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The Phosphoinositide-3-Kinase (PI3K)-Delta and Gamma Inhibitor, IPI-145, Overcomes Signals from the PI3K/AKT/S6 Pathway and Promotes Apoptosis in CLL

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Abstract

The functional relevance of the B-cell receptor (BCR) and the evolution of protein kinases as therapeutic targets have recently shifted the paradigm for treatment of B-cell malignancies. Inhibition of p110δ with idelalisib has shown clinical activity in CLL. The dynamic interplay of isoforms p110δ and p110γ in leukocytes support the hypothesis that dual blockade may provide a therapeutic benefit. IPI-145, an oral inhibitor of p110δ and p110γ isoforms, sensitizes BCR-stimulated and/or stromal co-cultured primary CLL cells to apoptosis (median 20%, n=57; p<0.0001) including samples with poor prognostic markers, unmutated IgVH (n=28) and prior treatment (n=15) (p<0.0001). IPI-145 potently inhibits the CD40L/IL-2/IL-10 induced proliferation of CLL cells with an IC_{50} in sub-nanomolar range. A corresponding dose responsive inhibition of $pAKT^{Ser473}$ is observed with an IC_{50} of 0.36 nM. IPI-145 diminishes the BCR-induced chemokines CCL3 and CCL4 secretion to 17% and 37% respectively. Pre-treatment with 1 µM IPI-145 inhibits the chemotaxis towards CXCL12; reduces pseudoemperipolesis to median 50%, inferring its ability to interfere with homing capabilities of CLL cells. BCR- activated signaling proteins AKT^{Ser473}, BAD^{Ser112}, $ERK^{Thr202/Tyr204}$ and $S6^{Ser235/236}$ are mitigated by IPI-145. Importantly, for clinical development in hematological malignancies, IPI-145 is selective to CLL B-cells, sparing normal B- and T-lymphocytes.

Keywords

CLL; PI3Kinase; Duvelisib; apoptosis; BCR; microenvironment; IPI-145; p110δ; p110γ

Introduction

For many years, chemo-immunotherapy has been the standard of care for CLL[1]. However, association with a variety of morbidities, including secondary malignancies has been a major drawback with conventional chemotherapies. The functional relevance of the B-cell receptor (BCR) pathway and the identification of protein and lipid kinases as therapeutic targets have recently shifted the paradigm for treatment of B-cell malignancies. Inhibitors of Bruton's Tyrosine Kinase (BTK) and Phosphatidylinositide-3-Kinase (PI3K)-Delta, ibrutinib and idelalisib, have shown promising activity in the clinic and are recently FDA-approved for CLL[2, 3].

Of the three classes of PI3K isoforms, class IA is comprised of the p110α, p110β and p110δ catalytic domains and class IB is made up uniquely of the p110γ.[4] These catalytic domains partner with p85 or p101/p84 regulatory subunits to make the holoenzymes that phosphorylate phosphatidylinositol (4,5)-bisphosphate (PIP_2) to Phosphatidylinositol (3,4,5)-trisphosphate (PIP_3); reversal of this reaction is catalyzed by a phosphatase PTEN[5]. Because PI3K and PTEN are among the most frequently mutated oncogene and tumor-suppressor gene in solid tumors, this pathway has become a desired axis to target[6]. In B-cell malignancies, while this pathway is not commonly mutated, the differential expression and function of p110 isoforms in BCR signaling provide the potential for targeted therapeutic intervention. Whether PI3K isoform-specific or pan-isoform PI3K inhibition constitutes the optimal therapeutic strategy in lymphoid malignancies is still under debate. However, the distinct role of the p110γ and p110δ support isoform specific inhibition in B-cell malignancies[7].

P110δ is a key isoform for B-cells as it plays a crucial role in mediating BCR signaling, proliferation/survival, antibody production and/or antigen presentation[8]. It is vital for B- and T-cell activation and function[9], Fc receptor signaling in mast cells[10], Th1-Th2 differentiation[11] and T-regulatory cell function[12]. Though p110γ is expressed in CLL[13], there is less evidence for the role of p110γ than for p110δ in the regulation of B-cell activation and/or function. However, studies highlight a critical function of p110γ in leukocyte chemotaxis[14], mast cell activation, chemokine-mediated trafficking, and microglial activation[15]. Isoform p110γ is integral to the integrin-dependent homing of progenitor cells and compounds reported to inhibit p110γ significantly reduced the CXCL12 (SDF-1α)-induced transmigration of human epithelial cells[16]. It is reported that p110γ is indispensable for constitutive migration of naive CD8 T-cells and subsequent activation and differentiation into effector CD8 T-cells, and their migration to inflammatory sites[17]. Dendritic cells obtained from p110γ deficient mice showed a reduced ability to respond to chemokines or to migrate to lymph node sites[18]. Studies in mice either lacking p110δ or p110γ reported that p110γ-deficient T-cells but not B-cells, showed reduced chemotactic responses to the lymphoid chemokines, CCL19, CCL21, and CXCL12. In contrast, p110δ-deficient B-cells showed a diminished chemotactic response to CXCL13. Together, these data establish the distinct roles of p110δ and p110γ in lymphocyte function and immune cell trafficking.

Clinical studies have demonstrated that inhibition of p110δ isoform has therapeutic value for CLL patients[2, 19, 20]. Given the multiple roles of p110δ and p110γ isoforms in lymphocyte function and their combined activity in mediating efficient trafficking of immune competent cells, we hypothesized that the dual blockade of isoforms p110δ and p110γ could present a unique therapeutic opportunity in the treatment of B-cell malignancies. IPI-145 is an orally bioavailable, highly potent small molecule inhibitor of p110δ and p110γ with K_D values of 0.023 nM and 0.24 nM, respectively[21, 22]. Importantly, inhibition of both isoforms is observed at physiologically relevant concentrations. IPI-145 has profound effects on adaptive and innate immunity inhibiting B- and T-cell proliferation, blocking neutrophil migration, inhibiting basophil activation and showed activity in collagen-induced arthritis, ovalbumin-induced asthma, and systemic lupus erythematosus rodent models. Inhibition of neutrophil and eosinophil recruitment and cytokine production in an asthma model was observed with doses of IPI-145 sufficient to block p110γ, but less so with lower doses predicted to inhibit only p110δ[21]. In addition, phase 1 studies in hematologic malignancies with IPI-145 (Duvelisib) have shown clinical activity in indolent NHL and CLL, and phase 2 and phase 3 studies in these indications are currently underway[23–28].

With accumulating evidence that B-CLL disease is dependent on interactions with the immune microenvironment, the effect of combined inhibition of p110δ and p110γ isoforms with IPI-145 was investigated in primary CLL cells. The data complements previous reports on the biological consequences and molecular changes in primary CLL cells induced by isoform-specific inhibition[29, 30]. IPI-145 showed direct cytotoxicity; cytokine-mediated induction of CLL cell proliferation was markedly reduced. Inhibition of BCR signaling, reflected by decreased activation of AKT, BAD, ERK, and S6, downstream markers of an active PI3K signaling pathway was observed. IgM-stimulated induction of chemokines such as CCL3 and CCL4 was mitigated by IPI-145 in primary samples. Furthermore, CLL cell chemotaxis and migration were diminished by IPI-145, implicating multiple roles of p110δ and p110γ isoforms in survival, lymphocyte trafficking and cell migration in B-cell malignancies.

Patients and methods

Drugs and chemicals

IPI-145, idelalisib, and ibrutinib for in vitro use were provided by Infinity Pharmaceuticals, Inc. Cambridge, MA[21]. Dimethyl sulfoxide (DMSO) was purchased from Sigma Aldrich (St. Louis, MO). The final concentration of DMSO in control and treated cultures was 0.1%. Anti-IgM (polyclonal goat F(ab′)2 fragments to human IgM) was obtained from MP Biomedicals (Santa Ana, CA) or from Jackson Immunoresearch. CXCL12 (SDF-1α) for chemotaxis experiments (200 ng/mL) was obtained from R&D systems. sCD40L and IL-10 were purchased from Life Technologies (Grand Island, NY) and IL-2 was from Peprotech (Rocky Hill, NJ). For FACScan, PerCP anti-human CD19 and CD5-PE were obtained from Biolegend. Alexa Fluor 488 anti-Human Ki67 and p-AKT (Ser[473]) (D9E) XP (Alexa Fluor 647) were purchased from BD Biosciences and Cell Signaling, respectively.

Patients and healthy donors

The present study was carried out using lymphocytes isolated from peripheral blood samples obtained from healthy donors or patients with CLL[31]. All participants signed written informed consent forms in accordance with the Declaration of Helsinki, and the laboratory protocols were approved by the Institutional Review Board at the University of Texas MD Anderson Cancer Center.

Isolation of leukemia cells

CLL or normal PBMCs were isolated from peripheral blood samples by Ficoll-hypaque procedure and plated at a density of 1×10^7 cells/ml in RPMI medium + 10% autologous plasma[31].

BCR triggering and marrow stromal cell (MSC) co-culture

CLL samples were pre-incubated in RPMI medium (containing 10% autologous plasma), stimulated with 10 μg/mL α-IgM and incubated without or with IPI-145[32]. For co-culture studies, CLL lymphocytes were cultured with or without confluent layers of human MSC (NKtert; RIKEN cell bank, Tsukuba, Japan) at a ratio of 100 CLL cells to 1 MSC[31]. The NKTert cell line was maintained and routinely tested for *Mycoplasma* infection and authenticated by short tandem repeat analysis at MD Anderson Cancer Center's characterized cell line core facility.

Measurement of cell viability

Cell viability was measured by the standard method of AnnexinV/PI binding assay[31].

Chemokine quantification

CCL3 and CCL4 concentrations in cell culture supernatants of α-IgM stimulated CLL cells and/ or stromal co-cultured CLL cells were measured in the absence or presence of IPI-145 using Quantikine ELISA kits (R&D Systems) according to the manufacturer's protocol[32]. A standard curve containing a blank was prepared for each experiment in the absence of chemokines, and its absorbance was subtracted from that obtained in the presence of sample. Results were expressed as concentration in pg/mL for each sample.

Chemotaxis toward CXCL12 (SDF-1α)

Chemotaxis assays across polycarbonate transwell inserts were performed as previously described[32]. Briefly, 10 million cells were incubated in RPMI medium (containing 10% autologous plasma) in the absence or presence of 1 μM IPI-145 for 1 hr and transferred into the top chambers of Transwell® cell culture inserts (Costar®) with a diameter of 6.5 mm and a pore size of 5 μm. Filters were placed onto wells containing medium (control) or medium with 200 ng/mL CXCL12 (SDF-1α) (R&D Systems), and CLL cells were allowed to migrate for 3 hrs at 37°C. Migrated cells in the lower chamber were collected and counted on a FACSCalibur for 20 seconds at 60 μL/min in duplicates.

Migration beneath marrow stromal cells (pseudoemperipolesis)

NKTert stromal cells were seeded the day before the assay onto collagen-coated 12-well plates at 5×10^4 cells/well. Next day, 10^7 CLL cells/mL were incubated for 4 hrs with or without IPI-145. Cells that had not migrated into the stromal cell layer were removed by vigorously washing with RPMI medium and the stromal cell layer containing transmigrated cells was detached by incubation for 1 minute with trypsin/EDTA. Cells were immediately resuspended and counted by FACSCalibur for 20 seconds at 60 μL/min in duplicate as described previously[33]. A lymphocyte gate was set according to the different relative size and granularity (forward scatter and side scatter) characteristics to exclude stromal cells from the counts.

Proliferation of CLL cells and AKT activation

CLL PBMCs were seeded at 1×10^6 cells/well in a 24-well plate and treated with either 10 μg/mL IgM or a cytokine cocktail containing 1μg/mL sCD40L, 10 ng/mL IL-10, and 10 ng/mL IL-2. Cells were harvested at various time points post stimulation, fixed in BD Cytofix Fixation Buffer, and stored at -80^0C for subsequent flow cytometry analyses. Untreated samples were collected at every time point as baseline controls. For CLL proliferation assays, cells were treated with the cytokine cocktail and harvested five days later. For both assays, intracellular expression of Ki-67 and pAKT^{Ser473} were measured in CD19$^+$/5$^+$ CLL cells by flow cytometry.

Immunoblot analysis

CLL cell pellets were washed with PBS, lysed on ice for 20 minutes in RIPA lysis buffer and the supernatant was removed and the protein content was determined using a DC protein assay kit (Bio-Rad Laboratories), loaded and transferred to nitrocellulose membranes (GE Osmonics Labstore) as described previously[31]. Membranes were blocked for 1 hr in licor blocking buffer, incubated with primary antibodies overnight at 4°C against the following: pAKT(Ser^{473}), t-AKT, p-ERK (Thr^{202}/Tyr^{204}), t-ERK (Cell Signaling, MA), p-Bad, t-Bad, p-S6, t-S6, Mcl-1, Bcl-xL, Bcl-2, (Santa Cruz, CA), and GAPDH (Abcam, Cambridge, MA). The antibodies to poly (ADP-ribose) polymerase (PARP) was from BIOMOL International (Plymouth Meeting, PA), and PI3K isoforms were from Millipore. After washing with PBS–Tween-20, membranes were incubated with infrared-labeled secondary antibodies (LI-COR Inc) for 1 hr, scanned and visualized using LI-COR Odyssey Infrared Imager.

Statistical analysis

Linear regression analysis and Student's t-tests (two tailed) were performed using the GraphPad Prism6 software (GraphPad Software, Inc. San Diego, CA).

Results

IPI-145 abrogates B-cell receptor- and bone marrow stromal cell- mediated survival in primary CLL cells

The effect of IPI-145 (structure - Figure 1A), on BCR- and MSC-mediated cell survival was determined. As expected, α-IgM crosslinking induced an increase in viability (median 90%, range 66% – 98%) in comparison to unstimulated CLL cells (median 85%, range 39% – 97%) (n=19; Figure 1B; p<0.0001; 24 hrs). Of note, samples with 17p deletion demonstrated high viability in cultures under unstimulated conditions (n=7; table 1). Incubation with 1 μM IPI-145 significantly reduced CLL cell viability to a median 80% (range 31% – 96%) in BCR-stimulated cells. Although there was heterogeneity in response to IPI-145 among various samples (Figure 1B; each symbol denotes a representative patient's sample), overall there was a moderate, yet significant decrease in viability of samples analyzed by a paired student's t test (controls are normalized to 100%, Figure 1C; p<0.0001; n=19). As has been shown previously, stromal co-cultures increased viability of CLL cells (median 95%, range 69% – 98%) in comparison to primary cells with no stromal support (median 80%; range 39% – 95%; Figure 1D; n=20). Incubation of co-culture samples with 1 μM IPI-145 for 24 hrs significantly reduced CLL cell viability to a median of 73% (range 42% – 96%, Figure 1D; p<0.0001; n=20). A list of patient samples (n=57), their prognostic factors, and percent viability of CLL cells without or with IPI-145 in BCR-stimulated and/or stromal co-cultured model systems is provided (Table 1). When a dose- (Supplemental Figure 1A–I; n=9) and time- (Supplemental Figure 2A–I; n=9) dependent activity of IPI-145 was tested in CLL cells, IPI-145 significantly reduced the CLL cell viability at concentration as low as 500 nM (p=0.009; n=11; 24 hrs; Supplemental Figure 3A) and at time point as early as 12 hrs (p=0.004; n=4; 1 μM; Supplemental Figure 3B).

Correlative response of IPI-145 with prognostic factors

Given the heterogeneity among samples, subgroup analyses were performed on established genetic subtypes of CLL (Figure 1E). Samples with 11pdel (n=6), 13qdel (n=19), trisomy 12 (n=6) and 17pdel (n=9) were equally sensitive to IPI-145 (p<0.0001). Importantly, 11qdel samples were comparatively more sensitive. No significant difference in responses was observed between CLL cells with IgVH mutated (n=19) or unmutated status (n=28) or CLL cells obtained from patients previously treated (n=15) or untreated (n=42) (Figure 1F and G; p<0.0001).

IPI-145 inhibits proliferation and activation of AKT in primary CLL cells

To mimic the proliferative state in lymph node pseudofollicles, CLL cell proliferation was induced with a CD40L/IL-2/IL-10 cytokine cocktail[34]. Cytokine stimulation led to a time-dependent induction of proliferation measured by Ki67 positivity at the indicated time points (Figure 2A). In parallel, there was a significant induction of pAKT[Ser473] following CD40L/IL-2/IL-10 incubation (Figure 2A). In addition, cells stimulated with α-IgM showed an early increase in pAKT levels, which then declined by 24 hrs (Figure 2A; middle panel). Induction of CLL cell proliferation, as measured by Ki67, was not observed after α-IgM treatment. In contrast, CLL cells stimulated with CD40L/IL-2/IL-10 had greater increases in both AKT phosphorylation and proliferation. At 72 hrs, there was a sustained increase in

pAKT ^{Ser473} (up to 50 fold Figure 2A; lower panel). IPI-145 significantly inhibited the CD40L/IL-2/IL-10 induced proliferation of primary CLL cells and pAKT ^{Ser473} expression (Figure 2B). The proliferation inhibition was in the low nanomolar range (IC_{50} 0.16 nM; n=3), suggesting a potent anti-proliferative effect of IPI-145 on CLL cell proliferation (Figure 2B). The IC_{50} of proliferation inhibition for IPI-145 was 0.16 nM in comparison to 2.9 nM for idelalisib, a specific p110δ inhibitor (Figure 2B; n=3). In addition, a corresponding dose-responsive inhibition of pAKT expression at Ser^{473} in CLL cells was also observed with an IC_{50} of 0.36 nM (Figure 2C; n=3).

Inhibition of CCL3 and CCL4 chemokine secretion

In response to BCR activation by α-IgM stimulation, there was a robust increase in the production of chemokines CCL3 (n=11) and CCL4 (n=5) in the cell culture supernatants (Figure 3A–B). The mean CCL3 and CCL4 concentrations in supernatants of CLL cells following α-IgM stimulation increased from 17 to 831 pg/mL and 347 to 2283 pg/ml, respectively (Figure 3A and B; 24 hrs; p<0.0001). Treatment of CLL cells with 1 μM IPI-145 abrogated the BCR-induced production of CCL3 and CCL4 significantly, reducing the respective mean values to 142 pg/mL and 840 pg/mL.

To determine if the chemokine production is induced through interactions with bone marrow stromal microenvironment, the levels of CCL3 and CCL4 were measured in the supernatants of CLL cells co-cultured with supporting stromal cells. In contrast to BCR-stimulated cells, co-cultured CLL cells exhibited no significant change in chemokine production (Figure 3C; n=4 and D; n=5; 24 hrs). These data suggests that the chemokine production is primarily mediated by BCR signaling and not through interactions with bone marrow stroma cells. These observations indicate a context-dependent production of chemokines, as previous studies showed that CLL cells co-cultured with nurse like cells (that represent a lymph node microenvironment) secreted significant levels of chemokines[35].

Inhibition of CLL cell chemotaxis and migration beneath stromal cells by IPI-145

Given the pivotal role of PI3K in leukocyte trafficking, inhibition of chemotaxis toward CXCL12 / SDF-1α was evaluated with IPI-145. Primary CLL cells incubated in trans-well inserts (for 3 hrs at 37°C) in media containing CXCL12 demonstrated an increase in migration towards CXCL12 to a median 2863 cells/20 sec (range 851 – 3649 counts) in comparison to control, median 612 (range 215 – 2569 counts) (Figure 4A; n=8). However, pre-treatment with 1 μM IPI-145 significantly inhibited the chemotaxis towards CXCL12 to a median of 2142 cells/20 sec (range 788 – 3572 counts); (Figure 4A). Similarly, migration of CLL cells beneath the marrow stromal cells (pseudoemperipolesis) was diminished by 1 μM IPI-145 (median 50%; range 6% – 70%) in comparison to control (normalized to 100%) inferring that IPI-145 potently interferes with the homing and migration capabilities of CLL cells (Figure 4B; n=4).

Inhibition of BCR- and MSC- activated signaling molecules by IPI-145

Given that IPI-145 is a potent inhibitor of p110δ and p110γ isoforms, we tested the inhibition of signaling proteins downstream BCR pathway. IPI-145 in suspension cultures significantly inhibited the phosphorylation of AKT at Ser^{473} and ERK at Thr^{202}/Tyr^{204}, and

decreased downstream anti-apoptotic protein Mcl-1 (Figure 5A and B; n=6; Mcl-1 - p=0.01; pAKT – p=0.02; pERK – p=0.01). Additionally, BCR engagement with α-IgM induced activation of signaling proteins AKT, BAD, ERK and S6 at sites Ser^{473}, Ser^{112}, Thr^{202}/Tyr^{204} and $Ser^{235/236}$, respectively and induction of Bcl-2 family protein Mcl-1 (Figure 5C; n=6; lane 2 for each patient). Pre-incubation with IPI-145 in BCR- activated cells, did not reverse the inhibition of signaling axis or PARP cleavage (Figure 5C and 5E; n=6; lane 4 for each patient; pAKT – p=0.006; pERK – p=0.011; pBAD – p=0.007). On the same note, quantitation of immunoblots of signaling molecules on co-cultured samples without and with IPI-145 revealed no statistical significance (Figure 5D and 5F; n=3), which could be due to variability in sample prognosis, in a small set of samples (n=3). Finally, differential expression of PI3K isoforms was observed in primary CLL cells; with abundant p110δ and p110β, modest p110γ and undetectable levels of p110α in the samples tested (Figure 5G; n=3). α-IgM stimulation or IPI-145 treatment did not affect the protein levels of isoforms.

Comparative studies of IPI-145 with idelalisib and ibrutinib

In addition to proliferation assays (Figure 2B), studies were conducted to compare activities of IPI-145 with other BCR pathway kinase inhibitors in apoptosis induction, migration inhibition and chemokine blockade. Comparison of IPI-145 to idelalisib and ibrutinib with respect to apoptosis induction (n=35) revealed that at an equivalent concentration, all three inhibitors had similar levels of cytotoxicity (Figure 6A; p<0.0001; all three agents) for primary CLL cells. Additionally, CLL cells stimulated with α-IgM (n=11) and/or co-cultured with stromal cells (n=7) in presence or absence of IPI-145, idelalisib and ibrutinib demonstrated statistically significant induction of apoptosis (Figure 6B; IPI-145 - p=0.007; IDE - p=0.009; IBR - p= 0.03) and (Figure 6C; IPI-145 - p=0.001; IDE – p=0.02; IBR – p=0.05), respectively. Inhibition of chemokine production (Figure 6D; n=4) and migratory abilities of CLL cells (Figure 6E; n=4) tested at an equivalent concentration were p=0.02; p=0.11; p= 0.07 and p=0.03; p=0.09 and 0.33 for IPI-145, idelalisib and ibrutinib, respectively.

Effect of IPI-145 on normal PBMCs

To evaluate the therapeutic index, we investigated the effect of IPI-145 on PBMCs isolated from peripheral blood of normal donors at concentrations that decreased the viability of CLL cells (Figure 7A and B; n=3; 1 µM; 24 hrs). The cytotoxicity assay using specific surface markers on the two lymphoid subsets revealed that IPI-145 is selective to CLL cells sparing normal T- and B-lymphocytes (Figure 7C).

Discussion

The B-cell receptor signaling pathway plays a key role in the pathogenesis of CLL. The PI3Kδ that is downstream of BCR regulates several cellular processes. Class IA PI3K (isoforms p110α and p110β) that are universally expressed in all cell types, regulate cell cycle entry in controlling cell division, DNA replication, insulin metabolism and tumorigenesis[36–39]. As p110δ is primarily restricted to leukocytes, drug development efforts have initially focused on synthesizing small-molecule inhibitors of p110δ for the treatment of B-cell malignancies[35, 40–43]. Idelalisib (formerly called CAL-101 and GS-1101) is a

potent, oral, selective small-molecule inhibitor of p110δ. In phase 1 studies, idelalisib both as a single agent and in combination with rituximab had clinical activity with a generally acceptable toxicity profile in patients with lymphoma and relapsed or refractory CLL[2, 19, 20, 44, 45]. Idelalisib is approved by the US Food and Drug Administration for the treatment of patients with relapsed CLL in combination with rituximab (http://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm406410.htm).

While clinical and preclinical results support p110δ as a rational target for inhibition in B-cell malignancies, p110γ is expressed in both malignant B-cells and in the cells of tumor microenvironment, where it plays an important role in the regulation of T-cell and innate immune cell function, including immune cell trafficking and chemo-attractant directed migration. It is also proposed to be involved in CLL due to its role in immune cell activation and trafficking via chemokines[46–48]. Our data demonstrated no cytotoxicity to CD3+ T-cell subsets with IPI-145 suggesting that the inhibition of gamma isoform with IPI-145 may have effect on specific T-cell subsets within microenvironment. IPI-145 is a potent inhibitor of p110δ and p110γ, (the isoforms predominantly expressed in immune cells) and is currently being investigated in hematological malignancies.

Cell death induction by IPI-145 is apparent in BCR-stimulated cells as well as in CLL cells co-cultured with supporting stromal cells (Figure 1). Identification of potential novel biomarkers in response to PI3K inhibition is in progress. Of these, the leading candidates are chemokines CCL3[49] and CCL4, secreted by both normal and malignant lymphocytes that act through the chemokine receptors CCR1 and CCR5. When the BCR pathway is activated, these chemokines are secreted in high levels by leukemic lymphocytes, particularly lymph-node derived CLL cells[50–52]. Experiments with IPI-145 displayed substantial inhibition of both CCL3 and CCL4 production in BCR-stimulated assays (Figure 3A and B). These chemokines are also among the chemokines and cytokines reduced in CLL patients receiving IPI-145[53].

Circulating CLL cells in peripheral blood are largely arrested in the G0/G1 phase of the cell cycle and undergo spontaneous apoptosis in vitro. However, heavy water labeling studies report a more significant amount of CLL B-cell proliferation in the pseudofollicles than was previously appreciated[52, 54, 55]. Both proliferation and resistance to apoptosis are believed to be governed by supporting stromal cells in the tissue microenvironment. Experiments with CLL cells and added chemokines that mimic the microenvironment to induce B-cell proliferation revealed the ability of IPI-145 to inhibit proliferation at low nM concentrations (Figure 2A and B). In parallel to growth inhibition, there was a marked decrease in the phosphorylation of PI3K regulatory protein, AKT (Figure 2C). While apoptosis induction in peripheral blood-derived primary CLL cells was moderate (Figure 1A), the effect of IPI-145 on cytokine-induced CLL cell proliferation was impressive. Because tumor burden and proliferation in lymph nodes are strongly and directly associated with disease progression[52], these results emphasize the potential clinical utility of IPI-145 in CLL.

The clinical activity of BCR pathway kinase inhibitors in patients with CLL is associated with marked lymphocytosis due to redistribution of tumor cells from the lymph node into the peripheral blood and/or due to disruption in the homing mechanisms of CLL cells[20].

Similar to other kinase inhibitors, IPI-145 inhibits the migration and chemotaxis of CLL cells in vitro, further supporting the significance of isoforms in the migration and homing capabilities of CLL cells (Figure 4A and B).

IPI-145 is distinct from idelalisib, as it is an inhibitor of both p110δ and p110γ isoforms. Phase 1 dose-escalation study of IPI-145 with dose expansion cohorts including CLL patients reported that IPI-145 is generally well tolerated in patients with hematological malignancies and clinical activity is observed at all dose levels. In CLL, including high-risk patients, responses are seen with rapid resolution of lymphocytosis, justifying its further development in CLL[23–28, 56].

In summary, the preclinical activity of IPI-145, an inhibitor of p110δ and p110γ isoforms of PI3Kinase in CLL is supported by the data generated. As p110γ is also predominantly expressed in T-cells[57], additional studies of IPI-145 on the function of T-cell subsets are warranted. T-cells have been reported to promote the survival and proliferation of B-CLL cells[58,59], therefore, abrogation of their ability to provide support for CLL cells through the inhibition of T-cell activation or migration could potentially provide a therapeutic benefit for CLL patients treated with IPI-145. The activity of IPI-145 is selective to primary CLL cells as the survival of normal PBMCs, B-cells or T-cells are unaffected (Figure 7). As p110δ is critical for CXCL13-driven migration of B-cells[60] and p110γ is important in the CXCL12-mediated migration of T-cells[61], the p110γ inhibition with IPI-145 may have effect on specific T-cell subsets within tumor microenvironment. The encouraging preclinical and clinical data with IPI-145 support the continued clinical evaluation of dual inhibition of p110δ and p110γ isoforms in CLL, and indicate that p110γ as may be another important target in this indolent disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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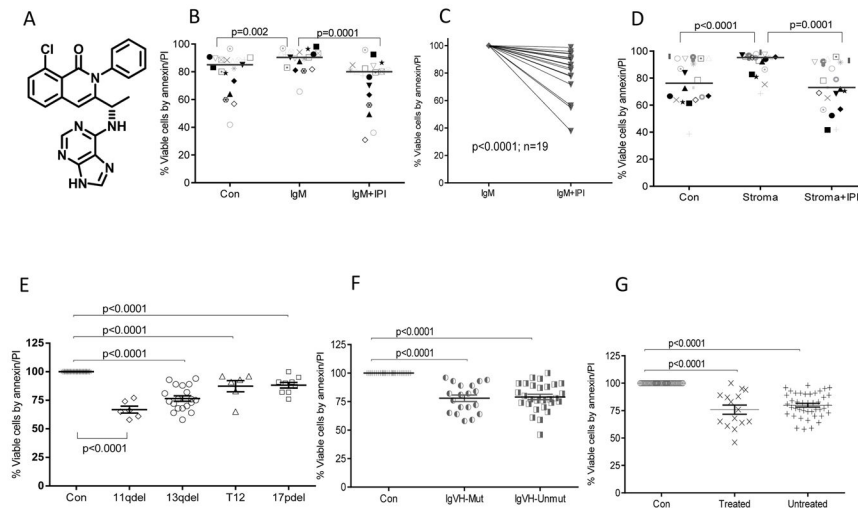
Figure 1. IPI-145 treatment disrupts the antigen- and stromal cell- mediated survival in primary cells

A. Structure of IPI-145. **B**. IPI-145 overcomes BCR- derived survival signals. Primary CLL cells from 19 patients are cultured in RPMI medium plus 10% autologous plasma in the absence (Con) or presence of α-IgM (10 μg), without or with 1 μM IPI-145 for 24 hrs. The CLL cell viability is assessed by FACSCalibur (Annexin/PI binding assay). Each symbol in the graph represents a CLL sample. **C**. IPI-145 overcomes α-IgM-induced survival signals. Data from Figure 1B and 1C are the same, but represented in different fashion. The viabilities are normalized to the relative viability of time-matched control samples (100%) to account for differences in spontaneous apoptosis in samples from different patients (n=19). **D**. IPI-145 overcomes stroma- derived survival signals. Primary CLL cells from 20 patients are co-cultured in RPMI medium plus 10% autologous plasma in the absence (Con) or presence of bone marrow stromal cells (NKTert stromal cells, Str) without or with 1 μM IPI-145 for 24 hrs. The CLL cell viability is assessed by FACSCalibur (Annexin/PI binding assay). Each symbol in the graph represents a CLL sample. **E**. Correlation between IPI-145 mediated cytotoxicity in suspension cultures and established genetic subtypes of CLL. 11qdel represents samples that have deletion of chromosome 11q (n=6), which is the locus of ATM gene, 13q14del (n=19) represents samples that lack miR15 and 16a, T12 (n=6) represents samples that are identified with trisomy 12 (three copies of chromosome 12) and 17pdel (n=9) represents samples that have deletion of chromosome 17p, the locus of p53, a tumor suppressor gene. **F**. Correlation between IPI-145 mediated cytotoxicity in suspension cultures and IgVH status in CLL (unmutated is defined as >98% homology to germline IgVH segments; unmutated - n=28; mutated – n=19). **G**. Correlation between IPI-145-mediated cytotoxicity in suspension cultures and prior treatment. The untreated sample is defined as samples obtained from patients with no previous therapy (untreated – n=42; prior treated – n=15). The treated sample is defined as samples obtained from patients who received one or more prior therapies. The error bars in **E**–**G** represent mean ± SEM for viable cells measured by Annexin/PI binding assay.
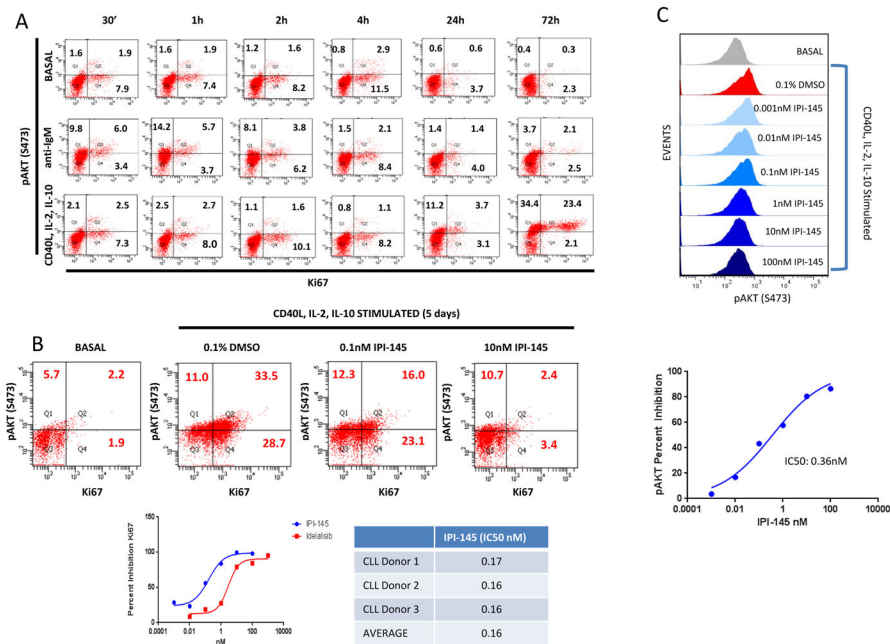
Figure 2. IPI-145 treatment blocks the phosphorylation of AKT at Ser^{473} and inhibits proliferation of primary CLL cells

A. Time-dependent induction of pAKT^{Ser473} and CLL cell proliferation (Ki67) with either α-IgM or CD40L/IL-2/IL-10 stimulation. CLL cells are cultured in RPMI medium plus 10% FBS in the absence (Con) or presence of either α-IgM or CD40L/IL-2/IL-10 stimulation for 72 hrs. The levels of AKT phosphorylation (Ser^{473}) and CLL cell proliferation (Ki67) are assessed by FACSCalibur at the indicated time points. **B**. Effect of IPI-145 on CD40L/IL-2/IL-10-induced CLL cell proliferation. CLL lymphocytes are cultured for 5 days in the presence of CD40L/IL-2/IL-10 cocktail plus a dose response of either IPI-145 or idelalisib. CLL cell proliferation (Ki67) and AKT phosphorylation (Ser^{473}) are assessed by FACSCalibur. Representative IC$_{50}$ curves for inhibition of CLL cell proliferation are shown for both IPI-145 and idelalisib. Individual IPI-145 IC$_{50}$ values are presented for 3 different samples. **C**. Dose responsive inhibition of pAKT (Ser^{473}) with IPI-145 treatment. CLL PBMCs are cultured for 5 days in the presence of CD40L/IL-2/IL-10 plus a dose response of IPI-145. AKT phosphorylation (Ser^{473}) is assessed by FACSCalibur on CD5/CD19 double positive CLL cells. Representative histograms and an IPI-145 IC$_{50}$ curve for inhibition of CLL cell AKT phosphorylation levels are shown.
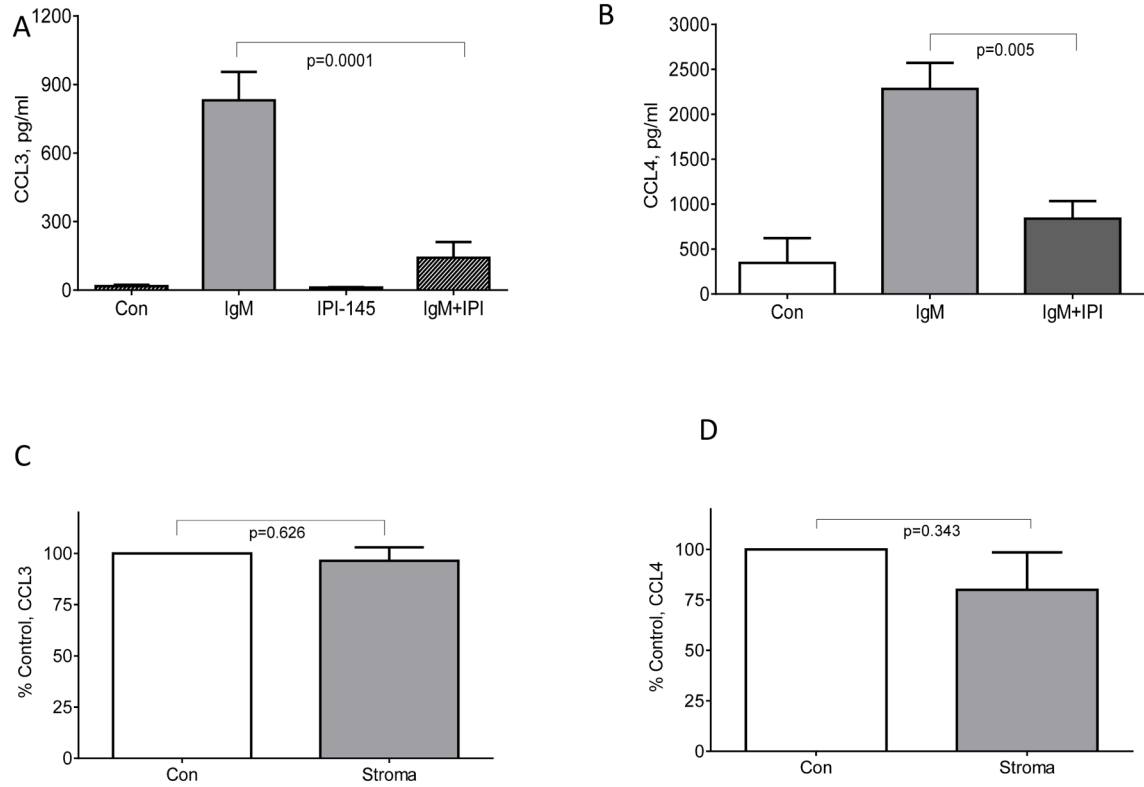
Figure 3. IPI-145 treatment down-regulates antigen receptor-mediated secretion of CCL3 and CCL4 in primary CLL cells

A–B. Primary CLL cells are cultured in RPMI medium plus 10% autologous plasma in the absence (Con) or presence of α-IgM, without or with 1 μM IPI-145 for 24 hrs. The CCL3 concentrations in supernatants of BCR-activated CLL cells from 11 different CLL samples (*p<0.0001*) (**A**) and CCL4 concentrations in supernatants of BCR-activated CLL cells from 5 different CLL samples (*p=0.005*) (**B**) are measured using ELISA assay. The error bars represent mean ± SEM. **C–D.** Primary CLL cells are co-cultured in RPMI medium plus 10% autologous plasma in the absence or presence of bone marrow stromal cells (NKTert stromal cells) without or with 1 μM IPI-145 for 24 hrs. The CCL3 (**C;** n=4) and CCL4 (**D;** n=5) concentrations in supernatants of co-cultured CLL cells are measured using Quantikine ELISA kits (R&D Systems). The error bars represent mean ± SEM.
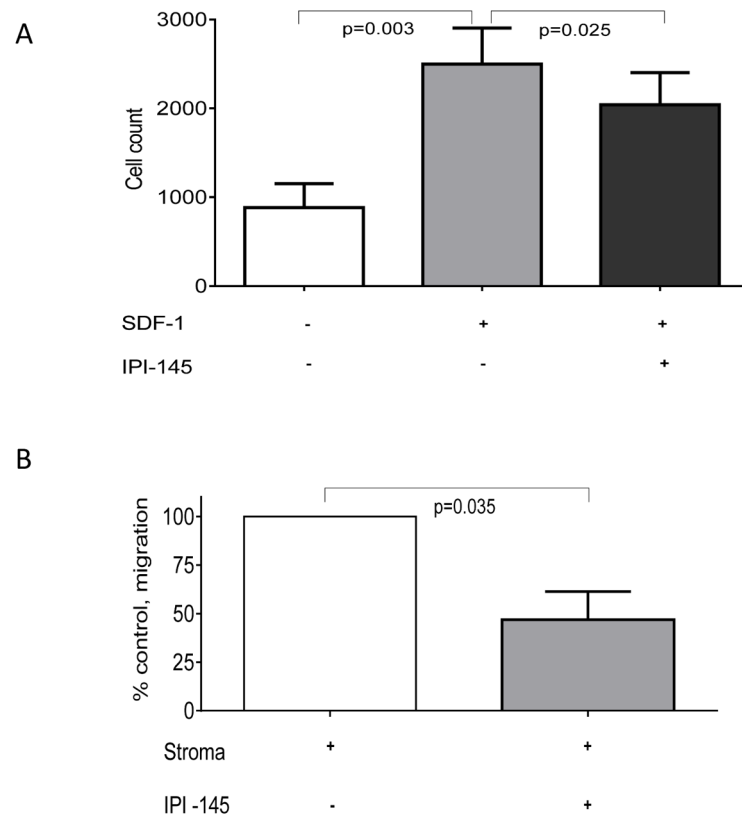
Figure 4. IPI-145 treatment inhibits CLL cell chemotaxis and pseudoemperipolesis
A. Migration of CLL cells toward CXCL12/SDF-1α is carried out as described in materials and methods section. Depicted is the mean ± SEM relative migration of CLL cells in 3 hrs from 8 different patients toward CXCL12/SDF-1α (200 ng/ml) in the absence (medium control) or presence of IPI-145 (1 μM). **B**. Pseudoemperipolesis of CLL cells in co-cultures is carried out as described in materials and methods section. Depicted is the mean ± SEM relative migration of CLL cells in 4 different patients (Pseudoemperipolesis; migration beneath the stromal cells in 4 hrs) in co-cultured CLL-Stroma in presence or absence (medium control) of IPI-145 (1 μM).
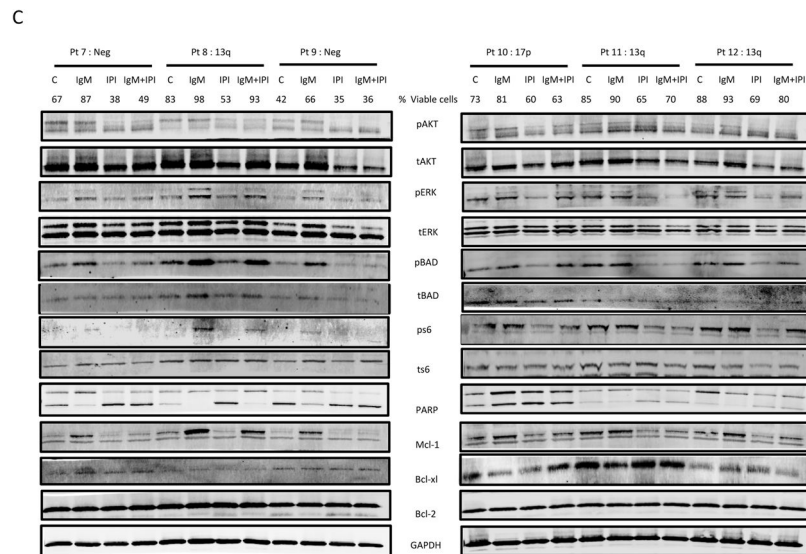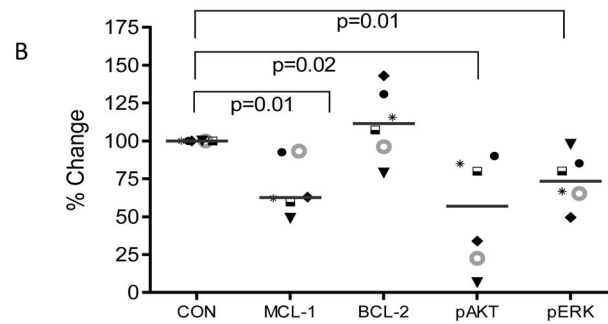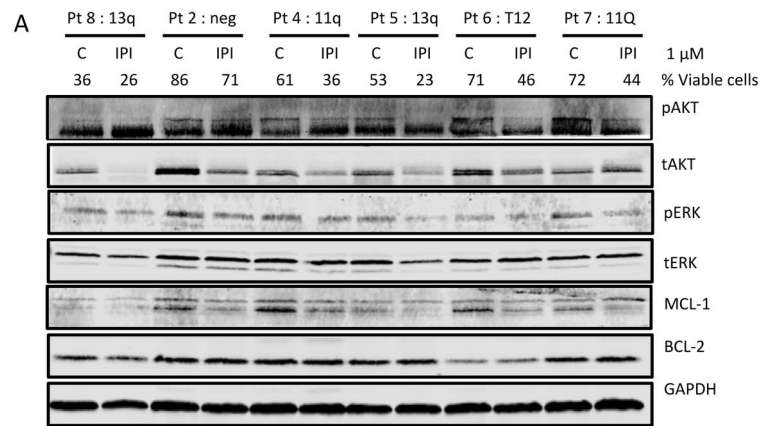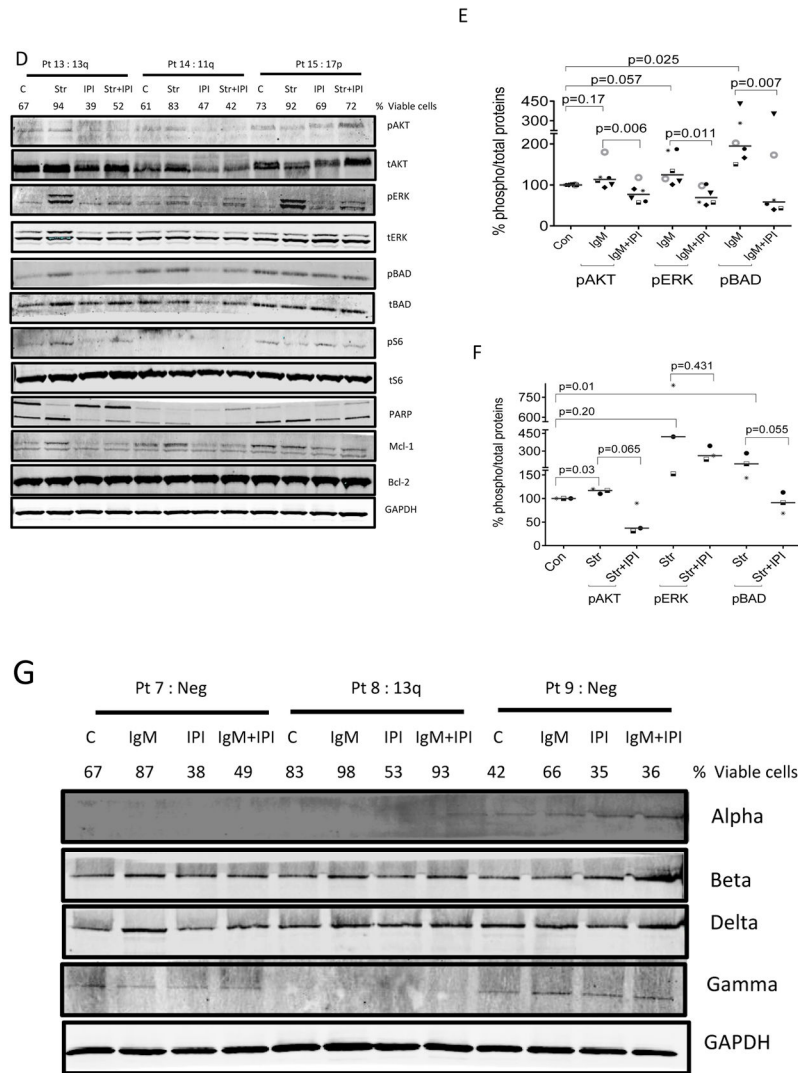
Figure 5. BCR- activated signaling molecules are inhibited by IPI-145 treatment
A. CLL primary cells are incubated with 1 μM IPI-145 and the total and phospho-proteins for AKT and ERK, and Bcl-2 family anti-apoptotic proteins are evaluated (C = Control; IPI = IPI-145). The patient cytogenetics and % viability of cells measured by Annexin/PI assay is provided for each sample. **B.** The immunoblots for Mcl-1, Bcl-2 and pAKT, pERK from Figure 5A are quantitated and normalized to either GAPDH or total proteins and provided as a percent change in expression (dot plot; each symbol denotes a CLL sample). **C–D.** CLL cells are either stimulated with α-IgM (C; n=6) or co-cultured with stroma (D; n=3) in the absence or presence of IPI-145 for 24 hrs and the total and phospho-proteins for AKT, ERK, BAD, S6 and Bcl-2 family anti-apoptotic proteins (Mcl-1, Bcl-2 and Bcl-XL) and PARP cleavage are evaluated by immunoblot analysis. The patient cytogenetics and % viability of cells measured by Annexin/PI assay is provided for each sample. **E–F.** The immunoblots from Figure 5C and 5D for pAKT, pERK and pBAD are quantitated and normalized to respective total proteins. **G.** Evaluation of PI3K isoforms in primary CLL samples. CLL

cells are stimulated with α-IgM in absence or presence of IPI-145 for 24 hrs and the protein levels of isoforms p110α, β, δ, and γ are evaluated by immunoblot analysis (n=3).
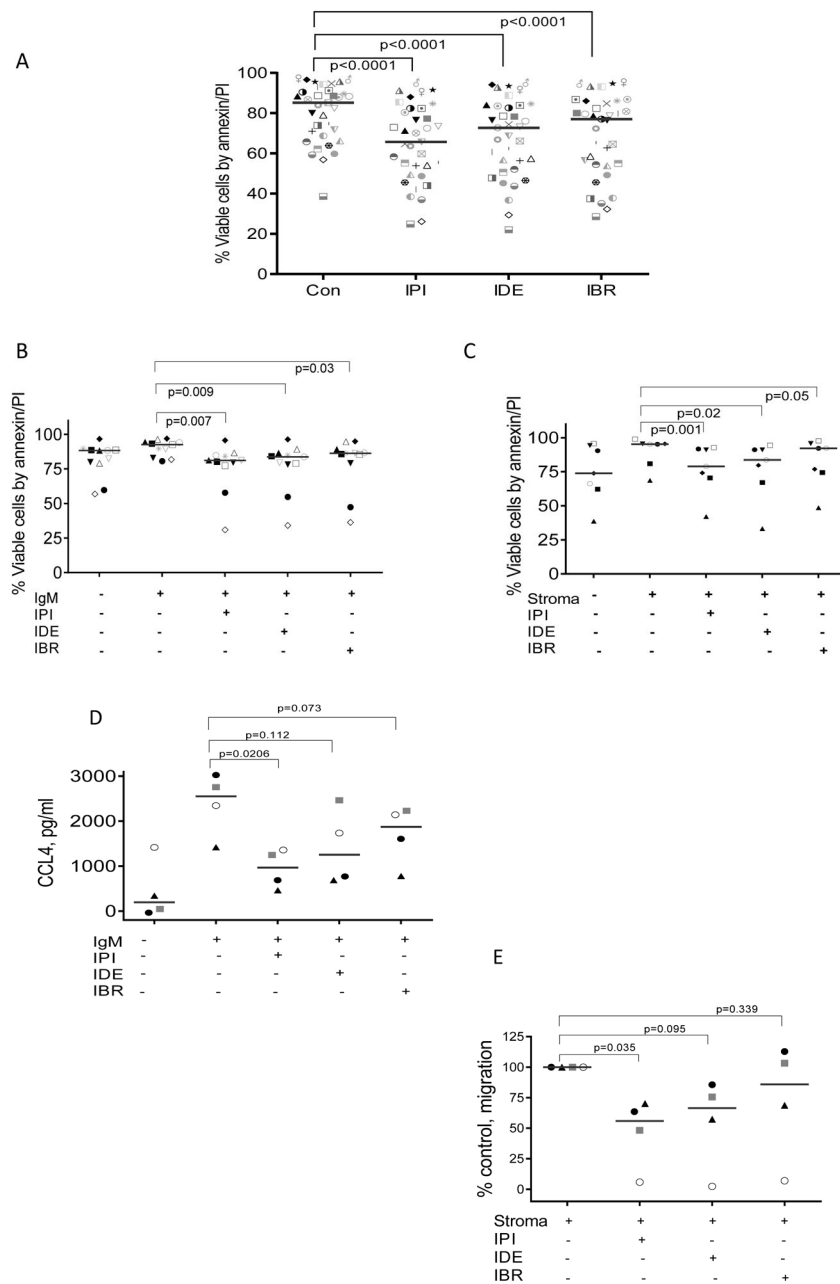
Figure 6. Comparative studies of IPI-145 with idelalisib and ibrutinib
A–C. Evaluation of apoptosis. Primary CLL cells are cultured in RPMI medium plus 10% autologous plasma (n=35), and/or in the absence (Con) or presence of α-IgM (n=11), and/or in stromal co-cultures (n=7) without or with IPI-145, idelalisib and ibrutinib (all 1 μM) for 24 hrs. The % viability is measured by Annexin/PI binding assay. Each symbol in the graph represents a CLL patient's sample. **D**. Evaluation of CCL4 on BCR inhibition. Primary CLL cells are cultured in RPMI medium plus 10% autologous plasma in the absence (Con) or presence of α-IgM, without or with IPI-145, idelalisib and ibrutinib (all 1 μM) for 24 hrs. The CCL4 concentration in supernatants of BCR-activated CLL cells from 4 different CLL samples is measured using Quantikine ELISA kits (R&D Systems). Each symbol in the

graph represents a CLL patient's sample. The lines represent grand median. **E**. Pseudoemperipolesis of CLL cells in co-cultures. Depicted is the relative % control, grand median migration of CLL cells in 4 different samples (Pseudoemperipolesis; migration beneath the stromal cells in 4 hrs) in absence or presence of IPI-145, idelalisib and ibrutinib (all 1 μM). (IPI = IPI-145; IDE = Idelalisib; IBR = Ibrutinib).
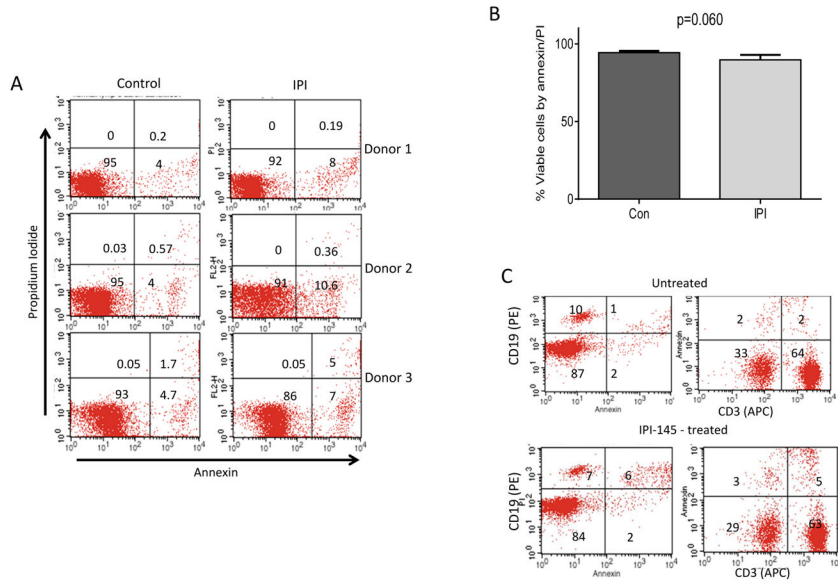
Figure 7. Effect of IPI-145 treatment on normal PBMCs

A. Normal PBMCs isolated from peripheral blood of healthy donors (n=3) are incubated with 1 μM IPI-145 for 24 hrs and the cell toxicity is measured by Annexin/PI binding assay. Representative dot plots for each patient sample are provided. **B**. The data from three donors (Figure 7A) is plotted against untreated time matched control and the statistical analysis is done by a paired Student's t test. **C**. The cytotoxicity (Annexin – FITC) of IPI-145 is tested specifically on two subsets of population, B-cells and T-cells in normal PBMCs using specific cell surface markers CD19 (PE) and CD3 (APC), respectively. Representative dot plot for one healthy donor is provided.

Table 1

Patient Charecteristics

P No.	Age	Sex	WBC	Rai	Prior Rx	FISH	IGVH	% Viability by Annexin/PI binding assay						
								Con	IPI	IgM	IgM+IPI	Str	Str+IPI	
1	80	F	53	1	0	13Q	UM	75	56					
2	65	M	36	0	0	NEG	M	86	72					
3	115	F	-	-	0	(MYELO)	-	96	94					
4	69	M	275	4	1	11Q	NR	60	39					
5	73	F	189	1	0	13Q	M	79	55					
6	79	M	91	2	2	T12	M	71	46					
7	52	F	59	1	1	11Q	UM	72	44					
8	46	F	22	4	1	13Q	UM	34	30					
9	63	M	183	1	0	13Q	M	69	64					
10	56	M	191	2	0	ND	UM	50	35					
11	60	M	115	4	0	ND	UM	84	65					
12	75	F	39	1	0	11Q	UM	58	43					
13	57	F	178	2	0	13Q	M	67	39			94	52	
14	66	M	110	2	0	11Q	UM	61	47			83	42	
15	62	M	263	4	1	17P	UM	73	69			92	72	
16	60	M	18	0	0	13Q	UM	84	61			97	68	
17	68	F	121	3	0	13Q	M	67	48			96	57	
18	62	M	60	2	5	11Q	NR	95	55			98	87	
19	65	M	19	0	0	13Q	M	79	60			93	78	
20	74	M	51	1	0	T12	M	95	88			96	94	
21	65	M	39	0	1	ND	UM	94	84			97	92	
22	67	F	35	0	0	11Q	UM	87	57			86	57	
23	78	M	86	3	2	17P	UM	95	95			96	96	
24	48	F	173	4	3	ND	NR	64	53			95	69	
25	62	M	263	4	0	17P	UM	90	82			95	92	
26	73	M	20	0	0	13Q	M	62	55			81	70	

P No.	Age	Sex	WBC	Rai	Prior Rx	FISH	IGVH	% Viability by Annexin/PI binding assay					
								Con	IPI	IgM	IgM+IPI	Str	Str+IPI
27	70	M	50	3	0	13Q	M	39	25			69	42
28	52	F	77	4	1	17P	UM	91	69	93	76		
29	74	M	75	4	1	13Q	M	83	53	98	93		
30	62	M	121	0	0	NEG	M	64	38	87	49		
31	56	F	27	3	0	13Q	UM	85	65	90	70		
32	68	M	15	1	0	17P	M	73	60	81	63		
33	62	F	76	4	0	NEG	NR	42	35	66	36		
34	55	M	103	0	0	NEG	UM	88	69	93	80		
35	57	M	14	0	0	UNK	ND	90	78	90	82		
36	54	F	21	1	0	17P	UM	91	82				
37	66	F	21	0	0	ND	UM	87	70				
38	77	M	59	0	2	13Q	UM	95	65				
39	68	M	59	0	0	T12	UM	88	80				
40	66	M	34	1	0	UNK	ND	71	54				
41	64	M	22	3	2	13Q	M	90	85	90	85		
42	54	M	57	1	0	T12	M	96	92				
43	54	F	50	1	0	NEG	M	64	45			75	65
44	67	F	42	4	1	UNK	UM	57	26	82	31		
45	84	M	24	0	0	13Q	M	82	73	89	81		
46	73	M	13	0	0	13Q	ND	79	54	96	87		
47	68	M	15	0	0	17P	UM	89	73	93	77		
48	67	F	33	1	0	T12	UM	88	73	94	85		
49	51	M	34	1	0	17P	UM	97	88	97	96		
50	69	M	18	3	0	T12	UM	80	77	83	80		
51	67	F	118	0	0	13Q	M	88	71	94	81		
52	52	F	26	2	2	17P	UM	89	77	93	80		
53	80	F	105	1	0	13Q	M	60	49	81	56		
54	70	F	53	0	0	UNK	ND	96	91			99	93

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P No.	Age	Sex	WBC	Rai	Prior Rx	FISH	IGVH	% Viability by Annexin/PI binding assay						
								Con	IPI	IgM	IgM+IPI	Str	Str+IPI
55	60	M	18	0	0	13Q	UM	66	49			95	79
56	71	M	256	3	0	NEG	UM	74	44			95	74
57	50	F	50	0	0	UNK	ND	94	85			95	91

Provided is the complete list of patient samples (n=57), their prognostic factors, and percent viability of CLL cells with or without IPI-145 in suspension cultures, or BCR-stimulated or stromal co-cultured model systems.

Abbreviations: WBC - White Blood Cells; Prior RX - Prior treatment; UM - Unmutated IgVH; M - Mutated IgVH; ND - not done, IPI- IPI-145; Str - Stroma; Con - time matched control at 24 hrs; UNK - Unknown