Supplementary Methods

Patient samples

PBMCs were obtained from CLL patients (n=7; 4/7 (57%) treatment naïve, 5/7 (71%) advanced Rai stage, 6/7 (86%) IGHV unmutated and 1/7 (14%) with del17p) in accordance with the Declaration of Helsinki and the IRB of the National Heart, Lung and Blood Institute (NHLBI; Bethesda, MD). PBMCs were isolated by density-gradient centrifugation (Ficoll Lymphocyte Separation Media; ICN Biomedicals, Irvine, CA, USA), followed by cryopreservation in 90% fetal bovine serum (FBS; Sigma, St Louis, MA, USA) and 10% dimethyl sulfoxide (DSMO; Sigma) using a slow rate freezing container (Thermo Fisher Scientific, Waltham, MA, USA) stored initially at -80°C and subsequently at -150°C.

Murine xenotransplant of CLL cells

Xenografting of PBMCs into 2-24 week old male NSG mice (Jax-5557; Jackson Laboratory, Bar Harbor, ME, USA) was carried out as previously described,[3] except that cells were thawed in PBS without staining prior to injection. Briefly, 10^8 PBMC's per mouse were injected retro-orbitally into 4-16 recipient mice, split equally between vehicle and combination treatment groups. Treatment for xenografted NSG mice was with drinking water containing vehicle or 0.15 mg/mL of acalabrutinib and 0.15 mg/mL of ACP-319 in vehicle. Xenografted NSG mice were sacrificed three weeks after cell injection and PB and spleens were harvested. Human anti- CD45, CD5 and CD19 or CD3 were used to identify human CLL cells and T cells.

NF-κB activity assay

NF-κB activity was measured using the TransAM NF-κB transcription factor assay kit (Active Motif, Carlsbad, CA, USA). Splenic TCL1-192 cells from mice treated with vehicle, acalabrutinib, ACP-319 or the combination of acalabrutinib and ACP-319 were collected after two weeks of treatment. Nuclear lysates were extracted via Nuclear Extract Kit (Active Motif). 4µg of nuclear lysates were applied in duplicate to 96-well plates coated with oligonucleotides containing NF-κB consensus

sequence (5′-GGGACTTTCC-3′). After incubation the plates were washed and incubated with an anti-p50 antibody followed by subsequent washes and incubations with anti-rabbit HRP-conjugated antibody and developing solution. The reaction was stopped by addition of a stop solution after 5 minutes and read at an absorbance of 450nm (Wallac Victor^3, PerkinElmer, Waltham, MA, USA).

Gene expression analysis

Total RNA was extracted from CLL PBMC's using RNeasy kit (Qiagen, Germantown, MD, USA), and cDNA was prepared using the High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA, USA). NF-κB specific gene signature score was determined as previously described.[36] Briefly, expression of six NF-κB target genes was quantified by real-time polymerase chain reaction (RT-PCR) on TaqMan Primers on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The difference in threshold cycle (ΔCt) for each gene of interest was calculated from the Ct of the housekeeping gene (VCP)—Ct of the gene of interest (e.g., CCL3). The ΔCt for the pathway-specific genes were averaged into an NF-κB signature score.

Supplementary Figure Legends

Supplementary Figure S1: Impact of acalabrutinib, ACP-319 or their combination on the proportion of proliferating and viable TCL1-192 cells in peripheral blood. (a) Mean ± SEM percentage change in the proportion of KI67+ TCL1-192 cells in each treatment group compared with vehicle treated mice (n=20 split evenly across treatment groups in two experimental cohorts). **(b)** Mean ± SEM percentage change in the proportion of viable TCL1-192 cells (Annexin-V and VIVID double negative) in each treatment group compared with vehicle treated mice (n=40 split evenly across treatment groups in two experimental cohorts). Abbreviations: Acala, acalabrutinib; Combo, combination treatment with acalabrutinib and ACP-319. All comparisons by an unpaired t-test using a linear model to take into account the random batch effect. Statistics comparing treatment to vehicle control are shown below the treatment bars; statistics comparing treatments are shown with comparison brackets: **P*<0.05, ***P*<0.01, ****P*<0.001 and *****P*<0.0001.

Supplementary Figure S2: Efficacy of combination therapy with acalabrutinib and ACP-319 on patient derived CLL and T cells in a CLL xenograft model.

All data shown are at three weeks after CLL cell injection and 11-18 days after treatment start. **(a)** Representative dot plots of CD19/CD5 staining in vehicle and combination treated mice, percentage of human CLL and T cells are indicated. **(b-c)** Symbols correspond to individual patients and the data points represent the mean of all mice injected with cells from the same patient; in **(b)** absolute human CLL cell count in the peripheral blood and in **(c)** percentage of CLL cells among human lymphocytes (CD45+) in the spleen. Line represents median. **(d-e)** Mean ± SEM of the ratio of human CD45+ cell to total nucleated cells in (d) PB and (e) SP. Circle represent the mean of all mice injected with cells from the same patient. **(f-g)** Symbols correspond to individual patients and the data points represent the mean of all mice injected with cells from the same patient; in **(f)** absolute human T cell count in the peripheral blood and in **(g)** percentage of T cells among human

lymphocytes (CD45+) in the spleen. Line represents median. Abbreviations: Veh, vehicle and Combo, combination treatment with acalabrutinib and ACP-319. Statistical analysis by paired Student's t-test.

Supplementary Figure S3: Impact of BTK and PI3Kδ inhibition on BCR signaling in TCL1-192 cells from peripheral blood or primary CLL cells harvest from the murine spleen.

(a) Mean ± SEM percentage change in the proportion of pERK positive TCL1-192 cells collected from the peripheral blood in single agent or combination treated mice as compared with vehicle-treated mice (n=40 split evenly across treatment groups in two experimental cohorts). **(b-c)** Mean ± SEM MFI (isotype MFI subtracted) of **(b)** pPLCγ2 and **(d)** pERK in CLL cells treated with the combination of acalabrutinib and ACP-319 or vehicle-control, harvested from murine spleens, analyzed by flow cytometry. Abbreviations: Acala, Acalabrutinib; Combo, combination treatment with acalabrutinib and ACP-319. Comparisons of TCL1-192 cells were by an unpaired t-test using a linear model to take into account the random batch effect. Comparisons of patient matched CLL cells were by paired Student's t-test. Statistics comparing treatment to vehicle control are shown; ***P*<0.01.

Supplementary Figure S4: Impact of acalabrutinib, ACP-319 or their combination on ERK and NF-κB signaling in TCL1-192 cells from peripheral blood.

(a) Mean ± SEM percentage change in the proportion of pNF-κB positive TCL1-192 cells collected from the peripheral blood in single agent or combination treated mice as compared with vehicle-treated mice (n=40 split evenly across treatment groups in two experimental cohorts). **(b)** Mean ± SEM percentage change in nuclear expression of NF-κB isoforms p50 determined by ELISA (N=7 per treatment group across two cohorts) versus vehicle controls. **(c)** Mean ± SEM change in average NF-κB signature score (N=5 per treatment group) versus vehicle controls. All comparisons by an unpaired t-test using a linear model to take into account the random batch effect. Statistics

comparing treatment to vehicle control are shown below the treatment bars; statistics comparing treatments are shown with comparison brackets; *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.