens is reduced at least 100-fold. PI3Kδ-deficient mice also display aberrant responses to T-cell–dependent antigens. This might be partly the result of defective antigen presentation by B cells (61) as well as defects in the follicular helper T-cell subset (62). PI3Kδ does not play a positive role in all B-cell responses. For example, PI3Kδ inhibition promotes immunoglobulin class switching in vitro (63) and specifically augments the production of IgE both in vitro and in vivo (64, 65).

The mechanism for diminished BCR responses in PI3Kδ-deficient B cells has been studied extensively [Fig. 3; for more detail, see review articles (46, 66)]. In response to BCR crosslinking, PI3Kδ-deficient B cells display a reduced Ca<sup>2+</sup> mobilization response and an almost complete loss of Akt phosphorylation, a common readout of PI3K activation in cells (57–59). Again, similar results were observed in wild-type cells treated with IC87114 (47). The reduced Ca<sup>2+</sup> response is likely to explain much of the functional deficits, because the Ca<sup>2+</sup>-ionophore controls early events in antigen recognition and capture as well as later events such as transcription factor activation and gene expression. NF-κB activation and FOXO inactivation are both impaired in PI3Kδ-deficient B cells (47, 57).

The survival of peripheral B cells in the absence of antigen is sustained by the cytokine BAFF (Fig. 3) as well as tonic (basal) signaling through the BCR (67, 68). Chronic BCR signaling also sustains survival in a subset of diffuse large B-cell lymphoma (DLBCL) (69) and is implicated in survival of CLL cells (70). PI3Kδ-deficient mice show only a modest decrease in the FO B-cell pool (57–59), suggesting that other mechanisms can compensate to maintain survival. Srinivasan and colleagues (71), have shown that constitutively active PI3Kα is sufficient to maintain FO B-cell survival after BCR inactivation. However, the converse experiment has shown that PI3Kα loss-of-function does not affect B-cell survival (72). Investigation of compound mutant mice showed that loss of both PI3Kα and PI3Kδ, but not PI3Kα or PI3Kδ alone, is required to eliminate peripheral FO B cells (72). One implication of this result is that selective pharmacologic PI3Kδ inhibition should not lead to disappearance of mature FO cells, a conclusion supported by studies of mice treated with IC87114 (48). Assuming that BCR-positive B-cell tumors rely on similar mechanisms for survival, these results also predict that cell-intrinsic survival mechanisms might be resistant to selective PI3Kδ inhibition. Preclinical data with CAL-101 support this prediction, as discussed subsequently.

**Figure 3.** Diverse stimuli converge on PI3Kδ in B cells. The B-cell receptor (BCR) for antigen, the B-cell co-receptor (CD19, part of a complex with CD21 and CD81), and Toll-like receptors for pathogen-associated molecular patterns all activate PI3Kδ. Cytokines derived from lymphoid stromal cells (BAFF) and T cells (IL-4, TNF-α) also activate PI3Kδ. Chemokines, which signal through GPCRs to PI3Kδ and PI3Kγ in most other cell types, activate PI3Kδ in B cells. The key outputs of PI3Kδ activity for B-cell proliferation and survival are shown below the red arrows. PI3Kδ, phosphoinositide 3-kinase; IL-4, interleukin-4; TNF-α, tumor necrosis factor-α; GPCR, G-protein-coupled receptors.
An unexpected and important phenotype of PI3Kδ-deficient B cells is their reduced response to chemokines (Fig. 3). Like other immune cells, B cells rely on chemokine signals to control their positioning within lymphoid tissue both in the absence and presence of antigen (73, 74). Resting FO B cells express high levels of the receptor CXCR5, which binds to CXL13 localized in B-cell follicles. Activated FO B cells upregulate CCR7, whose ligands CCL19 and CCL21 are abundant in T-cell zones, thus allowing activated B cells to meet up with cognate T cells. B cells at most developmental stages also express CXCR4, whose ligand CXCL12 is important during bone marrow development and peripheral B-cell recirculation. CXCR4, CXCR5, and CCR7 are all G-protein-coupled receptors, which in most cells activate the PI3Kγ isoform (and sometimes PI3Kδ) rather than PI3Kα or PI3Kδ (5). Nevertheless, chemokine-mediated signaling and migration are markedly impaired in PI3Kδ-deficient B cells (48, 75). The mechanism for chemokine receptor coupling to PI3Kδ in B cells remains mysterious, but likely has central relevance to the efficacy of CAL-101 as discussed subsequently.

PI3Kδ is also a signaling hub in T cells, mast cells, and other immune cells (Supplementary Fig. S1) (46, 76). However, PI3Kδ inactivation generally has more modest effects on these other cell types. This distinction is partly the result of functional overlap of PI3Kδ with other PI3K isoforms. For example, thymocyte development is severely compromised by combined loss of PI3Kδ and PI3Kβ but not by inactivation of PI3Kδ alone (77–79). PI3Kδ does play a significant role in clonal expansion and differentiation of CD4 T cells (80–82). With respect to chemokine responses in T cells, the role of class I PI3K as a whole is rather limited, and PI3Kδ seems to be the relevant isoform (83). Curiously, in some myeloid subsets, the PI3Kγ isoform couples not only to chemokine receptors, but also to tyrosine kinase-linked receptors that conventionally signal through the other class I isoforms (45). Overall, mouse models of PI3Kδ deficiency support the prediction that acute PI3Kδ inhibition should affect the function of B cells more dramatically than most other immune cell types but with selective effects on trafficking and antigen responses.

THE CAL-101 STORY

CAL-101 is a quinazoline—(S)-2-[1-[[9H-purin-6-yl]amino]propyl]-5-fluoro-3-phenylquinoxalin-4(3H)-1— that inhibits PI3Kδ with IC_{50} value of 2.5nM in vitro (84). The compound inhibits other class I PI3Ks only at much higher concentrations (40- to 300-fold higher IC_{50}) and has essentially no activity against other lipid kinases and protein kinases including the PTKK-family kinases mTOR and DNA-PK (84, 85). In cell-based assays, CAL-101 blocks PI3Kδ-dependent responses at greater than 240-fold lower concentrations than responses dependent on other isoforms (85).

For the first CAL-101 study in human disease, Calistoga selected allergic rhinitis (Clinical Trials identifier NCT00836914). This choice reflects the initial positioning of PI3Kδ inhibitors in the inflammation drug discovery pipelines (86). Although PI3Kδ remains undoubtedly an attractive target for immune disease therapy, the subsequent studies of CAL-101 in B-cell malignancies have established drugs of this class as an exciting new therapeutic strategy for the treatment of hematologic cancers. Therapeutic application in both inflammation and B-cell malignancies is reminiscent of the anti-CD20 antibody rituximab.

The first study of CAL-101 in cancer (NCT00710528) recruited patients with relapsed or refractory B-cell malignancies. These diseases included CLL, non-Hodgkin’s lymphoma (NHL), acute myeloid leukemia (AML), and multiple myeloma (MM). Although most of the data have not yet been published, some of the results have been presented at conferences over the past year (87, 88). The most impressive clinical responses were in CLL and certain subtypes of NHL. In this phase I dose-escalation study, primary goals were to study CAL-101 safety, pharmacokinetics (PK), and pharmacodynamics. Therefore, it was surprising and encouraging that CAL-101 caused lymph node shrinkage in all evaluable patients with CLL and across all dose levels. A significant percentage of subjects showed durable responses (approximately 25% overall response rate) and remained on study for many cycles of treatment. CAL-101 showed a favorable tolerability profile, even after sustained treatment. Mouse studies had suggested that PI3Kδ inhibition would greatly elevate levels of IgE (64, 65), but this has not yet been reported in the human patients. It will be important to determine whether reported dose-limiting toxicities are mechanism-based and predictive of PI3Kδ inhibitors as a class (not only for oncology, but also to support development in other disease settings). It is worth pointing out that the CLL subjects were mostly older adults with comorbid conditions, who had been heavily pretreated and had failed previous therapies. Hence, achieving meaningful single-agent clinical responses with manageable side effects was a significant feat. Applying a PI3Kδ inhibitor in front-line therapy might produce even better results.

A key observation in the patients with CLL was that lymph node responses were accompanied by a transient elevation in circulating lymphocyte counts, known as lymphocytosis. This phenomenon is likely the result of lymphocytes being released from lymphoid tissue microenvironments and/or failing to home from the blood into lymph nodes. The observation that lymphocytosis occurs, but is transient, suggests that CAL-101 acts by dislodging CLL cells from the protective niche of the lymph node, rendering them susceptible to gradual apoptosis from loss of survival signals. It is also possible that CAL-101 has some direct proapoptotic effect on CLL cells both in lymphoid tissue and after release into the blood.

There is growing consensus that PI3K inhibitors, like most targeted therapies, will be most effective when used in combination (7, 89). One current study (NCT01088048) is testing combinations of CAL-101 with two commonly used agents in CLL and indolent NHL (NHL): rituximab (anti-CD20) and bendamustine. The results that have been presented, albeit preliminary, indicate high response rates with all combinations in both diseases. Interestingly, patients with CLL treated with CAL-101 plus rituximab or bendamustine did not display lymphocytosis. One interpretation is that the combination treatment is directly toxic to malignant cells even in their microenvironmental niche. Another possibility is that CAL-101 releases the cells from these niches to increase vulnerability to toxic effects of the companion agent. Regardless of the
mechanism, these results provide encouragement for testing PI3Kδ inhibitor combinations in other disease settings, including blood cancers that do not respond to single-agent PI3Kδ inhibition. Combining CAL-101 with lenalidomide in CLL also shows promise based on preclinical studies (90). An important objective for such combination studies is to establish appropriate long-term dose levels and safety margins.

The initial clinical data from CAL-101 trials has created tremendous excitement in the field of PI3K drug development. Thousands of biologists and chemists have been working for the past decade to validate targets and bring compounds forward, and CAL-101 provides proof-of-concept that these efforts will pay off with significant advances in clinical management of cancer. At the same time, there is considerable surprise that the biggest success to date has come in indolent blood cancers (CLL, iNHL) and that so far the most effective drug is selective for PI3Kδ. Leukemia and lymphomas generally express all class I PI3K isoforms, rarely carry mutations in PI3K genes (91), and have lower incidence of PTEN loss compared with epithelial tumors (92, 93). CLL and iNHL do not generally express activated receptor tyrosine kinases or oncogenic Ras, yet these patients responded better than those with AML, a disease with high frequency of receptor tyrosine kinase activation (Flt3, Kit) or activated Ras and associated with elevated p110δ expression (94, 95).

In addition, the first set of preclinical studies of CAL-101 did not clearly predict the dramatic efficacy of the drug in human trials (84, 85, 96). Cytotoxic effects on human CLL samples in tissue culture are inconsistent and generally achieved only at CAL-101 concentrations approaching 10 μM. This is approximately 1,000 times the IC50 value for CAL-101 in PI3Kδ-selective cell-based assays and is well within the range where PI3Kδ and PI3Kγ isoform inhibition is observed. These results suggest that selective PI3Kδ inhibition has minimal impact on cell-intrinsic survival signals in B-lineage cancer cells. It should be noted that clinical exposures to CAL-101 are greater than 2 μM even at the lowest doses tested, suggesting that partial inhibition of non-PI3Kδ isoforms may contribute to cytotoxic effects in patients.

Further clues to the efficacy of CAL-101 emerge when examining data from CLL cells cultured in the presence of cytokines or stromal cells. The cytokines BAFF and TNF-α boost survival of primary CLL cells in vitro but this effect is attenuated by CAL-101 at the relatively low concentration of 0.1 μM (96). Similarly, the survival advantage conferred by growth on fibronectin or stromal cell layers is reversed by 0.1 μM CAL-101 (96). At a concentration of 0.5 μM, CAL-101 completely blocks survival signaling from BCR engagement and significantly reduces survival of CLL cells cultured on specialized “nurse-like cells” (NLC) derived from peripheral blood monocytes (97). Together these observations suggest that PI3Kδ is essential for CLL cell survival pathways initiated by extrinsic factors in the microenvironment. This mechanism could also contribute to the impressive efficacy of CAL-101 combination therapies. Indeed, CAL-101 potentiates the prodeath effects of bendamustine, fludarabine, and dexamethasone in CLL cells grown on stromal cells (97).

A recent publication from Calistoga, in collaboration with MD Anderson Cancer Center, provided additional key insights into the mechanism of action of CAL-101 (97). One important finding was that CAL-101 blocks CLL cell chemotaxis in response to CXCL12 and CXCL13. In accord, CAL-101 inhibits chemokine-dependent increases in AKT phosphorylation. These results are consistent with studies of PI3Kδ-deficient mouse B cells mentioned previously and provide a likely explanation for the release of CLL cells from the lymph node environment into the circulation. CAL-101 also suppresses the secretion of cytokines by both the CLL cells (CCL2, CCL3) and stromal cells (CXCL13) in coculture.

**Figure 4. Inhibiting PI3Kδ targets both malignant B cells and the tumor microenvironment.** (Left) CLL cells are drawn from the vasculature into lymphoid organs by chemotactic signals from chemokines. In the lymph node microenvironment, CLL cells signal bidirectionally with nurse-like cells (NLC) to generate a suite of cytokines and chemokines to promote survival. T cells in the lymphoid tissue can also secrete factors to promote CLL survival. Not shown: adhesion receptors promote maintenance of CLL cells in the protective niche of the lymph node and stabilize interactions with NLC, T cells, other stromal cells, and extracellular matrix. (Right) On treatment with the PI3Kδ inhibitor, loss of survival cell-intrinsic survival signals and disruption of the protective niche causes some CLL cells to die in the lymph node. Most of the CLL cells are released into the efferent lymph and eventually into the blood, causing lymphocytosis. This eventually resolves as circulating CLL cells die from niche exclusion.
The coculture of CLL and NLC triggers release of additional factors (CCL7, CCL17, CCL22, soluble CD40L, IL-6, and TNF-α) whose secretion is reduced by CAL-101. An important element of this study was the analysis of cytokine levels in peripheral blood of patients with CLL treated with CAL-101. The data show a uniform decrease in CCL2, CCL3, and CXCL13 in patient plasma after 28 days of treatment compared with pretreatment measurements (97). Assessment of AKT phosphorylation in circulating CLL cells was used as a pharmacodynamics marker and showed a strong correlation with reduced chemokine concentrations. This indicates that at pharmacologically active doses, CAL-101 disrupts the CLL microenvironment, resulting in reduced production of chemokines.

Putting all the data together, one can now propose a model to explain the efficacy of CAL-101 in patients with CLL (Fig. 4). PI3Kδ inhibition reduces both the production of chemokines and the response of CLL cells to these factors. This leads to release of CLL cells from the protective niche in lymphoid tissue. Concurrently, those cells remaining in the niche receive weakened survival signals from microenvironmental factors such as BAFF, CD40L, adhesion molecules, and extracellular matrix. Some of the tumor cells may die rapidly but the majority of cells seem to exit lymphoid tissue and circulate in the blood. Gradually, CLL cells excluded from the lymphoid tissue niche undergo apoptosis.

When rituximab or bendamustine is included in the treatment regimen, the destruction of these circulating B cells is accelerated.

**OUTLOOK**

It seems that PI3K inhibitors are now destined to enter clinical practice in CLL and possibly other blood cancer settings. Calistoga became part of a larger pharma company (Gilead), and plans are in place for accelerated clinical trials and rapid registration of CAL-101 (now named GS-1101). Several other companies have PI3K inhibitor programs (Table 1) and some are no doubt diverting resources from inflammation to oncology. At the same time, the CAL-101 data raise several interesting and important questions for further consideration.

One question is whether targeting additional PI3K isoforms would provide even greater benefit. The pan-PI3K inhibitors and PI3K/mTOR inhibitors currently in clinical trials have been tested mainly in patients with solid tumors. Would a broad-spectrum PI3K inhibitor provide more rapid elimination of CLL cells, and/or durable responses in patients refractory to CAL-101? This possibility is supported by data showing greater inhibition of CXCR4-mediated CLL chemotaxis by pan-PI3K inhibitors relative to PI3K-selective inhibitors (37). It is possible that pan-PI3K inhibitors will

### Table 1. Phosphoinositide 3-kinase inhibitors in development

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*Eli Lilly patent applications: WO2009064802; WO2008064018.
†Merck Serono patent application: WO2011058149.
‡Incyte patent applications: WO2011075630; WO2011008487.
§Res pivert patent applications: WO2011048111.
*The company has phosphoinositide 3-kinase program. Status and patent information are not available.
**The structure of XL-499 (Exelixis) is not disclosed. The patent information is not available.
††The structure of KAR-4141 (Karus) is not disclosed. The patent information is not available.
§§The development candidate ID and structure are not disclosed. Incozen Patent application: WO2011055215.
| The structure of CAL-263 is not disclosed. Calistoga/Gilead patent application: WO2010129331.
| The development candidate ID and structure are not disclosed. The patent information is not available.
be more effective than selective PI3K inhibitors in leukemias
arising from immature B cells (B-ALL), terminal center B
cells (DLBCL), plasma cells (MM), or myeloid progenitors
(AML). If pan-PI3K inhibitors cause unmanageable side
effects, an alternative strategy would be to design compounds
that target PI3K along with another class 1 isoform. A dual
PI3Kδ/PI3Kε inhibitor would be predicted to suppress sur-
vival more completely in malignancies derived from mature B
cells. Alternatively, a dual PI3Kδ/PI3Ky inhibitor might block
chemokine signaling across a broader range of lymphoid ma-
lignancies. Dual PI3Kδ/PI3Ky inhibitors were found to sup-
press inflammatory markers more effectively than selective
PI3K inhibitors using in vitro assays of human lymphocytes
(49). A molecule that inhibits PI3K and mTOR might also be
useful. Targeting mTOR with an allosteric (Rapalog) or
ATP-site kinase inhibitor (TORC1/2) could further suppress
survival signals and metabolic responses to environmental
cues including growth factors and nutrients.

The mechanism of action of CAL-101 also raises the ques-
tion of whether blocking microenvironmental signals is a
general key to success in targeted cancer therapies. Many
drugs that kill cancer cells in vitro fail to suppress tumor
growth in vivo because of cell-extrinsic survival signals.
Conversely, the CAL-101 story teaches us that a drug with
limited activity on isolated tumor cells can be highly effective
in vivo by disrupting the microenvironment. Altering the in
vitro conditions to include stromal support, thereby model-
ing the microenvironment more faithfully, provided a sensi-
tized system that revealed potent antileukemic activity. Such
systems are therefore valuable even at early stages of drug
screening. An intriguing possibility is that PI3K inhibition
could also provide benefit in solid tumors in which B cells
contribute to the supportive microenvironment. For exam-
ple, B cells are part of the inflammatory infiltrate supporting
survival of prostate cancer cells (98).

It is also important to understand more about the micro-
environment in malignancies that do not respond to isoform-
selective compounds. For example, CAL-101 has shown lesser
efficacy in MM versus CLL. Both are relatively slow-growing
malignancies arising in B-lineage cells, and patient samples cul-
tured in vitro showed similar degrees of growth arrest and apop-
tosis in the presence of CAL-101 (84, 85, 96). However, the MM
patient population did not respond as favorably to CAL-101
treatment in clinical trials. Whereas the protective niche for
CLL cells is in lymph nodes and the spleen, the primary niche for
MM cells is in the bone marrow. In this case, in vitro models
were not predictive of in vivo efficacy because CAL-101 was able
to block the prosurvival effects of IL-6 or bone marrow stromal
cells in MM cell lines in culture (84).

Selectively targeting PI3K isoforms to promote rather than
disrupt immune responses to tumors is an important goal.
CAL-101 is not cytotoxic to normal lymphocytes even at 10 μM.
However, it is important to keep in mind that PI3K inhibitors
are likely to impact significantly the differentiation and migra-
tion of T cells, thereby influencing the immune response to
tumor cells. IC87114 suppresses proliferation of both murine
and human T cells (82). CAL-101 strongly suppresses cytokine
production by human T cells, even when the drug is present at
a low concentration (0.1M) (96). PI3K inhibition also promotes
the induction of a regulatory T-cell fate but can also inhibit
the function of these cells (66, 76). PI3K inhibition affects the
activation of natural killer cells (99) with possible detrimental
effects on immunosurveillance and antibody-dependent cel-
lular cytotoxicity (ADCC) (100). Because rituximab and other
monoclonal antibody therapies require ADCC for maximal
efficacy, interfering with ADCC will limit the efficacy of com-
bination approaches. PI3K isoforms (including PI3Kδ) regulate
maturation and migration of macrophages and dendritic cells
(101–103), which play important roles in the microenviron-
ment of many tumors. In summary, understanding how PI3K
inhibitors influence the function of T cells, natural killer cells,
and myeloid cells will be key to choosing the optimal pharma-
alogic profile.

Discussions of PI3K inhibitor efficacy in cancer often touch
on the issue of cancer stem cells. Most normal stem cells in tis-
sues exhibit low PI3K activity, and artificial elevation of PI3K
or AKT, or inactivation of FOXO proteins, promotes exit from
quiescence and stem cell exhaustion (104–107). Consequently,
there is concern that cancer stem cells will be resistant to PI3K
inhibition, analogous to the imatinib-resistance of leukemic
stem cells (LSC) in CML. Although some studies suggest that
LSCs have elevated PI3K/AKT activity relative to normal he-
matopoietic stem cells (105), a recent article showed that LSCs
in AML have elevated FOXO function that is necessary to
maintain this compartment (108). Therefore, PI3K inhibitors
such as CAL-101 might be less effective in AML because such
agents would enforce FOXO activity in the LSC compartment.
It will be interesting to examine the differentiation state of re-
sistant cells in patients who exhibit incomplete responses to
PI3K inhibitors. This can be done using multiparameter flow
cytometry or through genomewide studies of transcriptional or
epigenetic signatures.

A broader question is whether the success of CAL-101 should
alter the development path for other PI3K inhibitors. Current
dogma is that candidate therapeutics should be validated
extensively in cell-based assays and then rodent tumor mod-
els before initiating human trials. However, cell lines and xe-
nograft models have significant limitations for predicting drug
response of human tumors growing in their natural host (14).
As discussed previously, coculture systems to mimic the micro-
environment are helpful but do not always provide predictive
value. Genetically engineered mice have advantages for mod-
ing cancer development and maintenance but have a high
cost and do not recapitulate the genetic complexity of bona
fide human tumors. In the case of CAL-101, no data have been
published from mouse tumor models (probably as a result of
poor PK of this drug in mice) and the data from isolated cell
lines did not predict a strong cytotoxic effect. On the other
hand, the clinical data from human patients demonstrated
clear efficacy even in a relatively small (and therefore relatively
inexpensive) phase I study. The clinical trial was initiated before
much of the mechanistic data on chemokines and microen-
environmental signals was generated. These considerations sup-
port the merit of moving compounds quickly into the clinic as
soon as favorable tolerability can be shown in preclinical tox-
icity studies. Contrary to early predictions, most PI3K inhibitors
(including pan-PI3K and PI3K/mTOR) appear to be reasonably
well tolerated and there is strong justification for evaluating
their efficacy in patients with cancer, ideally selected (enriched)
cancer types.
Regardless of the path to the clinic, it is clear that trials should incorporate extensive analysis of biomarkers of treatment response. These can be biomarkers of intracellular signaling (phospho-AKT, phospho-S6) as well as immune activation (cytokines, chemokines). Correlation of biomarker changes with treatment response will help reveal the mechanism of drug action in vivo. In the case of the CAL-101 trial in CLL, the dramatic reductions in circulating cytokines and chemokines provided important clues to explain drug efficacy (97). Measuring chemokines and cytokines in plasma might help establish a blood-based clinical biomarker for correlation with drug dose/exposure and therapeutic efficacy.

In summary, CAL-101 has opened up many new avenues of research for both laboratory and clinical scientists. As more PI3K pathway inhibitors are tested in human patients, we anticipate further exciting advances in disease management along with new biologic insights.

Disclosure of Potential Conflicts of Interest
D.A. Fruman is a scientific advisor to Intellikine and receives research support from Intellikine. C. Rommel is an employee of Intellikine.

Acknowledgments
We thank Kevoi Shokat, Klaus Okkenhaug, Vito Palombillo, Yi Liu, and Pingda Ren for helpful comments on the manuscript. We also thank Pingda Ren and Liansheng Li for assistance preparing figures and tables regarding inhibitor compounds.

Grant Support
D.A. Fruman was supported by a Discovery Grant from the University of California Industry-University Cooperative Research Program.

Received September 26, 2011; revised October 21, 2011; accepted October 21, 2011; published OnlineFirst November 30, 2011.

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PI3Kδ Inhibitors in Cancer: Rationale and Serendipity Merge in the Clinic

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Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-11-0249

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