Comparison of Acalabrutinib, A Selective Bruton Tyrosine Kinase Inhibitor, with Ibrutinib in Chronic Lymphocytic Leukemia Cells

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Abstract

Purpose: Ibrutinib inhibits Bruton tyrosine kinase (BTK) by irreversibly binding to the Cys-481 residue in the enzyme. However, ibrutinib also inhibits several other enzymes that contain cysteine residues homologous to Cys-481 in BTK. Patients with relapsed/refractory or previously untreated chronic lymphocytic leukemia (CLL) demonstrate a high overall response rate to ibrutinib with prolonged survival. Acalabrutinib, a selective BTK inhibitor developed to minimize off-target activity, has shown promising overall response rates in patients with relapsed/refractory CLL. A head-to-head comparison of ibrutinib and acalabrutinib in CLL cell cultures and healthy T cells is needed to understand preclinical biologic and molecular effects.

Experimental Design: Using samples from patients with CLL, we compared the effects of both BTK inhibitors on biologic activity, chemokine production, cell migration, BTK phosphorylation, and downstream signaling in primary CLL lymphocytes and on normal T-cell signaling to determine the effects on other kinases.

Results: Both BTK inhibitors induced modest cell death accompanied by cleavage of PARP and caspase-3. Production of CCL3 and CCL4 chemokines and pseudoemperipolesis were inhibited by both drugs to a similar degree. These drugs also showed similar inhibitory effects on the phosphorylation of BTK and downstream S6 and ERK kinases. In contrast, off-target effects on SRC-family kinases were more pronounced with ibrutinib than acalabrutinib in healthy T lymphocytes.

Conclusions: Both BTK inhibitors show similar biological and molecular profile in primary CLL cells but appear different on their effect on normal T cells.

Introduction

The B-cell receptor (BCR) pathway is critical for the proliferation, maintenance, and survival of B cells (1). Bruton tyrosine kinase (BTK) is pivotal in the BCR axis (2) and is activated when BCR is stimulated. BTK is a cytoplasmic protein that is expressed in hematopoietic cells in general and B-lymphoid cells in particular. Because of its critical role in BCR axis signaling, importance of the BCR pathway in B-cell proliferation and maintenance, and its selective expression in B cells, BTK is an attractive therapeutic target. The concept of inhibiting BTK to treat B-cell malignancies stems from the observations that agammaglobulinemia (3) and immunodeficiency diseases (4) in pediatric patients are associated with a decreased number of B cells and their function (5). These attributes were due to the loss of activity of a cytoplasmic kinase, which was cloned and termed BTK (2, 6). Similar to humans, mice lacking BTK activity develop X chromosome–linked immunodeficiency syndrome (7).

BTK is expressed in normal and malignant B cells. Furthermore, upon crosslinking and activation of BCR, BTK protein levels have been demonstrated to increase (8) in murine normal B cells and through CXCR4 and CXCL12 signaling in murine chronic lymphocytic leukemia (CLL) lymphocytes (9). In human specimens, BTK mRNA levels are higher in CLL lymphocytes compared with normal B cells, although protein levels vary (2, 10). An increase in BCR signaling pathway proteins, such as LYN, SYK, and BTK, was observed in CLL cells derived from lymph node tissue samples that had activated NF-κB signatures (11). BTK plays a pivotal role in the BCR pathway, promoting proliferation, survival, maintenance, and migration of malignant B cells. Collectively, these observations provide a strong rationale for targeting BTK in B-cell malignancies, including CLL.

Ibrutinib is a first-in-class BTK inhibitor that irreversibly binds to cysteine (Cys)-481 in the kinase domain and potently blocks its enzymatic activity (12). Ibrutinib decreases proliferation, moderately increases apoptosis, attenuates survival signals in stroma or nurse-like cells, and reduces cell adhesion and chemokine production in preclinical models (10, 12, 13). These effects reflect BCR pathway inhibition, which has also been demonstrated by reduced levels of phospho-BTK, diminished downstream ERK–MAP kinase pathway signaling, and altered the expression of antiapoptotic proteins (10, 12, 14, 15). Clinical and laboratory...
Acalabrutinib Compared with Ibrutinib in CLL Cells

Translational Relevance

Ibrutinib is an oral, first-in-class Bruton tyrosine kinase (BTK) inhibitor that is approved by the US Food and Drug Administration for the treatment of patients with previously treated and untreated chronic lymphocytic leukemia (CLL). This drug irreversibly binds to the Cys-481 residue of BTK; however, ibrutinib also binds to several other cysteine-containing kinases, which could result in off-target toxicities. Acalabrutinib, a more selective BTK inhibitor developed to minimize off-target effects, has shown promising clinical activity in patients with relapsed/refractory CLL. Preclinical comparisons of ibrutinib and acalabrutinib are needed to better understand potential differences between the biological and molecular effects of these BTK inhibitors in primary CLL cells and normal T cells.

Overall, these clinical response data with ibrutinib strongly validated BTK as a target and ibrutinib as a therapeutic intervention for CLL. Toxicity, albeit limited, and inhibition of other cysteine-containing kinases, underscored the need for more selective BTK inhibitor (35).

Acalabrutinib (ACP-196) is a selective BTK inhibitor in clinical development for the treatment of hematologic malignancies. Acalabrutinib has a unique reactive butyramide group, which forms a covalent bond with the Cys-481 residue in BTK (Supplementary Fig. S1A), as does the acrylamide group of ibrutinib (Supplementary Fig. S1B). The half maximal inhibitory concentration (IC50) of ibrutinib for the BTK protein is 1.5 nmol/L compared with 5.1 nmol/L for acalabrutinib, indicating stronger BTK inhibition with ibrutinib in biochemical assays. However, acalabrutinib is a more selective BTK inhibitor. For example, the IC50 values for inhibition of 8 of 9 kinases that contain cysteine residues that align with Cys-481 in BTK were less than 10 nmol/L for ibrutinib; however, none of the 9 kinases had IC50 values less than 10 nmol/L for acalabrutinib. Indeed, a recent clinical report demonstrated that toxicities, including atrial fibrillation and bleeding, which are associated with ibrutinib, were not observed in patients with relapsed/refractory CLL treated with acalabrutinib for a median of 14 months. Acalabrutinib monotherapy achieved an ORR of 95% in patients with relapsed/refractory CLL (n = 60 evaluable), including patients with del(17p) (n = 18; ref. 32).

In the current study, we compared ibrutinib and acalabrutinib to determine their effects on biologic and molecular activities, including inhibition of BCR/BTK axis and downstream signaling, induction of apoptosis, chemokine production, and migration in CLL cells, and off-target signaling in healthy T cells. Our data suggest that acalabrutinib and ibrutinib demonstrate similar molecular and biologic consequences in primary CLL cells but different effects on signaling events, including LCK and SRC kinase phosphorylation, in primary T-lymphocytes. While ibrutinib inhibited LCK and SRC phosphorylation in normal T cells, acalabrutinib did not reach IC50 values for these enzymes at physiologically relevant concentrations. Collectively, our data establish a similar preclinical activity profile of acalabrutinib and ibrutinib in primary CLL cells but a differentiated one in T cells.

Materials and Methods

Patient sample collection and cell culture

Peripheral blood was obtained from patients with CLL who provided written informed consent as part of a protocol approved by the Institutional Review Board of The University of Texas MD Anderson Cancer Center in accordance with the Declaration of Helsinki. Baseline characteristics of patients are summarized in Supplementary Table S1. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density centrifugation (Atlanta Biologicals) and suspended in RPMI1640 media supplemented with 10% human serum (Sigma-Aldrich). PBMCs
Consisted primarily (>90%) of CLL lymphocytes based on CD19 and CD5 positivity. BCR signaling was stimulated by incubating CLL cells with goat F(ab')2 fragments of human immunoglobulin M (IgM; MP Biomedicals).

**Drugs**

Acalabrutinib was provided by Acerta Pharma. Ibrutinib was purchased from Selleck Chemicals. Stock solutions of both drugs were made in DMSO and used generally at equimolar concentrations. Time-matched, DMSO-treated (i.e., vehicle-treated) cells were used as controls.

**Cytotoxicity assays**

CLL cells were treated with 1 or 3 μmol/L acalabrutinib or ibrutinib for 24, 48, or 72 hours. Cells were then stained with annexin V and propidium iodide and counted by flow cytometry as described previously (36). Cell death in drug-treated samples was normalized by subtracting the cell death of control vehicle-treated, time-matched CLL samples, which ranged from 5% to 20%.

**Chemokine assays**

CCL3 (Mip-1α) and CCL4 (Mip-1β) levels in the media of CLL cell cultures exposed to α-LMg stimulation and BTK inhibitors were measured using a Quantikine ELISA Kit (R&D Systems; ref. 37). Concentrations were extrapolated from a standard curve and expressed in pg/mL.

**Migration assays**

For pseudoemperiploesis, NK-Tert stromal cells were seeded onto collagen-coated plates. The next day, CLL cells were incubated with or without drug and layered on stroma. After 4 hours, cells that had not migrated into the stromal cell layer were removed by vigorously washing with RPMI1640. The stromal cell layer containing transmigrated cells was detached with trypsin/ethylenediaminetetraacetic acid. CLL cells were resuspended in media and counted by flow cytometry for 20 seconds at 60 μL/minute (37).

**Immunoblot analyses**

Immunoblots were performed with cellular protein extracts and visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences; ref. 37). Antibodies for specific proteins are listed in Supplementary Table S2.

**Effect on phospho-ITK and PLC γ1 in T-cell line**

Jurkat cells were treated with acalabrutinib, ibrutinib, or DMSO control for 2 hours and stimulated with 3.3 mmol/L H2O2 for 10 minutes. Following stimulation, cells were lysed with Cell Extraction Buffer (Thermo Fisher #ENN0011) containing protease inhibitors on ice for 30 minutes. Lysates were precleared and incubated with Protein A sepharose beads (GE Healthcare #17-5138-01) and ITK antibody (Abcam #ab32507) overnight at 4°C. The next day, supernatant from the beads was collected, and a Western blot analysis for phospho-PLCγ1 (Cell Signaling Technology #14008S) was performed on the supernatant. ITK bound to the beads was eluted with Sample Buffer (Life Technologies #B0007) containing reducing reagent (Life Technologies #B0009), and a Western blot analysis was performed with 4G10 antibody (Millipore #05-321) to detect phospho-ITK, with total ITK (Abcam #ab32507) as control. Ponceau staining was used to verify even loading after transfer. Densitometry was performed using GelQuant software (version 1.8.2).

**Statistical analysis**

Paired two-tailed Student t tests were performed using GraphPad Prism 6 software (GraphPad Software, Inc.) to compare DMSO-treated cells with drug-treated cells. Similarly, paired two-tailed Student t tests were used to compare ibrutinib-treated cells with acalabrutinib-treated cells at either 1 or 3 μmol/L concentration of the drug.

**Results**

Ibrutinib and acalabrutinib induce apoptosis

In vitro experiments were performed with 1 or 3 μmol/L ibrutinib or acalabrutinib. The peak plasma concentrations of ibrutinib in CLL patients following an oral dose of 560 mg ranges from 150 to 200 ng/mL (340 nmol/L–450 nmol/L; total ibrutinib levels). The peak plasma concentrations of acalabrutinib following a single 100 mg dose are 520 ± 286 ng/mL (1,118 nmol/L total acalabrutinib). Hence, the concentration of drug in the experiments (1 or 3 μmol/L) was selected to span a range similar to, or a half-log greater than, total concentrations of ibrutinib or acalabrutinib achieved during clinical trials (17, 32).

Without stimulation of the BCR pathway, at 1 and 3 μmol/L, compared with controls, ibrutinib and acalabrutinib induced modest yet statistically significant (P values range from 0.05 to <0.0001) increases in apoptosis rates in primary CLL cells at 24, 48, and 72 hours of treatment (Fig. 1A–C). Median cell viability for samples treated with 1 μmol/L ibrutinib were 95%, 90%, and 88% at 24, 48, and 72 hours, respectively. Median cell viability rates for samples treated with 1 μmol/L acalabrutinib were 98%, 96%, and 93% at 24, 48, and 72 hours, respectively. While the differences between treatment groups were only 3% to 6% at each time point, they were statistically significant. For example, the P values were 0.0048, 0.0041, and 0.0065 at 24, 48, and 72 hours. Similar small differences between ibrutinib- and acalabrutinib-induced apoptosis were also observed at 3 μmol/L of the inhibitors. In general, at each concentration and time point, ibrutinib induced consistently and significantly higher apoptosis of CLL cells than acalabrutinib. As expected, IgM stimulation resulted in a survival advantage, with moderate cell death due to both inhibitors (Fig. 1D–F). Prognostic factors such as IGVH mutation status (9 mutated vs. 5 unmutated),
ZAP-70 positivity (6 positive and 9 negative), B2M level (9 less than 2.5 and 8 more than 2.5), and other characteristics such as prior therapy (7 treated and 12 previously untreated), absolute lymphocyte count (11 less than 100,000 and 7 more than 100,000 ALC/μL), age (10 less than 60 and 10 more than 60 years old), and gender (13 males and 7 females), did not appear to affect acalabrutinib-mediated cell death (P value always <0.2; data not shown).

PARP cleavage increased from 0.8- to 5.3-fold in CLL samples (n = 5) treated with 1 or 3 μmol/L ibrutinib or acalabrutinib compared with time-matched vehicle-treated control (Fig. 2A). There were no significant differences in PARP cleavage between cells treated with 1 μmol/L (P = 0.14) or 3 μmol/L (P = 0.57) ibrutinib versus acalabrutinib (Fig. 2B). Compared with time-matched, vehicle-treated controls, cleaved caspase-3 levels increased from 0.8- to almost 3-fold without significant differences between ibrutinib- and acalabrutinib-treated cells (P = 0.50 and P = 0.08 at 1 and 3 μmol/L, respectively, Fig. 2C). Collectively, these data indicate that the BTK inhibitors ibrutinib and acalabrutinib inhibit moderate levels of apoptotic cell death (<15%).

Ibrutinib and acalabrutinib inhibit chemokine production and migration

BCR activation increases production of the chemokines CCL3 and CCL4 in CLL cells (37, 40). Conversely, inhibition of BCR signaling by ibrutinib decreases the levels of these chemokines (12). As shown in Fig. 3, IgM-induced production of CCL3 was inhibited by ibrutinib and acalabrutinib (Fig. 3A–D). CCL3 levels did not significantly differ between ibrutinib-treated and acalabrutinib-treated cells without IgM (P = 0.355) or with IgM (P = 0.170). CCL4 levels (Fig. 3E–H) were reduced in cells treated with ibrutinib or acalabrutinib compared with vehicle control without significant differences between drug treatment groups (P = 0.246). Both drugs showed comparable inhibition (P = 0.747) of IgM-stimulated CCL4 production.

Activation of BCR signaling by chemokines promotes cell migration beneath the stroma, also known as pseudomorphosis (13, 40–42). BTK inhibition impairs the expression and function of the chemokine CXCR4, resulting in retention of CLL cells in niches (9); similarly, chemokine-controlled adhesion and migration of CLL lymphocytes are inhibited by ibrutinib (15). In
Ibrutinib and acalabrutinib inhibit BTK phosphorylation and downstream signaling in CLL cells

Ibrutinib and acalabrutinib reduced phosphorylation of BTK protein levels, as shown by immunoblots (Fig. 4A). Both drugs appear to impede the BTK signaling pathway by decreasing the phosphorylation of ERK and S6 (Fig. 4A). However, phosphorylation of AKT at Thr308 was not affected by ibrutinib or acalabrutinib. Quantitation of immunoblots representing five patient samples demonstrated that in the absence of IgM stimulation, BTK phosphorylation was significantly ($P < 0.0001$ with ACP at both concentrations; $P = 0.033$ and $P = 0.0005$ with IBT at 1 and 3 μmol/L, respectively) reduced by both drugs relative to control and to similar extents (Fig. 4B). Stimulation with IgM mitigated the BTK-inhibitory effects of these drugs (not shown). ERK phosphorylation was reduced by 50% to 60% by both drugs with both concentrations ($P < 0.002$). Similarly, S6 phosphorylation, which was measured in samples from 3 patients, was decreased by 50% upon drug treatment. In conclusion, the inhibitory signature of ibrutinib and acalabrutinib on BTK pathway was similar in CLL cells.

Ibrutinib and acalabrutinib treatment decrease Bcl-2 and Mcl-1 total protein levels

We previously reported that ibrutinib decreases Mcl-1 protein levels in primary CLL cells without changing Bcl-2 protein expression (14). Consistent with these data, ibrutinib and acalabrutinib decreased Mcl-1 ($P < 0.002$) without IgM stimulation (Fig. 5A and B). The decrease in Mcl-1 total protein was also significant when culture conditions included IgM stimulation ($P = 0.008$; Fig. 5A and C). In contrast, Bcl-2 total protein level did not change in either culture conditions ($P > 0.11$) protein levels (Fig. 5A–C). Collectively, these data establish that acalabrutinib is similar to ibrutinib in its action on Bcl-2 and Mcl-1 proteins in CLL cells.

Ibrutinib and acalabrutinib treatments differ in inhibiting SRC family kinases

Next, we examined the effects of these two BTK inhibitors on SRC family kinases in T cells obtained from healthy donors. SRC family kinases play an important role in platelet activation, and their inhibition may contribute to adverse bleeding events (26). To help ensure that any observed dephosphorylation was due to the BTK inhibitors, and not due to endogenous phosphatase activity, a low dose of the phosphatase inhibitor H2O2 was added during cell stimulation (38). While both drugs decreased the levels of phospho-LCK (Y505; Fig. 6A) and phospho-SRC (Y418; Fig. 6B) in a dose-dependent manner, the extent of inhibition was very different, with ibrutinib demonstrating a...
more potent inhibitory effect on the phosphorylation of LCK and SRC. The EC50 for ibrutinib was less than 0.2 μmol/L, whereas the EC50 for acalabrutinib was not reached at 10 μmol/L. These data suggest that ibrutinib may have greater off-target effects on SRC kinase inhibition in healthy T cells than acalabrutinib.

Ibrutinib and acalabrutinib show differential inhibition of ITK

To further characterize the effects of both drugs in T cells, we measured the phosphorylation of ITK and its substrate, PLCγ1, in stimulated Jurkat T cells treated with ibrutinib or acalabrutinib. We observed a dramatic difference in phospho-ITK and phospho-PLCγ1 at 1 μmol/L concentrations of each drug, with neither enzyme affected by acalabrutinib compared with >90% inhibition with ibrutinib treatment (Fig. 6C–E). In addition, ibrutinib reduced phosphorylation of both enzymes even at lower (0.25 and 0.5 μmol/L) concentrations. These data show that ibrutinib, but not acalabrutinib, inhibits ITK and its downstream target PLCγ1 in T cells as an off-target effect.

Discussion

The overall aim of this project was to compare ibrutinib and acalabrutinib in CLL patient samples to determine similarities and differences of these two agents. We focused on four different
approaches. First, we compared the biological effect of each drug in CLL patient samples. Second, we evaluated the impact of these BTK inhibitors on chemokine production and migration. Third, we investigated the impact on substrate (BTK) phosphorylation and downstream events after on-target inhibition in CLL cells. Finally, we compared the effect of these agents on signaling in healthy T cells, which could be considered off-target effects.

Similar to what has been reported for ibrutinib (10, 12), acalabrutinib treatment resulted in cell death in less than 15% of CLL lymphocytes. The extent of cell death was 3% to 6% higher with ibrutinib versus acalabrutinib, which could be due to off-target activities of ibrutinib (32). Patient characteristics, including prognostic factors and prior therapy, were not associated with the degree of cell death induced by acalabrutinib and ibrutinib. These observations suggest that at equimolar free drug concentrations, both acalabrutinib and ibrutinib would be expected to have similar efficacy in patients with CLL.

BCR activation promotes the secretion of proinflammatory chemokines, including CCL3 and CCL4, in CLL cells (40). Ibrutinib treatment has been shown to decrease CCL3 and CCL4 levels in CLL cell cultures. This finding of decreased chemokine levels due to BTK inhibition by ibrutinib was also observed during separate clinical trials with either ibrutinib (12) or acalabrutinib (32). Consistent with these data, the current study showed that

![Figure 4. Comparison of ibrutinib- and acalabrutinib-mediated inhibition of BTK phosphorylation and downstream signaling. A, CLL cells were incubated with DMSO only (controls) or with 1 or 3 μmol/L ibrutinib or acalabrutinib for 48 hours without IgM stimulation (lanes 1-5) or with IgM stimulation (lanes 6-10). The protein lysates were immunoblotted for total and phosphorylated BTK, AKT, ERK, and S6 proteins. Vinculin was used as a loading control. B, Immunoblots representing five different patient samples without IgM stimulation were quantitated. Values represent fold change in phosphorylated protein relative to total protein.](image)

![Figure 5. Comparison of ibrutinib and acalabrutinib-mediated changes in the levels of Bcl-2 and Mcl-1 antiapoptotic proteins. A, CLL cells were incubated with DMSO only (controls) or with 1 or 3 μmol/L ibrutinib (IBT) or acalabrutinib (ACP) for 48 hours without IgM stimulation (lanes 1-5) or with IgM stimulation (lanes 6-10). The protein lysates were immunoblotted for total Bcl-2 and Mcl-1 proteins. Vinculin was used as a loading control. B and C, Immunoblots were analyzed from 5 different patients, and changes in Mcl-1 and Bcl-2 in CLL cells treated with BTK inhibitors without (B) or with (C) IgM stimulation were quantitated. Values represent fold change relative to DMSO controls.](image)
ibrutinib and acalabrutinib similarly reduced CCL3 and CCL4 production in the presence of BCR pathway stimulation (Fig. 3). This was also reflected in the migration of CLL cells toward stroma, presumably mediated in part by CXCL12 (SDF1), consistent with a previous study (15).

**BTK** mRNA levels have been reported to be higher in CLL cells than in normal B cells; however, BTK protein levels vary among patients (10). Because ibrutinib and acalabrutinib are potent, covalent, and irreversible inhibitors of BTK, both drugs reduced phosphorylation of BTK. Consistent with this inhibition of the kinase, phosphorylation of downstream proteins, such as ERK and S6, was similarly blunted with ibrutinib and acalabrutinib (Fig. 4B). In our samples, at this 24-hour time point, phosphorylation of AKT was not inhibited, possibly due to sustained activation of other signaling pathways (43). Downstream of BCR signaling pathway is an effect on the NF-κB activity. We were not able to observe a clear effect on downstream phospho-p65 levels in this study (data not shown).

Our prior preclinical and clinical investigations demonstrated that ibrutinib decreased Mcl-1 protein levels while Bcl-2 levels remain the same or increase (14). This finding was also recently reported by other investigators (44). Our current data indicate that acalabrutinib and ibrutinib cause similar reductions in Mcl-1 protein expression even in the presence of BCR stimulation with IgM (Fig. 5). CLL cell survival is associated with the expression of Bcl-2 family antiapoptotic proteins, such as Mcl-1 and Bcl-2. Hence, a decline in Mcl-1 protein levels in response to treatment with BTK inhibitors provides rationale for combining ibrutinib or acalabrutinib with the Bcl-2 antagonist venetoclax (45, 46).

Indeed, such combination strategies have been tested and found to augment ibrutinib-induced cytotoxicity in CLL (14, 44) and other B-cell malignancies (47).

BTK belongs to the Tec family of tyrosine kinases, which has five members. While BTK is expressed specifically in B cells and not in T cells, other Tec family members, such as ITK and TXK, are expressed in T cells. T-cell receptor–mediated signal transducers downstream of ITK were inhibited by ibrutinib (30, 48) but not acalabrutinib in the Jurkat T-cell line (32). Similarly, phosphorylation of TEC was decreased by ibrutinib (32). Such off-target effects have the potential to lead to toxicities, such as bleeding (24, 49). The SRC family, mostly FYN and LYN, plays a critical role in platelet activation via the collagen receptor GPVI–FcRy complex (50). Autophosphorylation of Y418 and dephosphorylation of Y530 on SRC are critical events that lead to activation of the protein. The latter is phosphorylated by CSK, which inhibits

![Figure 6](image-url)
LCK because the protein folds up and binds its own SH2 domain. LCK (Y505) was inhibited only by ibrutinib (Fig. 6A). Our data demonstrate that ibrutinib reduces phosphorylation of SRC and LCK. In contrast to ibrutinib, acalabrutinib showed minimal inhibition of SRC and LCK. Differential effect of these two drugs on T cells was further evidenced in Jurkat T-lymphoblastic cell line for ITK and PLCy1 (Fig. 6C–E). Consistent with these data, previous studies with acalabrutinib demonstrated minimal effects on EGFR, TEC, or ITK signaling, and no inhibition of thrombus formation in vivo at clinically relevant concentrations (32, 51).

In conclusion, our investigations demonstrate that the selective BTK inhibitor acalabrutinib produces biological effects in CLL cells that are comparable with those exerted by ibrutinib but that the molecular impact on healthy T cells appeared different.

Disclosure of Potential Conflicts of Interest
V. Gandhi reports receiving commercial research grants from Acerta. No potential conflicts of interest were disclosed by the other authors.

Disclaimer
The authors directed the development of the manuscript and were fully responsible for all content and editorial decisions for this manuscript.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Patel, E. Bibikova, W. Wierda, V. Gandhi
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