Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Byrd JC, Harrington B, O'Brien S, et al. Acalabrutinib (ACP-196) in relapsed chronic lymphocytic leukemia. N Engl J Med 2016;374:323-32. DOI: 10.1056/NEJMoa1509981

SUPPLEMENTARY APPENDIX

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PHARMACODYNAMIC ANALYSES METHODS

Immunoblotting

Primary CLL cells were isolated using RosetteSep™ Human B Cell Enrichment Cocktail (Cat. #15064; Stemcell Technologies; Vancouver, BC, Canada) followed by Ficoll Paque Plus™ density separation method (G.E. Healthcare Biosciences AB; Uppsala, Sweden) per manufacturer instructions. Primary T cells were isolated in a similar fashion using RosetteSep™ Human T Cell Enrichment Cocktail (Cat . #15061; Stemcell Technologies).

All primary cells and cell lines were treated with indicated concentrations of acalabrutinib or ibrutinib (Acorn Pharma Tech, LLC), washed twice with phosphate-buffered saline (PBS), then replenished with RPMI media supplemented with 10% fetal bovine serum prior to stimulation (RPMI/10% FBS). Primary CLL cells were then stimulated for 15 minutes with plate-bound anti-human IgM (MP Biomedicals; Santa Ana, CA). Plates were prepared by incubating anti-IgM in PBS at 10 µg/mL overnight at 4°C. Wells were washed with PBS once immediately prior to plating cells. Primary T cells and Jurkat cells were stimulated using plate-bound anti-human CD3 antibody (Cat. #16-0037-85; eBiosciences; San Diego, CA) prepared in a similar fashion to anti-IgM and soluble anti-human CD28 antibody at 1 µg/mL (Cat. #16-0288; eBioscience) for 45 minutes. H460 cells were stimulated with soluble epidermal growth factor at 50 ng/mL (Cat. #PHG011; ThermoFisher Scientific; Grad Island, NY) for 10 minutes. Whole cell lysates were prepared immediately after stimulation. Proteins were detected using the following antibodies: anti-phospho-IKBA (Ser32, Cat. #2859), anti-IKBA (Cat. #4812), anti-phospho-ERK1/2 (Thr202/Tyr204, Cat. #9101), anti-ERK1/2 (Cat. #9102), anti-phospho-AKT (Thr308, Cat. #9257), and anti-AKT (Cat. #9272), anti-phospho-EGFR (Tyr1173, Cat. #4407) anti-phospho-EGFR (Tyr1068, Cat. #2234), anti-EGFR (Cat. #2646), anti-JunB (Cat. #3753), and anti-NFAT (Cat. #4389) (Cell Signaling Technologies; Danvers, MA).

Tec Phosphorylation Assay

Commercially purchased human platelets (0.5 mL at a concentration of 3×10^8 platelets/mL) were treated with 50 µg/mL of collagen or buffer (unstimulated control) for 5 minutes. Each test article was dissolved in dimethyl sulfoxide. Platelets were incubated with either dimethyl sulfoxide or increasing concentrations of test article for 30 minutes. Platelets were then lysed in detergent buffer containing 15 mmol/L Hepes (pH 7.4), 150 mmol/L NaCl, 1 mmol/L phenyl methyl sulfonyl fluoride (PMSF), 10 mmol/L EGTA (ethylenegylcotetraacetic acid), 1 mmol/L sodium orthovanadate, 0.8 µg/mL leupeptin, and 2% Triton X-100 (wt/vol). Replicate pairs of the lysates were analyzed using a Phosphotyrosine TEC ELISA assay (RayBiotech, Norcross, GA). Unstimulated control values were subtracted from readings before calculation of percent phospho-Tec relative to DMSO-treated, collagen-stimulated platelet levels. Ibrutinib was purchased from Chembest (Shanghai, China).

Kinase Inhibition Assays

Recombinant enzymes were used to profile the in vitro activity of acalabrutinib compared with ibrutinib (Chembest; Shanghai, China). Each drug was dissolved in dimethyl sulfoxide at a stock concentration of 10 mM and 10-point kinase inhibitory activities were measured with adenosine triphosphate at a concentration consistent with the Michaelis constant (Km) of each enzyme, with a 1h preincubation of the compound with the kinase before start of the assay through the addition of ATP. Data are listed for the experiments where acalabrutinib and ibrutinib were evaluated head-to-head in the same experiment. BTK and ITK kinase activity was measured using the IMAP (immobilized metal ion affinity-based fluorescence polarization) assay at Acerta Pharma BV, Netherlands. BMX, TXK, EGFR, ERBB2, ERBB4, JAK3, BLK, FGR, FYN, HCK, LCK, LYN, SRC, YES1 kinase activity was measured using the Z'-LYTE assay at Thermo Fisher (Madison, WI). Inhibitory activity on TEC was measured using the LanthaScreen assay from ThermoFisher, with total incubation of compound with kinase for 2 hours, similar to total incubation time of compound with kinase in IMAP and Z'-LYTE assay.

Sample Collection and Peripheral Blood Mononuclear Cell (PBMC) Isolation

Blood for pharmacodynamic analysis was drawn in heparin-coated vacutainer tubes just prior to dosing on days 1, 2, 8 and 28 of cycle 1; and 4 hours after dosing on days 1, 8, and 28 of cycle 1. Samples were shipped at ambient temperature overnight to Acerta Pharma analytical labs and immediately purified to obtain PBMCs using Ficoll Paque Plus™ density separation method (G.E. Healthcare Biosciences AB; Uppsala, Sweden; Product insert instructions 71-7167-00 AG), followed by cryopreservation in liquid nitrogen. Plasma was cryopreserved for cytokine analysis.

Cell Preparation for Btk Target Occupancy and Signaling Assays

Samples from all time points from each patient were analyzed together in batch with a control PBMC sample from a healthy volunteer (IRB approved protocol number: ACE-RES-001). Cryopreserved cells were thawed (37°C water bath), washed in RPMI + 1% FBS RPMI. Five million cells per sample were washed with 1 mL cold PBS and cell pellets were snap frozen in liquid nitrogen for occupancy assay. Remaining cells were resuspended in RPMI + 10% FBS for signaling assays.

Btk Target Occupancy ELISA

Ninety-six-well Optiplate (PerkinElmer; Waltham, MA) plates were coated overnight with 125 ng/well anti-Btk antibody (BD Biosciences) and blocked with bovine serum albumin (BSA). Frozen cell pellets were lysed in ice cold lysis buffer containing 50 mM Tris-HCl pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.05% digitonin, and protease inhibitor cocktail (Sigma-Aldrich; St. Louis, MO). Cell lysates were incubated for 1 hour on ice in the presence or absence of acalabrutinib (1 µM). At this concentration, Btk was shown in preclinical studies to be saturated and completely bound by acalabrutinib. The cell lysates were incubated for 1 hour on ice with a biotinylated derivative of acalabrutinib (ACP-4016; 10^{-7} M). The equivalent of 5×10^5 cells of lysate/well, in replicates of four, were incubated for 2 hours at ambient temperature on the anti-Btk coated 96-well OptiPlate. Plates were washed with PBS + 0.05% Tween four

times. Streptavidin-HRP (Invitrogen; ELISA grade) was added at 100 µL/well (120 ng/mL) and incubated for 1 hour at room temperature. Plates were washed with PBS + 0.05% Tween three times and then washed two times with PBS. One hundred µL/well of SuperSignal ELISA Femto Substrate (ThermoFisher Scientific; Waltham, MA) was added and then chemiluminescence was measured after 1 minute (EnVision® plate reader; PerkinElmer). The percent of Btk occupancy for each sample time point was calculated relative to the day 1 predose sample for each patient. The signal from the day 1 predose sample without exogenous acalabrutinib represents 100% free Btk (or 0% occupied Btk), whereas the signal from the day 1 predose sample with exogenous acalabrutinib represents 0% free Btk (or 100% occupied Btk). The incubation of each cell lysate with 1 µM acalabrutinib was used to correct for background signal not related to free Btk.

$$\%\ \text{Free Btk sample X} = \left[\frac{\text{Sample X} - \text{Sample X}^{+\text{ACP196 [1uM]}}}{\text{Day1 Predose} - \text{Day1 Predose}^{+\text{ACP196 [1uM]}}}\right] \text{X } 100$$

$$\%\ \text{Occupied Btk} = 100\% - \%\ \text{Free Btk}$$

Phosphoflow Cytometry for Measuring Phosphorylated Btk (Y223)

The set of PBMC samples for each patient consisting of all sample time points were analyzed together in deep well plates (Nunc™; ThermoScientific) at 100,000 cells/well. A subset of wells containing cells from the day 1 predose sample was treated with acalabrutinib (1 µM) and incubated for 2 hours in a 37°C, 5% CO_2 incubator. This concentration of acalabrutinib was determined in preclinical studies to completely inhibit B-cell receptor-induced phosphorylated Btk (p-Btk; Y223) signaling. A method of signaling via the B-cell receptor was adapted from previously described methods.[2] B-cell receptor signaling was initiated with the addition of goat anti-human IgM F(ab')2 (10 µg/mL) + H_2O_2 (3.3 mM), and incubated at 37°C for 10 minutes. PBMCs were fixed with paraformaldehyde (1.6%) for 10 minutes at 37°C, followed by cell

permeabilization with ice cold 100% methanol. Plates were stored at –80°C overnight. Cells were washed and stained with an antibody cocktail consisting of unconjugated rabbit anti-human p-Btk (Y223) (Abcam; Cambridge, United Kingdom) and fluorescently labeled mouse antibodies against human CD20 PerCP-Cy5.5, CD19 PE-Cy7, CD3 BV421, CD5 BV510, and cleaved PARP (all from BD Biosciences; Franklin Lakes, NJ). Cells were washed with PBS + 0.5% BSA two times, followed by a secondary staining step using goat anti-rabbit -IgG Alexa 647 antibody (1:1000 dilution; LifeTechnologies; Carlsbad, CA) for 30 minutes at 4°C. Cells were washed two times. Phosphoflow cytometry was performed with a FacsVerse flow cytometer (BD Biosciences). The p-Btk median fluorescence values (MFI) were collected in the cleaved PARP negative, CD19+, CD20+/-, CD5+, CD3 negative gate. This population consists of CLL cells. The magnitude of p-Btk signal was calculated based on a metric of fold change over control, where control is the p-Btk MFI from the stimulated day $1^{+\text{ exogenous acalabrutinib 1}\mu M}$ sample. All conditions were run in duplicate wells and the results were calculated from the average of both wells.

Natural Killer (NK) Cell Activity Assay

PBMCs from patients were evaluated in NK cell activity assays using previously described methods.[3] K562 cells were labeled with ^{57}Cr and exposed to no effector cells (spontaneous release), effector cells at various ratios (10:1 to 100:1) and to 0.5% Triton X 100 (maximal lysis). Percent specific lysis was calculated as (experimental well-spontaneous lysis)/(maximal lysis-spontaneous lysis) x 100.

In Vivo Thrombosis Model

Blood samples for platelet isolation from healthy volunteers and ibrutinib-treated patients were collected under IRB protocol #1302013582. Freshly isolated platelets—from 5 patients with CLL treated with commercially available ibrutinib (420 mg once per day [QD]) and 3 patients with CLL treated with acalabrutinib (100 mg twice per day [BID])—were fluorescently labeled and infused into mice possessing the human A1 domain of VWF (designated VWF^{HA1}) as

previously described.[4] Using confocal microscopy, intravital measurements of thrombus formation can be evaluated in real time after laser-induced injury to the vessel wall of arterioles (~40–65 mm diameter) in VWF^{HA1} mice continuously infused with the fluorescently labeled human platelets. The size of resulting thrombi was calculated using image analysis software (Slidebook 6; Intelligent Imaging Innovations Inc; Denver, CO). Freshly isolated platelets from healthy volunteers were used as normal controls (n=5).

Cytokine Analyses

Cytokine measurements were done using Luminex xMAP® technology using MILLIPLEX® reagents (Cat. #HCYTMAG-60K-PX38, HCP2MAG-62K-PX23, HCD8MAG-15K; EMD Millipore; Billerica, MA).

PHARMACOKINETIC ANALYSES METHODS

Sample Collection

Blood samples were collected for pharmacokinetic analysis in lithium heparin tubes, centrifuged and plasma was stored at –80ºC. Samples for cycle 1 day 1 were collected at predose and 0.25, 0.5, 0.75, 1, 2, 4, 6 and 24 hours (before dose on day 2) post-dose. Samples for cycle 1 day 8 were drawn predose and 0.25, 0.5, 0.75, 1, 2, 4, and 6 hours post-dose. On cycle 1 day 15, 22, and 28, a pharmacokinetic sample was drawn predose and 1 hour post-dose.

Analytical Methods

Plasma concentrations of acalabrutinib were determined using a validated analytical liquid chromatography-tandem mass spectrometry (LC-MS/MS) method against a stable labeled internal standard at Basi (West Lafayette, IN) in lithium heparin plasma. The quantification range was 1.00 to 1000 ng/mL.

Pharmacokinetic Analysis

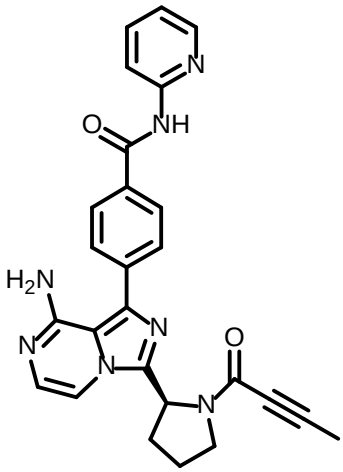
Pharmacokinetic analyses were done by noncompartmental methods by 7th Wave Laboratories LLC (Chesterfield, MO) using validated WinNonlin® software (Certara USA; Princeton, NJ). Pharmacokinetic parameters for acalabrutinib were calculated using noncompartmental analysis. Only plasma concentrations greater than or equal to the validated lower limit of quantification (LLOQ) were used in the pharmacokinetic analyses. Nominal blood sampling times were used in all analyses. The maximum concentration (C_{max}) and time to maximum concentration (T_{max}) were taken directly from the individual's data. The area under the concentration-time curve ($AUC_{0–6}$, $AUC_{0–12}$, $AUC_{0–24}$ and $AUC_{0–inf}$; where data permit) were calculated using the linear trapezoidal method (log / linear rule). As data permit, the elimination rate constant (λ_z) was calculated as the negative of the slope of the terminal log-linear segment of the plasma concentration-time curve. The range of data used for each subject and study drug administration was determined by visual inspection of a semi-logarithmic plot of

concentration versus time and was comprised of ≥3 data points along the elimination phase. The terminal elimination half-life ($t_{1/2}$) was calculated according to the following equation: $t_{1/2} = 0.693/\lambda_z$. Oral clearance (CL/F) and volume of distribution (Vz/F) were calculated according to: $CL/F = (dose/AUC_{0\text{-}inf})$ and $Vz/F = dose/\lambda_z \times AUC_{0\text{-}inf.}$

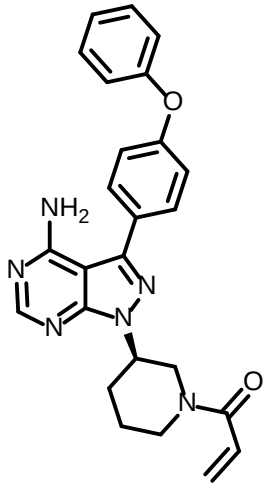
Plasma concentrations and derived pharmacokinetic parameters were summarized using descriptive statistics. Graphs of individual subject and mean plasma concentrations versus time data, by cohort, are presented on linear and semi-logarithmic axes.

SUPPLEMENTARY FIGURES AND TABLES

Figure S1. Chemical Structures of Acalabrutinib and Ibrutinib

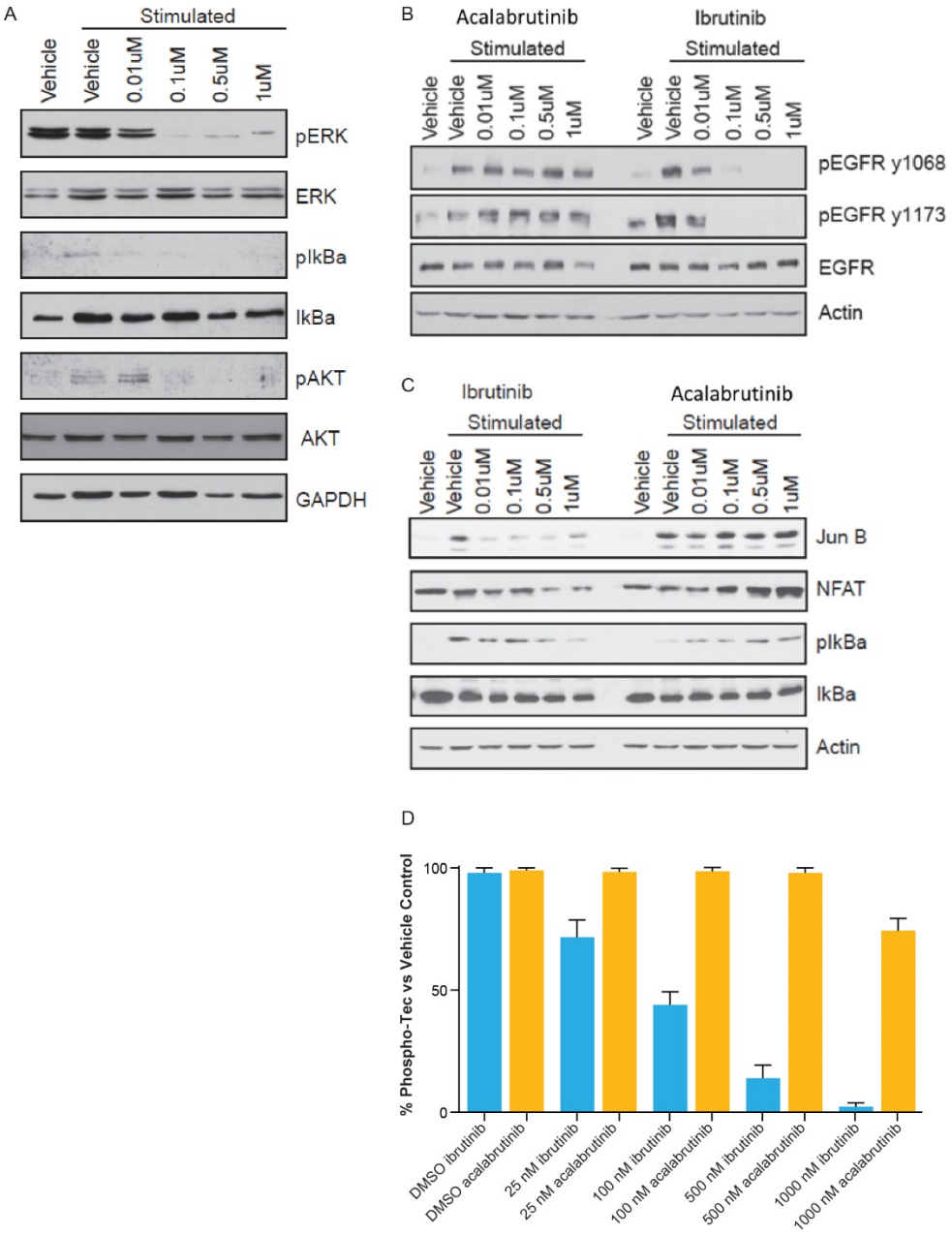


Acalabrutinib



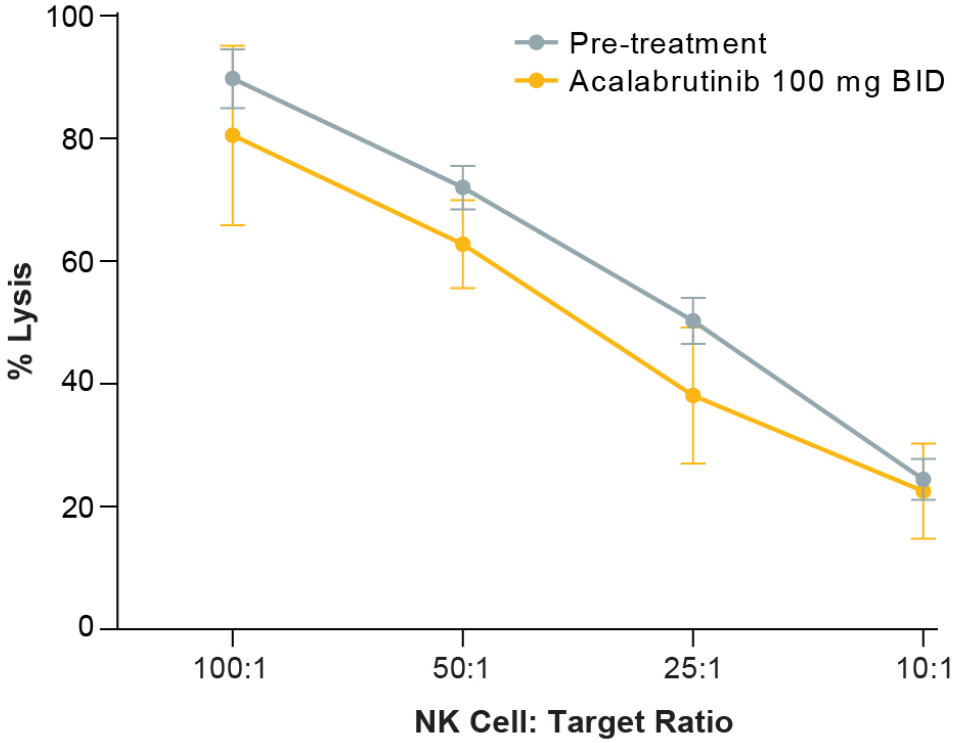
Ibrutinib

Figure S2. In Vitro Signaling Assays



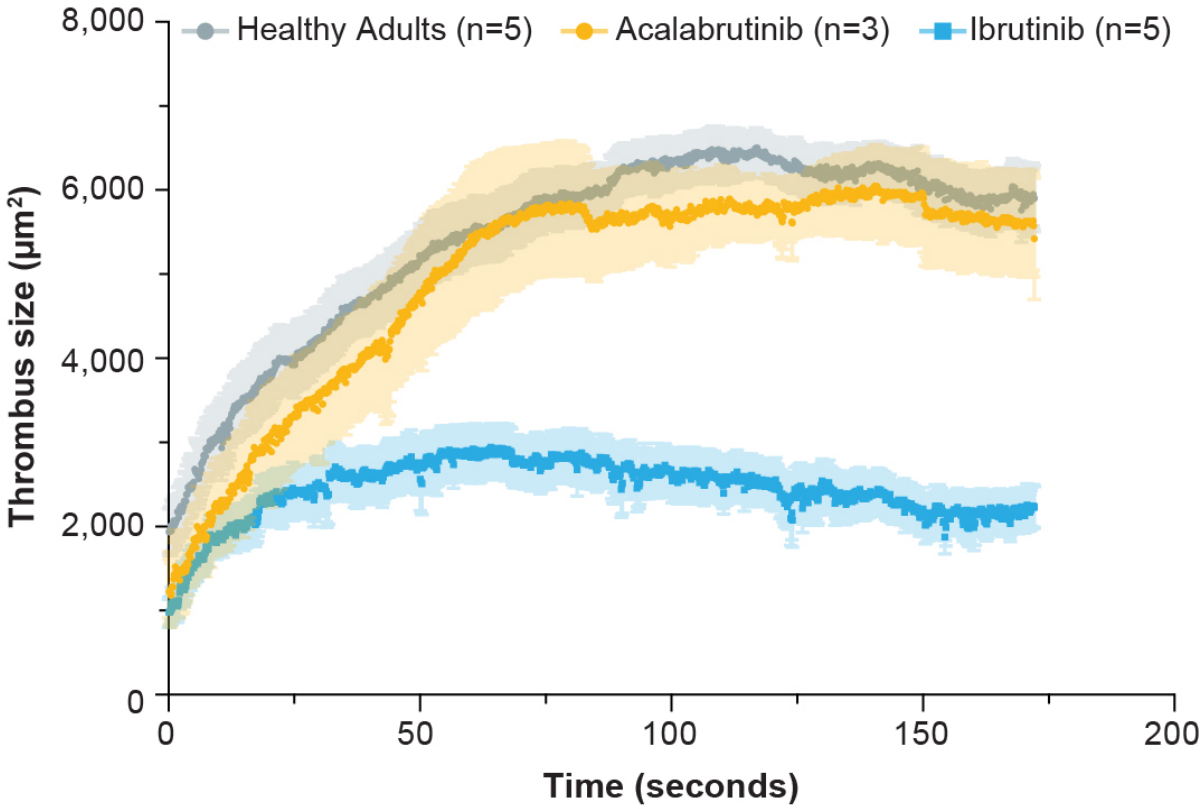
Panel A shows a representative Western blot of primary CLL cells from 7 patients treated with increasing concentrations of acalabrutinib and then stimulated with IgM. Panel B shows effect of increasing concentrations of ibrutinib versus acalabrutinib on EGFR phosphorylation in H460 lung cancer cells. Panel C shows the effect of increasing concentrations of ibrutinib versus acalabrutinib on T-cell receptor signal transducers downstream of Itk in Jurkat cells. Panel D shows the effect of ibrutinib versus acalabrutinib on Tec phosphorylation.

Figure S3. Natural Killer (NK) Cell Non-antibody-dependent Cell-mediated Cytotoxicity Assay



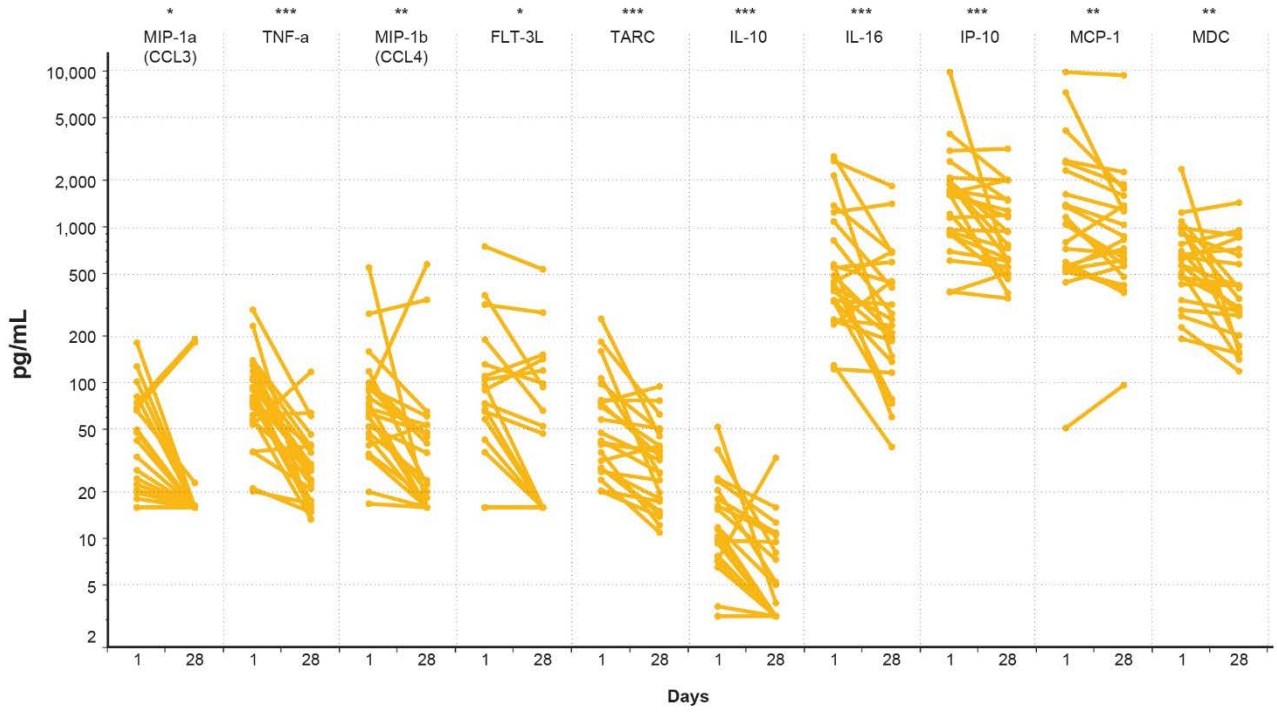
NK cells from patients (n=5) before and after acalabrutinib treatment (100 mg BID) were evaluated in a standard NK cell activity assay. BID denotes twice daily.

Figure S4. In Vivo Thrombosis Formation Model



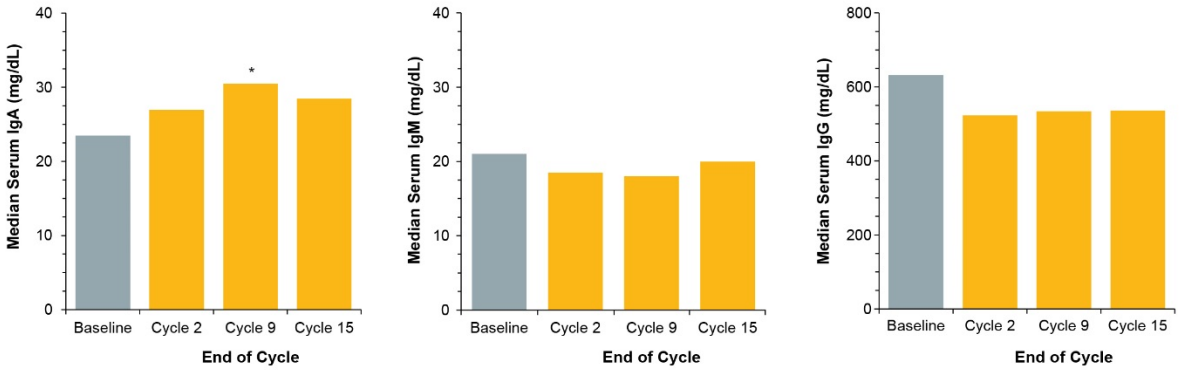
Platelets from patients treated with ibrutinib 420 mg once per day (QD) (n=5) or acalabrutinib 100 mg twice per day (BID) (n=3) were evaluated for their ability to support thrombus formation in laser injured arterioles of VWF^{HA1} mice. Freshly isolated platelets from healthy volunteers (n=5) were used as non-drug treated controls. A minimum of 4 arterioles per mouse was used to assess thrombus formation for each patient/volunteer sample. Median fluorescence intensity as a function of time is provided in the figure (shading denotes standard error of the median).

Figure S5. Cytokine Analyses (n=23)



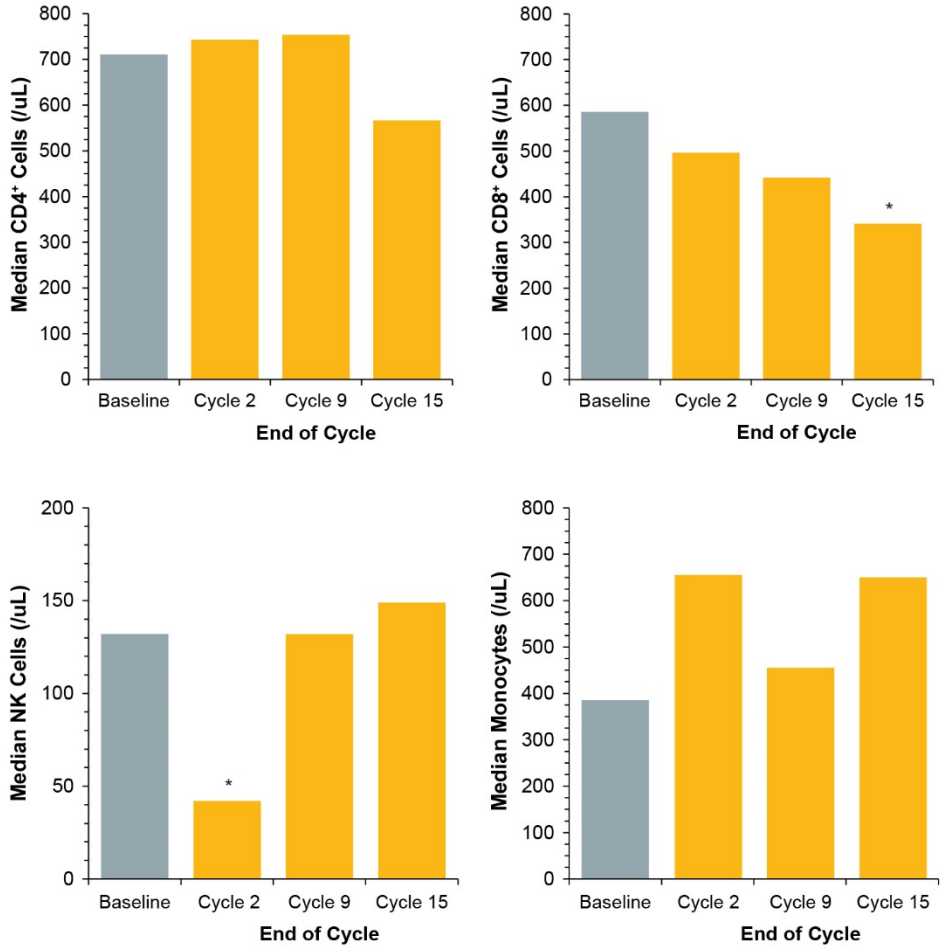
*P<0.05; **P<0.01, ***P<0.001. P-values based on Wilcoxon Signed-Rank test.

Figure S6. Change in Median Serum Immunoglobulin A (n=16), Immunoglobulin M (n=16) and Immunoglobulin G (n=11) Over Time in Patients Treated With Acalabrutinib.



The serum IgG plot excludes patients who received intravenous immunoglobulin. *P-value less than 0.05 by Wilcoxon Signed-Rank test compared with baseline.

Figure S7. Change in T Cell ($CD4^+$ [n=10] and $CD8^+$ [n=10]), Natural Killer (NK) Cell (n=9), and Monocyte (n=8) Counts Over Time in Patients Treated With Acalabrutinib



*P-value less than 0.05 by Wilcoxon Signed-Rank test compared with baseline.

Table S1. Recombinant Kinase Inhibition Assays

Kinase	IC_{50} (nM)	
	Acalabrutinib	**Ibrutinib**
BTK	5.1 ± 1.0 (N=4)	1.5 ± 0.2 (N=4)
BMX*	46 ± 12 (N=3)	0.8 ± 0.1 (N=3)
ITK*	>1000 (N=4)	4.9 ± 1.2 (N=4)
TEC*	93 ± 35 (N=2)	7.0 ± 2.5 (N=2)
TXK*	368 ± 141 (N=3)	2.0 ± 0.3 (N=3)
EGFR*	>1000 (N=3)	5.3 ± 1.3 (N=3)
ERBB2*	~1000 (N=3)	6.4 ± 1.8 (N=3)
ERBB4*	16 ± 5 (N=3)	3.4 ± 1.3 (N=3)
JAK3*	>1000 (N=3)	32 ± 15 (N=3)
BLK*	>1000 (N=3)	0.1 ± 0.0 (N=3)
FGR	>1000 (N=2)	3.3 ± 1.1 (N=2)
FYN	>1000 (N=2)	29 ± 0 (N=2)
HCK	>1000 (N=2)	29 ± 0 (N=2)
LCK	>1000 (N=2)	6.3 ± 1.3 (N=2)
LYN	>1000 (N=2)	20 ± 1 (N=2)
SRC	>1000 (N=2)	19 ± 1 (N=2)
YES1	>1000 (N=2)	4.1 ± 0.2 (N=2)

*Kinases that contain a cysteine residue aligning with Cysteine-481 in Btk.
Acalabrutinib and ibrutinib comparison of in vitro activity inhibitory profiles (IC_{50} values, in nM) for recombinant enzymes of Tec, ErbB, Src family kinases, and other related kinases. For experiments with N=3 or N=4, plus/minus standard deviation is shown. For experiments with N=2, plus/minus the error/range over the two independent experiments is shown. IC_{50} denotes half-maximal inhibitory concentration.

Table S2. Summary of Day 8 Acalabrutinib Steady-State Plasma Pharmacokinetic Parameters

Group	Day	Dose Level (mg)	Interval		T_{max} (hr)	C_{max} (ng/ml)	AUC_{0-6} (hr•ng/ml)	AUC_{0-12} (hr•ng/ml)	AUC_{0-24} (hr•ng/ml)	AUC_{0-inf} (hr•ng/ml)	λ_z (1/hr)	$t_{1/2}$ (hr)	CL/F (L/hr)	Vz/F (L)
1	8	100	QD	N	8	8	7	7	7	7	7	7	7	7
				Mean	0.81	529	584	602	603	603	0.612	1.16	180	308
				SD	0.26	286	180	180	179	179	0.100	0.190	59.4	141
				CV%	31.8	54.2	30.8	29.8	29.7	29.7	16.4	16.4	32.9	45.6
2a	8	175	QD	N	7	6	7	7	7	7	7	7	7	7
				Mean	0.64	805	1140	1160	1160	1160	0.700	1.02	213	317
				SD	0.24	757	733	738	738	738	0.131	0.207	148	233
				CV%	38.0	94.1	64.1	63.4	63.4	63.4	18.8	20.2	69.7	73.3
2b	8	100	BID	N	28	26	28	24	24	24	24	24	24	24
				Mean	1.0	827	826	926	1850	927	0.677	1.13	165	361
				SD	1.1	841	652	663	1330	662	0.142	0.612	139	730
				CV%	104	102	78.9	71.6	71.6	71.4	21.0	54.0	84.0	202
3	8	250	QD	N	7	7	7	5	5	5	5	5	5	5
				Mean	1.1	1350	1990	2310	2310	2310	0.743	0.941	130	179
				SD	0.63	933	1080	1090	1090	1090	0.0772	0.0945	64.9	105
				CV%	54.8	68.9	54.1	47.0	47.0	47.0	10.4	10.0	50.0	58.5
4a	8	400	QD	N	6	5	6	6	6	6	6	6	6	6
				Mean	0.92	902	1730	1850	1870	1870	0.579	1.39	312	677
				SD	0.56	638	966	1050	1060	1060	0.228	0.620	223	698
				CV%	61.4	70.8	55.7	56.6	56.8	56.7	39.4	44.5	71.6	103

λ_z denotes terminal elimination rate constant, AUC area under the curve, BID twice per day, CL/F oral clearance, C_{max} maximum concentration, CV% coefficient of variation, INF infinity, QD once per day, SD standard deviation, $t_{1/2}$ terminal half-life, T_{max} time to maximum concentration, and Vz/F volume of distribution.

Table S3. Treatment-emergent Serious Adverse Events (N=61)*

	Grade 2	Grade 3	Grade 4	All Grades†
	No. (%)			
Pneumonia	0 (0.0)	3 (4.9)	2 (3.3)	6 (9.8)
Autoimmune hemolytic anemia	0 (0.0)	2 (3.3)	0 (0.0)	2 (3.3)
Pyrexia	1 (1.6)	1 (1.6)	0 (0.0)	2 (3.3)
Abdominal pain	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Acute kidney injury	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Acute respiratory failure	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Acute sinusitis	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Anemia	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Aortic stenosis	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Atrioventricular block	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Bradycardia	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Cellulitis	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Diarrhea	1 (1.6)	0 (0.0)	0 (0.0)	1 (1.6)
Dizziness	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Dyspnea	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Febrile neutropenia	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Furuncle	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Gastroenteritis	1 (1.6)	0 (0.0)	0 (0.0)	1 (1.6)
Gingivitis	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Hypertension	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Lung infection	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Lymphangitis	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Metastases to meninges	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Neutropenia	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Neutropenic sepsis	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Plasmablastic lymphoma	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Respiratory alkalosis	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)

	Grade 2	Grade 3	Grade 4	All Grades†
Respiratory failure	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Sepsis	0 (0.0)	0 (0.0)	1 (1.6)	1 (1.6)
Squamous cell carcinoma of skin	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Syncope	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)
Viral myocarditis	1 (1.6)	0 (0.0)	0 (0.0)	1 (1.6)
Wound infection	0 (0.0)	1 (1.6)	0 (0.0)	1 (1.6)

*Serious adverse events reported for all patients, regardless of cause, through October 1, 2015.

†The all-grade column includes one patient with a grade 5 event of pneumonia.

Table S4. Improvement in Baseline Cytopenia

Hemoglobin	
Baseline hemoglobin ≤11 g/dL – no of patients	21
On-treatment hemoglobin > 11 g/dL – no. of patients (%)	16 (76)
Absolute Neutrophil Count (ANC)	
Baseline ANC ≤ 1,500/µL – no of patients	15
On-treatment ANC > 1,500/µL – no. of patients (%)	12 (80)
Platelet Count	
Baseline platelet count ≤ 100,000/µL – no of patients	32
On-treatment platelet count > 100,000/µL – no. of patients (%)	20 (62)

Table S5. B Symptoms Before and On Treatment*

Patients with any B symptom at baseline – no. of patients	16
Patients with no B symptoms at the indicated time point – no./total no. of patients (%)	
end of cycle 2	12/14 (86)
end of cycle 3	14/16 (88)
end of cycle 4	13/16 (81)
end of cycle 5	13/15 (87)
end of cycle 6	12/14 (86)
end of cycle 9	15/15 (100)
end of cycle 12	11/13 (85)
end of cycle 15	8/8 (100)
end of cycle 18	4/4 (100)
end of cycle 21	1/1 (100)

*Evaluable patients included all patients who had at least one B symptom at baseline.

Table S6. Response Assessment Criteria[5]

Response	Peripheral Blood	Bone Marrow if done	Nodes, Liver, and Spleen[a]
CR*	Lymphocytes < 4 x 10^9/L ANC > 1.5 x 10^9/L[b] Platelets > 100 x 10^9/L[b] Hemoglobin > 11.0 g/dL (untransfused)[b]	Normocellular < 30% lymphocytes No B-lymphoid nodules	Normal (eg, no lymph nodes > 1.5 cm)
CRi	Lymphocytes < 4 x 10^9/L Persistent anemia, thrombocytopenia, or neutropenia related to drug toxicity	Hypocellular < 30% lymphocytes	Normal (eg, no lymph nodes > 1.5 cm)
PR*	Lymphocytes ≥ 50% decrease from baseline ANC > 1.5 x 10^9/L Or Platelets > 100 x 10^9/L or 50% improvement over baseline[b] Or Hemoglobin > 11.0 g/dL or 50% improvement over baseline (untransfused)[b]	Not assessed	≥ 50% reduction in lymphadenopathy[c] and/or in spleen or liver enlargement
PD*	Lymphocytes ≥ 50% increase over baseline Or Platelets decrease of ≥ 50% from baseline secondary to CLL Or Hemoglobin decrease of > 2 g/dL from baseline secondary to CLL	Not assessed	Increase ≥ 50% in lymphadenopathy or Increase ≥ 50% in hepatomegaly or Increase ≥ 50% in splenomegaly

ANC = absolute neutrophil count; CR = complete remission; CRi = CR with incomplete blood count recovery; PD = progressive disease; PR = partial remission; SD = stable disease

*CR: all of the above CR criteria have to be met, and patients have to lack disease-related constitutional symptoms; PR: at least two of the above PR criteria for lymphadenopathy, splenomegaly, hepatomegaly, or lymphocytes plus one of the criteria for ANC, platelets or hemoglobin have to be met; SD is absence of PD and failure to achieve at least a PR; PD: at least one of the above PD criteria has to be met. Note: Isolated elevation of treatment-related lymphocytosis by itself will not be considered PD unless patient becomes symptomatic from this per Cheson et al.[6]

a Computed tomography (CT) scan of abdomen, pelvis, and chest is required for this evaluation.

b Without need for exogenous growth factors.

c In the sum products of ≤ 6 lymph nodes or in the largest diameter of the enlarged lymph node(s) detected before therapy and no increase in any lymph node or new enlarged lymph nodes.

REFERENCES

1. Kohrt HE, Houot R, Goldstein MJ, et al. CD137 stimulation enhances the antilymphoma activity of anti-CD20 antibodies. Blood 2011;117:2423-32.

2. Irish, JM, Czerwinski DK, Nolan GP, Levy R. Kinetics of B Cell Receptor Signaling in Human B Cell Subsets Mapped by Phosphospecific Flow Cytometry. J of Immunol 2006;177: 1581-89.

3. Sierich, H and Eiermann, T (2013) Comparing Individual NK cell activity in vitro. Current Protocols in Immunology. Unit 14.32

4. Chen JC, Zhou H, Diacovo A, Zheng XL, Emsley J, Diacovo TG. Exploiting the kinetic interplay between the GPIbα–VWF binding interfaces to regulate hemostasis and thrombosis. Blood 2014:124;3799-807.

5. Hallek M, Cheson BD, Catovsky D, et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood 2008;111:5446-5456.

6. Cheson BD, Byrd JC, Rai KR, et al. Novel targeted agents and the need to refine clinical end points in chronic lymphocytic leukemia. J Clin Oncol 2012 30:2820-2822.