LYMPHOID NEOPLASIA

The PI3K/mTOR inhibitor PF-04691502 induces apoptosis and inhibits microenvironmental signaling in CLL and the Eμ-TCL1 mouse model

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Key Points

- PF-04691502 induces potent apoptosis in CLL cells and suppresses prosurvival anti–immunoglobulin M signaling and CXCL12-induced migration.
- PF-04691502 displays powerful antitumor effects in vivo in the Eμ-TCL1 mouse model.

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of CD5+CD19+ cells in the peripheral blood, lymph nodes, and bone marrow. Several prognostic markers are associated with progressive disease, including unmutated immunoglobulin heavy chain variable regions (IGHV) and high expression of CD38 and ZAP-70. Current treatment strategies involve the combination of fludarabine, cyclophosphamide, and rituximab; however, CLL largely remains incurable, with drug resistance and treatment relapse being common occurrences.

Recently, inhibitors targeting key signaling molecules such as Bruton tyrosine kinase (BTK; ibrutinib) and phosphatidylinositol 3-kinase (PI3K; idelalisib) have been approved due to exceptional clinical responses. In this regard, the PI3K/mTOR pathway is particularly attractive because it is activated upon ligation of various chemokine and cytokine receptors expressed by CLL cells, as well as after B cell–receptor (BCR) engagement. Class 1 PI3Ks are divided into class 1A (PI3Kα, PI3Kβ, and PI3Kδ) and class 1B (PI3Kγ) isoforms. The expression of PI3Kδ and PI3Kγ have crucial roles in a plethora of leukocyte functions, including proliferation, antibody secretion, survival, migration, and reactive oxygen species generation. Functional redundancy between PI3K isoforms is evident and supported by the fact that multiple isoforms require inhibition to fully reverse the neutrophil survival induced by granulocyte macrophage–colony-stimulating factor. PI3K exists in a complex signaling network with multiple partners, including those regulating mTOR. mTOR complex 1 (mTORC1) is a serine/threonine kinase activated downstream of PI3K that controls protein translation, growth, and proliferation in part via modulation of S6 ribosomal subunit activity. In contrast, mTOR complex 2 (mTORC2) phosphorylates AKT and is required for maximal activation of protein kinase B (AKT) (supplemental Figure 1A-B, available on the Blood Web site).

PI3K signaling is known to be overactive in CLL, with patients with unmutated IGHV having increased PI3K gene expression compared with patients with mutated IGHV. The PI3Kγ selective inhibitor idelalisib has been approved by the US Food and Drug Administration for the treatment of chronic lymphocytic leukemia.

Current treatment strategies for chronic lymphocytic leukemia (CLL) involve a combination of conventional chemotherapeutics, monoclonal antibodies, and targeted signaling inhibitors. However, CLL remains largely incurable, with drug resistance and treatment relapse a common occurrence, leading to the search for novel treatments. Mechanistic target of rapamycin (mTOR)-specific inhibitors have been previously assessed but their efficacy is limited due to a positive feedback loop via mTOR complex 2 (mTORC2), resulting in activation of prosurvival signaling. In this study, we show that the dual phosphatidylinositol 3-kinase 3-kinase (PI3K)/mTOR inhibitor PF-04691502 does not induce an mTORC2 positive feedback loop similar to other PI3K inhibitors but does induce substantial antitumor effects. PF-04691502 significantly reduced survival coincident with the induction of Noxa and Puma, independently of immunoglobulin heavy chain variable region mutational status, CD38, and ZAP-70 expression. PF-04691502 inhibited both anti–immunoglobulin M–induced signaling and overcame stroma-induced survival signals and migratory stimuli from CXCL12. Equivalent in vitro activity was seen in the Eμ-TCL1 murine model of CLL. In vivo, PF-04691502 treatment of tumor-bearing animals resulted in a transient lymphocytosis, followed by a clear reduction in tumor in the blood, bone marrow, spleen, and lymph nodes. These data indicate that PF-04691502 or other dual PI3K/mTOR inhibitors in development may prove efficacious for the treatment of CLL, increasing our armamentarium to successfully manage this disease. (Blood. 2015;125(26):4032-4041)
Administration to treat CLL patients with relapsed disease in combination with rituximab.4 Idelalisib inhibits chemokine- and BCR-induced signaling and sensitizes CLL cells to standard genotoxic agents.19,21 In patients, idelalisib has a dual mechanism of action: (1) it directly reduces CLL cell viability and (2) it disrupts stromal cell interactions and releases CLL cells from their protective microenvironments into the blood, where they are more susceptible to chemotherapy-induced apoptosis. However, single-isof orm inhibitors against PI3Kδ/δ6 and pan-class-1 PI3K inhibitors in CLL cells have all been reported to induce apoptosis.22,23 Pharmacologic inhibition of mTOR induces cell cycle arrest and apoptosis in CLL cells24–26; however, prolonged inhibition of mTOR is known to disrupt negative feedback loops and cause increased AKT inactivation in other malignancies.27,28

Given the important role of both PI3K and mTOR in CLL cell survival, the dual pharmacologic inhibition of both class-1 PI3K and mTOR signaling may offer new therapeutic potential, with the possibility of deeper remissions or as an alternative after resistance to idelalisib. PF-04691502 is a potent selective dual-class-1 pan-PI3K/mTOR inhibitor29 shown to inhibit tumor growth and to promote apoptosis in solid tumors.29-33 PF-04691502 inhibits PI3Kδ, PI3Kε, PI3Kγ, and PI3Kδ at 50% inhibitory concentration values (IC50) of 1.6 nM (Ki), 1.8 nM (Ki), 1.9 nM (Ki), and 2.1 nM (Ki), respectively. In cells, inhibition with PF-04691502 has resulted in IC50 values of 3.8 nM and 7.5 nM for P-AKT and P-AKT, respectively. During phase I clinical trials, PF-04691502 was shown to have a similar safety profile to other PI3K inhibitors.34

In this study, we showed that in primary CLL cells, PF-04691502 induced caspase-dependent apoptosis. In contrast, little effect on the viability of normal B and T lymphocytes was evident. BCR- and CXCR4-stimulated signaling was inhibited by PF-04691502, and chemokine-mediated motility was potently reduced. Similarly impressive antitumor effects were seen with the Eμ-TCL1 murine CLL-like cells in vitro and in vivo. Together, these data indicate that concomitant targeting of mTOR and PI3K is a powerful approach for treating CLL.

Materials and methods

Patients and cells

Diagnosis of CLL was according to the International Workshop on CLL 2008 criteria.35 Forty-nine CLL isolates were studied after informed written consent and in accordance with ethics committee approvals under the Declaration of Helsinki (reference 228/02/t) (supplemental Table 1). Procedures for the isolation of malignant cells and the determination of their purity have been described previously.36 All isolates contained >90% CD19+CD5+ cells. Normal B and T lymphocytes from peripheral blood were taken from healthy age-matched donors after written consent, and organs were harvested for further analysis. Total organ tumor burden was calculated by CD5+ B220+ flow cytometry using Quantibrite counting beads (Life Technologies).

Reagents

Tissue culture materials were from Life Technologies (Paisley, United Kingdom). ZVAD.fmk was from Enzo Life Sciences (Exeter, United Kingdom). PF-04691502 was provided by Pfizer and purchased from Selleck Chemicals (Houston, TX) for in vivo studies. Idelalisib, BYL719, everolimus, GSK2636771, and AS-605240 were from Selleck Chemicals. Annexin V was from the South-ampton Cancer Research United Kingdom core proteomics facility. Anti-IgM was from Cambridge Bioscience (Cambridge, United Kingdom) and CXCL12 was from Miltenyi Biotec (Bisley, United Kingdom). HFFF2 cells were a kind gift from Professor Thomas (University of Southampton).

Cell culture and protein extraction

CLL cell culture and protein extraction for immunoblot analysis were performed as previously described.38,39 Soluble anti-immunoglobulin (Ig)M was used at 20 µg/mL; bead-bound immobilized anti-IgM was added at a 2:1 ratio of beads to CLL cells. Densitometry of immunoblots is depicted in supplemental Figures 6 and 7.

Results

mTOR inhibition of CLL cells augments AKT signaling

Previous studies in other tumor systems have shown that inhibition of mTOR with everolimus/rapamycin results in augmentation of the mTORC2 target, AKT.27,28 Here, we show that this augmentation of AKT also occurs in CLL cells, both in resting conditions and more effectively after BCR engagement with soluble anti-IgM antibodies (Figure 1A-B), with the expected effects on the mTOR target, phosphorylated S6 kinase (pS6K) (Figure 1C). In contrast, the dual PI3K/mTOR inhibitor PF-04691502 does not induce hyperphosphorylation of AKT and inhibits the pathway, as shown with other PI3K inhibitors (supplemental Figure 1C). pS6K was completely abrogated with PF-04691502, whereas phosphorylated S6 ribosomal protein (pS6S235/236) is regulated by both the mitogen-activated protein kinase and PI3K/mTOR signaling pathways and was therefore only partially affected by both everolimus and PF-04691502 (Figure 1D). AKT is also phosphorylated at AKTThr308 in a PI3K-dependent manner by 3’-phosphoinositide-dependent kinase-1 (PDK-1), and was not significantly augmented by everolimus treatment before or after anti-IgM treatment. However, PF-04691502 completely abrogated AKTThr308 phosphorylation (Figure 1E). Together, these data indicate that the same negative feedback loop involving mTORC2 is in operation in CLL cells but is circumvented by PF-04691502.
PF-04691502 reduces viability in CLL cells independently of prognostic markers

CLL samples were treated with PF-04691502, and viability was assessed using annexin V/PI staining (supplemental Figure 2A). PF-04691502 reduced viability of CLL cells after 24, 48, and 72 hours (Figure 2A-B), with an IC50 value of 0.96 μM (median 0.65 μM), 0.32 μM, and 0.25 μM, respectively. Each of the same CLL samples treated with idelalisib did not reach its IC50 value up to 40 μM (Figure 2A), in agreement with the literature.19,20 Normal B and T cells from age-matched controls were largely unaffected by PF-04691502 over a similar dose range. No significant differences in mean IC50 values for PF-04691502 were observed between unmutated and mutated IGHV genes (Figure 2C) or between high and low ZAP-70 (>.30%) (Figure 2D) or CD38 expression (>.30%) (Figure 2E). However, a trend for lower IC50 values was observed in unmutated compared to mutated IGHV genes. Furthermore, 3 CLL samples with 17p del (2 of 3 samples >70% 17p del by fluorescence in situ hybridization) were as susceptible to PF-04691502-induced death as wild-type samples (supplemental Figure 2B), indicating that PF-04691502 may also have activity independent of 17p del status.

PF-04691502 induces apoptosis in a caspase-dependent mechanism

To determine the mechanism of death induced by PF-04691502, we first investigated expression of the key proapoptotic BH3-only proteins Noxa, Puma, Bad, Bim, and Bax, and the antiapoptotic Bcl-2 family proteins Mcl-1 and Bcl-2, which regulate apoptosis within CLL cells. Incubation with PF-04691502 (0.31-1.25 μM) induced Noxa and Puma expression (protein and messenger RNA) in a dose-dependent manner (Figure 3A; data not shown). No significant changes were observed for Bim, Bad, Bcl-2, or Mcl-1 (data not shown). Basal pAKT S473 and pS6K T389 were inhibited by PF-04691502, as expected. In response to proapoptotic stimuli, Bax undergoes a conformational change and subsequently becomes inserted into the outer mitochondrial membrane where it facilitates cytochrome c release and subsequent

PF-04691502 reduced viability of CLL cells in combination with the mTOR inhibitor everolimus (mTORC1 inhibitor). Each inhibitor alone (1 μM) reduced viability by no more than ~10% (supplemental Figure 2C). This result was in contrast to PF-04691502, which at the same concentration reduced CLL cell viability by >50%. The combination of all 5 selective inhibitors at 1 μM significantly reduced CLL cell viability more than any of the inhibitors alone, albeit significantly less than PF-04691502 (P = .004; n = 11). Lastly, we showed that a combination of idelalisib with everolimus was significantly more efficacious than either agent alone (P = .01 and P = .02, respectively) (supplemental Figure 2D) but significantly less so than PF-04691502 (P = .03). Together, these data suggest that PF-04691502 likely achieves its robust killing effects through simultaneous inhibition of mTORC1, mTORC2, and PI3Kδ.

Combination of isoform-specific PI3K inhibitors and an mTOR inhibitor mimic PF-04691502

PF-04691502 reduced viability of CLL cells to a greater extent compared to idelalisib. Therefore, we sought to determine whether we could replicate its efficacy using a combination of isoform-specific PI3K inhibitors, including BYL719 (PI3Kα inhibitor), GSK2636771 (PI3Kβ inhibitor), idelalisib (PI3Kδ inhibitor), and AS-605240 (PI3Kγ inhibitor), in combination with the mTOR inhibitor everolimus (mTORC1 inhibitor). Each inhibitor alone (1 μM) reduced viability by no more than ~10% (supplemental Figure 2C). This result was in contrast to PF-04691502, which at the same concentration reduced CLL cell viability by >50%. The combination of all 5 selective inhibitors at 1 μM significantly reduced CLL cell viability more than any of the inhibitors alone, albeit significantly less than PF-04691502 (P = .004; n = 11). Lastly, we showed that a combination of idelalisib with everolimus was significantly more efficacious than either agent alone (P = .01 and P = .02, respectively) (supplemental Figure 2D) but significantly less so than PF-04691502 (P = .03). Together, these data suggest that PF-04691502 likely achieves its robust killing effects through simultaneous inhibition of mTORC1, mTORC2, and PI3Kδ.
apoptosis. Using the conformational change–specific 6A7 antibody, we showed that CLL cells incubated with PF-04691502 produced a marked increase in the active, mitochondrially resident conformation of Bax (Figure 3B). Furthermore, treatment of CLL cells with PF-04691502 (0.31-5 μM) for 18 hours induced caspase-3 cleavage and subsequent appearance of the p85 cleaved subfragment of poly(ADP-ribose) polymerase (PARP) (Figure 3C). To confirm PF-04691502-induced apoptosis of CLL cells in a caspase-dependent manner, we treated cells with the pan-caspase inhibitor ZVAD.fmk. ZVAD.fmk significantly prevented the PF-04691502-induced decrease in cell viability, even at the highest concentrations of PF-04691502 (20 μM; \( P < .001; n = 7 \)) (Figure 3D).

**PF-04691502 reduced viability of CLL cells in coculture with stromal cells**

CLL cells are known to receive key survival signals from stromal cells in the bone marrow and lymph nodes, reducing CLL apoptosis and promoting drug resistance. Therefore, we investigated the effect of PF-04691502 on CLL cells cocultured with human stromal fibroblast cells (HFFF2), which we have observed can provide antiapoptotic signaling to CLL cells (Dias et al, manuscript in preparation). PF-04691502 significantly inhibited signaling induced by HFFF2 (supplemental Figure 3A) and reduced CLL cell viability (\( P = .016 \)), thus showing its ability to overcome stromal support (Figure 3E). To preclude that its activity was due to a direct cytotoxic effect on the fibroblasts (supplemental Figure 3B), we also performed “wash-out” experiments whereby PF-04691502 was added to the CLL cells for 1 hour and washed off prior to addition to the HFFF2 cells. Cell viability was still significantly reduced by PF-04691502 (\( P = .016 \)), and downstream signaling was inhibited in this setting (Figure 3F; supplemental Figure 3C).

**Dual PI3K/mTOR inhibition decreases prosurvival BCR signaling**

Ligation of the CLL BCR by antigen/autoantigen is thought to primarily occur in the lymph nodes, acting to enhance CLL survival and
resistance to chemotherapy. Antigen in lymph nodes may be present in both soluble and membrane-bound forms. Therefore, we used both soluble and bead-immobilized anti-IgM to investigate the effect of PF-04691502 (2.5-2500 nM) on BCR signaling. Analysis of downstream signaling showed that both the soluble (Figure 4A) and bead-immobilized (Figure 4B) anti-IgM induced strong activation of the pAKT and pS6 ribosomal subunit. PF-04691502 significantly inhibited pAKT and pS6 in CLL samples after soluble and immobilized anti-IgM treatment and at concentrations below maximum achievable plasma drug concentrations (100-200 nM). In contrast, pERK was not affected by PF-04691502 pretreatment, potentially due to downstream signaling from Syk/Ras being unaffected by PF-04691502. Next, we determined the effect of PF-04691502 on CLL motility in an in vitro transwell migration assay. Treatment of CLL cells with PF-04691502 showed no significant effect on basal CLL motility; however, CXCL12-induced CCR-derived migration was significantly abrogated after pretreatment with PF-04691502 for only 30 minutes (P < .02 and P < .025 at 1.25 and 2.5 µM, respectively) (Figure 5B) and was not dependent on reduced expression of CXCR4 (supplemental Figure 4B). These data indicate that PF-04691502 inhibits CXCL12 signaling and subsequent migration, which has potential importance in vivo, CXCL12 signaling enables CLL cells to enter and become retained within protective lymph node microenvironments. Therefore, disruption of CXCL12/CXCR4 signaling provides an attractive therapeutic target. Here, CLL cells treated with CXCL12 (200 ng/mL) for 10 minutes showed increased pAKT, pS6, and pS3, which was reversed after treatment with PF-04691502 (Figure 5A). 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for CLL cells accessing the protective lymph node microenvironment in vivo.

**PF-04691502 has efficacy in murine Eμ-TCL1 CLL cells ex vivo**

Next, we examined whether PF-04691502 displayed activity against murine Eμ-TCL1 tumor cells isolated and cultured ex vivo. Eμ-TCL1 tumor cells were isolated from the spleens of tumor-bearing mice and then treated with anti-IgM or CXCL12 in the presence of PF-04691502, and the effects on downstream signaling were investigated by immunoblot analysis. PF-04691502 substantially blocked signaling induced by both stimuli, reducing pAKT S308, pAKT S473, pS6K T389, and pS6 S235/236 to below background levels, with lesser effects

![Figure 4. PF-04691502 inhibits anti-IgM–induced signaling.](image)

CLL cells were treated with (A) soluble anti-IgM beads (n = 9) and (B) immobilized (Imm) anti-IgM beads (n = 5) prior to evaluation of downstream signaling in the presence and absence of PF-04691502 (0.0025-2.5 μM) by immunoblot analysis. Representative cases are shown. CLL cells were treated for 1 hour with PF-04691502 prior to soluble/immobilized anti-IgM addition. pAKT S308 and pS6K T389 were used as markers of PI3K signaling, pAKT S473 and pS6 S235/236 were used as markers of mTOR signaling, and pERK T202/Y204 was used as a marker of mitogen-activated protein kinase signaling. HSC70 was used as a loading control.

![Figure 5. PF-04691502 inhibits CXCL12 signaling and subsequent migration.](image)

(A) CLL cells were treated with 200 ng/mL CXCL12 for the times indicated in the presence or absence of PF-04691502 (0.25-2.5 μM). PF-04691502 was added 30 minutes prior to the addition of CXCL12. Immunoblotting was performed for pAKT S308, pS6K T389, pS6 S235/236, pERK T202/Y204, total proteins, and the loading control HSC70. Blot is representative of 6 independent experiments. (B) Using a transwell migration assay, we evaluated the migration of CLL cells toward 200 ng/mL CXCL12 in the presence or absence of PF-04691502 (1.25-2.5 μM). Flow cytometry was used to count the number of CD5 CD19 cells that had passed through the transwell filter (n = 5) (see supplemental Methods). Error bars represent SEM. *P < .03.
on pERK202/Y204 at 2.5 to 25 nM PF-04691502 (Figure 6A-B). These effects translated into an ability of PF-04691502 to efficiently kill Eµ-TCL1 tumor cells (n = 8 different tumors) (Figure 6C) and inhibit migration toward CXCL12 (Figure 6D). These data recapitulated those seen with primary human CLL cells above.

In vivo efficacy of PF-04691502

Lastly, we explored the activity of PF-04691502 in vivo. Cohorts of mice were inoculated with Eµ-TCL1 tumor cells and, when tumor was visible in the blood (~21 days), mice were randomized to receive 5 or 10 mg/kg/d PF-04691502 or vehicle control by oral gavage. The leukemic burden in the blood was monitored over the following 14 days (Figure 7A). After receiving the drug, a small but significant lymphocytosis was observed, which persisted for ~4 to 7 days. Subsequently, PF-04691502 elicited a marked reduction in leukemic burden compared to the vehicle control (P = .0001), indicating a profound antitumor effect. Both drug doses demonstrated the same efficacy, indicating that the minimum effective dose was achieved. Fourteen days posttreatment, vehicle-recipient animals exhibited a terminal disease and were euthanized alongside mice receiving 10 mg/kg/d PF-04691502, and the effects of the drug on multiple tissues, including spleen, lymph nodes, and bone marrow (Figure 7B-D), were determined. Mice receiving 5 mg/kg/d were maintained on drug for survival experiments. Visual assessment and weighing of the spleen illustrated the ability of the drug to prevent tumor expansion at this site (Figure 7B-C), which was confirmed by enumerating the number of tumor cells (Figure 7D). No increase in splenic mass was observed as compared to normal healthy C57BL/6 mice, whereas vehicle-treated mice exhibited a fourfold to eightfold increase in splenic mass, in addition to a tumor cell count an order of magnitude higher than that in PF-04691502-treated mice. Next, we examined the macroscopic appearance of the spleen using hematoxylin-and-eosin staining (supplemental Figure 5A). These images indicate that the Eµ-TCL1 leukemias destroy the typical splenic architecture, whereas this was largely prevented by
PF-04691502 treatment. In addition to the spleen, we also observed similar effects of the drug reducing tumor expansion in the lymph nodes and bone marrow (Figure 7D). Cumulatively, these effects significantly enhanced survival in 5 mg/kg/d–recipient animals ($P_{\leq} .0001$) (supplemental Figure 5B). Furthermore, mice showed a small but significant reduction in normal B-cell number but no significant reduction in normal T-cell number (data not shown). These data indicate that PF-04691502 has substantial efficacy in vivo, warranting further investigation of dual PI3K/mTOR inhibitors for the treatment of CLL.

**Discussion**

Treatment of CLL patients with ibrutinib and idelalisib have produced impressive clinical responses; however, the agents are not curative and only suppress the disease. Therefore, identifying an agent that can purge CLL cells from the protective lymph node and bone marrow niches in combination with substantive tumor toxicity may prove essential for an eventual cure. Our results showed that PF-04691502 inhibited BCR- and CXCL12-induced signaling, resulting in caspase-dependent apoptosis of CLL cells in vitro at nanomolar concentrations, whereas treatment of normal B and T cells resulted in little to no death. Caspase-dependency was confirmed with the pan-caspase inhibitor ZVAD.fmk and was preceded by the proapoptotic conformational change in Bax. Bax activation correlated with the induction of the BH3-only Bcl-2 family members Noxa and Puma, suggesting activation of the intrinsic apoptotic pathway. However, because 17p del cases displayed equivalent sensitivity to PF-04691502 as wild-type samples, the pathway leading to Noxa and Puma induction after PF-04691502 treatment may be TP53 independent. The mechanism behind the Noxa and Puma induction is currently unknown and the subject of our ongoing studies. PF-04691502 induced apoptosis independently of known prognostic markers, which is consistent with findings with idelalisib in CLL. However, an intriguing finding was the enhanced apoptotic potency of PF-04691502 in comparison to idelalisib. This increased cytotoxicity against CLL cells by PF-04691502 may be due to inhibition of >1 PI3K isoform or more likely due to the co-inhibition of PI3K and mTORC1/2. Evidence in mantle cell lymphoma indicates that constitutive PI3Kα signaling limits the efficacy of PI3Kδ selective inhibitors. Functional redundancy between PI3K isoforms in the survival of neutrophils has previously been shown; therefore, it may be theorized that in CLL cells, other PI3K isoforms may compensate for the selective inhibition of PI3Kδ with idelalisib, resulting in lower levels of cytotoxicity with this drug. However, the γδ
inhibitor PI-145 did not increase CLL cell apoptosis more than idelisib in vitro.44 In contrast, the dual PI3Ka/y inhibitor PIK90 and the pan-PI3K inhibitor NVP-BKM120 (buparlisib) induce substantially more CLL cell apoptosis23,45 than single-isofrom inhibitors.19 The potential caveat to inhibition of more than a single PI3K isoform is the possibility of greater toxicity to normal tissues. However, a number of pan-PI3K inhibitors and dual PI3K/mTOR inhibitors are currently progressing through clinical trials for various malignancies with promising results.46 Interestingly, long-term PI3Kα inhibition was not detrimental to mice and, in fact, protected against a reduction in insulin sensitivity, glucose tolerance, and fat accumulation.47 During our study, we combined inhibitors that targeted specific isoforms of PI3K and mTOR, replicating our findings with PF-04691502. Although combining the PI3Kα/β/δ/γ inhibitors or idelisib with the mTOR inhibitor everolimus induced substantial apoptosis, PF-04691502 alone still induced significantly more. This may be because everolimus inhibits predominately mTORC1, whereas PF-04691502 inhibits both mTORC1 and mTORC2, which is essential to inhibit the positive feedback and subsequent phosphorylation of AKT44,45. However, these data suggest that an mTORC1 inhibitor, and maybe more importantly, a dual mTORC1/2 inhibitor, in combination with idelisib may be more therapeutically beneficial in the treatment of CLL than PI3K inhibition alone.

Ligation of the BCR occurs primarily in the protective lymph node microenvironment48 and is thought to be crucial for the survival of the malignant clone.40 Furthermore, CXCL12 induces migration of the CLL cells from the periphery into the lymph node, promoting their retention within protective microenvironments.12 CXCL12/CXCR4 signaling has previously been shown to inhibit spontaneous cell death and chemotherapy-induced apoptosis.29 Therefore, our finding that PF-04691502 could overcome prosurvival anti-IgM-induced signaling and substantially inhibit CXCL12 signaling and subsequent CLL migration is of clear importance. This finding is also in agreement with what has been described for idelisib.20 These results indicate that PF-04691502 may have similar clinical characteristics to other kinase inhibitors, where the inhibition of BTK, PI3Kδ, or SYK results in reduced interaction of CLL cells with their protective microenvironments, redistribution of the tumor cells from tissues to the blood, and some cellular toxicity.19,50 These previous effects resulted in clinical activity whereby patients underwent rapid lymph node shrinkage and lymphocytosis within weeks of treatment initiation.7,11,20 Due to the relatively low toxicity of these agents alone on CLL cells, combining them with Bcl-2 antagonists (ABT-199) has been proposed. However, given the trend for greater Mcl-1 expression in CLL patient lymph nodes,31 which is regulated by microenvironmental factors through PI3K, PI3K inhibition may have greater efficacy. These latter results raise the question as to whether targets downstream of PI3K, such as AKT, are equally tractable. However, current data suggest not, because CLL cells treated with AKT inhibitors (MK2206 and AZD5363) required concentrations in excess of 10 μM to elicit relatively small amounts of apoptosis, and clinical trials have stopped recruiting (NCT01369849).

Tumor cell redistribution was also evident in the Eµ-TCL1 model, with a transient lymphocytosis seen in the blood concomitant with reduced tumor load in the secondary lymphoid organs and bone marrow upon PF-04691502 treatment. Such lymphocytosis is likely a consequence of reduced chemokine receptor expression or signal inhibition. Interestingly, although PF-04691502 treatment prevented accumulation of tumor in the spleen and caused protection of the global splenic architecture, tumor was still evident, even in the presence of PF-04691502. Therefore, these tumor deposits likely reflect treatment-resistant reservoirs that require additional interventions with other treatment modalities, which is the basis for our ongoing studies.

The results from this study show that simultaneous inhibition of all 4 class-1 PI3K isoforms in combination with the inhibition of mTOR kills CLL cells more potently than selective PI3Kδ inhibition alone. The data generated with systems mimicking ongoing BCR stimulation and stromal support indicate the ability of PF-04691502 to overcome protective microenvironment signaling in vivo. These results are supported by in vivo experimentation using the Eµ-TCL1 mouse model that further indicates that PF-04691502 may prove therapeutically useful for the treatment of CLL. Further studies are now required to investigate the key modes of action of PF-04691502 and explore its utility in combination with other treatment modalities.

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Authorship
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The PI3K/mTOR inhibitor PF-04691502 induces apoptosis and inhibits microenvironmental signaling in CLL and the Eµ-TCL1 mouse model

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