Treatment with Ibrutinib Inhibits BTK- and VLA-4-Dependent Adhesion of Chronic Lymphocytic Leukemia Cells In Vivo

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Abstract

Purpose: Ibrutinib leads to a transient lymphocytosis in patients with chronic lymphocytic leukemia (CLL) that develops within hours of starting drug and is due to the efflux of cells from lymphoid tissues into the blood. We therefore sought to investigate the in vivo effect of ibrutinib on migration and adhesion of CLL cells.

Experimental Design: Patients received single-agent ibrutinib (420 mg daily) on an investigator-initiated phase II trial. Serial blood samples were collected pretreatment and during treatment for ex vivo functional assays.

Results: Adhesion of CLL cells to fibronectin was rapidly (within hours) and almost completely inhibited (median reduction 98% on day 28, P < 0.001), while the effect on migration to chemokines was more moderate (median reduction 64%, P = 0.008) and less uniform. Although cell surface expression of key adhesion molecules such as CD49d, CD29, and CD44 were modestly reduced, this was only apparent after weeks of treatment. Stimulation of CLL cells from patients on ibrutinib with PMA, which activates PKC independent of BTK, restored the ability of the cells to adhere to fibronectin in a VLA-4–dependent manner. Finally, the addition of ibrutinib to CLL cells adhered to fibronectin in vitro caused the detachment of 17% of the cells, on average, consistent with in vivo observations of an increasing lymphocytosis within 4 hours of starting ibrutinib.

Conclusions: Inhibition of BTK and VLA-4–dependent adhesion of CLL cells to stroma and stromal components provides a mechanistic explanation for the treatment-induced lymphocytosis and may reduce CD49d-dependent prosurvival signals in the tissue microenvironment.

Introduction

CLL is a malignancy of mature autoreactive B cells (1, 2). The tumor microenvironment in secondary lymphoid tissues and the bone marrow plays a key role in the pathogenesis of the disease (3–5). This is underscored by the observation that CLL cells undergo apoptosis in vitro unless surrogates of survival signals found in the tumor microenvironment are provided. Furthermore, Herishanu and colleagues presented direct in vivo evidence that CLL cells are activated through the B-cell receptor (BCR) in the lymph node microenvironment (4). The BCR signaling pathway has emerged as a pivotal pathway in the pathogenesis of CLL and as a target for novel therapies (6–8). Bruton tyrosine kinase (BTK), a cytoplasmic TEC kinase, couples BCR activation to intracellular calcium release and NFκB signaling (9). BTK plays an essential role in B cells as demonstrated by the severe defect in B-cell development due to loss-of-function mutations in this kinase in patients with X-linked agammaglobulinemia. Furthermore, deletion of BTK in murine models supports a critical role for BTK in the development and progression of CLL (10, 11).

Ibrutinib, an orally active covalent BTK inhibitor, was recently approved in the United States and Europe for the treatment of patients with CLL who have received at least one prior therapy as well as of previously untreated patients who carry a deletion of chromosome band 17p13.1. Several clinical trials have shown that single-agent ibrutinib is well tolerated and can induce objective clinical responses in CLL, including in patients with high-risk disease features (12–14). Even as a single agent, ibrutinib induced durable responses in CLL patients with TP53 aberrations, including deletion 17p13.1 [del(17p)] and TP53 mutation, contrasting with the inferior responses and relatively early relapses when these high-risk patients are treated with chemoimmunotherapy (14). In a randomized phase III study of ibrutinib versus ofatumumab for relapsed or refractory CLL, patients treated with ibrutinib had a 78% reduction in the risk of tumor progression or death compared with patients in the ofatumumab arm (15).

A transient increase in lymphocytosis is commonly observed when treatment with ibrutinib or other BCR-directed kinase inhibitors is initiated (13, 16). In our investigator-initiated phase II study of ibrutinib in CLL, the average peak increase in the absolute lymphocyte count was greater than 65%, with more than 40% of...
Ibrutinib Inhibits VLA-4–Dependent Adhesion in CLL

Translational Relevance

Ibrutinib, as well as other BCR-directed kinase inhibitors, leads to a transient, treatment-induced lymphocytosis. The increase in CLL cells in the blood develops within hours of the first drug dose due to an efflux of cells out of the lymphoid tissues. Given that redistribution lymphocytosis is a characteristic observation when treating CLL with ibrutinib, we sought to better understand the underlying biologic mechanism by evaluating the in vivo effect of ibrutinib on the ability of CLL cells to migrate and adhere to stromal cells and components of the extracellular matrix. While we found an overall reduction in both migration and adhesion, only the ability of CLL cells to adhere to fibronectin was rapidly and almost completely inhibited by ibrutinib in all patients evaluated. The adhesion defect induced by ibrutinib was VLA-4 dependent, providing a mechanistic explanation for the treatment-induced lymphocytosis and suggesting a role for ibrutinib in disrupting CD49d-dependent prosurvival signals.

patients reaching the peak on or before day 2 of therapy and 78% by day 28 (16). In addition, we found that the ibrutinib-induced increase in lymphocytosis, at least early on treatment, is due to the release of tumor cells from the tissue compartment into the peripheral blood (16). This is considered to be an important drug effect as the microenvironment protects tumor cells from apoptosis through various stimuli such as chemokines, cytokines, and direct interactions with accessory cells and adhesion molecules (3, 17). The hypothesis that ibrutinib, through a disruption of these tumor–microenvironment interactions, could sensitize the cells to cytotoxic or antibody therapy is currently being investigated in clinical trials.

In vitro studies with ibrutinib have demonstrated reductions in both CLL cell migration and adhesion; key mechanisms in the trafficking of cells between the peripheral blood and secondary lymphoid tissues. It has been previously shown that treatment with 1 μmol/L ibrutinib inhibits CLL cell chemotaxis toward the chemokines CXCL12, CCL13, and CCL19 (18, 19). In addition, in vitro treatment with ibrutinib diminishes BCR-activated CLL cell adhesion to fibronectin and vascular cell adhesion molecule-1 (VCAM-1; ref. 18). However, data on drug effects in vivo are limited.

Within the microenvironment, integrins play a pivotal role in establishing contact of CLL cells with their surroundings and other cells (3, 17). A molecule of central importance for these tumor–microenvironment interactions is CD49d (integrin α4) which heterodimerizes with CD29 (integrin β1) to form very late antigen 4 (VLA-4) (20, 21). VLA-4 on CLL cells binds to fibronectin and VCAM-1 in the tissue compartments. CD49d is one of the most powerful prognostic markers in CLL; with high expression of CD49d on CLL cells identifying a more aggressive disease subset with inferior survival (22, 23). To characterize the in vivo drug effect on cell adhesion and migration, we performed functional assays in vivo using peripheral blood samples collected from CLL patients treated on an investigator-initiated phase II single agent ibrutinib trial.

Materials and Methods

Patient samples

This clinical trial of ibrutinib was registered at clinicaltrials.gov as NCT01500733. Patients with CLL or SLL who were either over the age of 65 years or had TP53 aberrations were enrolled from December 2011 to January 2014. A total of 86 patients were enrolled, 30 of which were evaluated in this study. A few (n = 4) untreated CLL patients enrolled on a natural history study (registered as NCT00923507 at clinicaltrials.gov) were also included in this study. For each set of analyses, 7 to 20 patients were evaluated; Supplementary Table S1 denotes which patients were analyzed in each experiment. Written informed consent was obtained in accordance with the Declaration of Helsinki, applicable federal regulations, and requirements of local institutional review boards. Peripheral blood samples were obtained pretreatment (pre) and on days 2 (after one dose of drug), 14, and 28 of ibrutinib treatment. Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation (Ficoll Lymphocyte Separation Media; ICN Biomedicals) and viably frozen in 90% FBS, 10% dimethyl sulfoxide (DMSO; Sigma) in liquid nitrogen. Plasma supernatant from bone marrow aspirates was collected at baseline, prior to initiation of treatment. Clinical patient characteristics are summarized in Supplementary Table S2.

Chemotaxis assay

Chemotaxis assays across polycarbonate Transwell inserts were performed as previously described (24). Briefly, 106 CLL PBMCs in AIM-V medium (Gibco) were transferred into the top chambers of Transwell culture inserts (Corning Incorporated) with a diameter of 6.5 mm and a pore size of 3 μm. Filters were then placed into wells containing medium (control), medium with 200 ng/mL CXCL12 and 100 ng/mL CCL19 (Prospec), or a mixture of 50% medium and 50% bone marrow supernatant collected pretreatment. Where indicated, cells were preincubated with 1 μmol/L ibrutinib (Sellekchem) in vitro for 1 hour. CLL PBMCs were allowed to migrate for 3 hours at 37°C. Migrated cells in the bottom chamber were collected, stained with CD19 and CD3, and counted using AccuCount blank particles (Spherotech) as previously described (25). The migration index was determined by subtracting the percent migration of the medium control from the percent migration of the chemotactrant wells and normalizing. Migration experiments were performed in triplicate plates; results are reported as the average.

Flow cytometry

Cells were stained as previously described (26) with the following antibodies: anti-CD19-APC and CD3-FITC and in select experiments one of the following PE-conjugated antibodies: IgG1-isotype control, anti-CXCR4, CCR7, CD38, CD49d, CD29, and CD44 (BD Biosciences). Cells were analyzed on a FACS Canto II flow cytometer (BD Biosciences) using FACS DIVA 6.1.1 and FlowJo (Version 10, TreeStar).

Ex vivo adhesion assay

PBMCs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen). PBMCs were plated at 107 cells/ml in 500 μL into 24-well fibronectin-coated plates (Corning Inc). Where indicated, cells were incubated with 1 μmol/L ibrutinib, 500 nmol/L fragerat (VLA-4 antagonist; MedChem Expression), 50 ng/mL Phorbol 12-myristate 13-acetate (PMA; Sigma), 10 μg/mL rCD18 (Abcam Inc.), 10 μg/mL rCD29 (R&D Systems), or 10 μg/mL rCD49d (LifeSpan BioSciences, Inc.) for 1 hour at 37°C. The plates were centrifuged at 400 x g for one minute and cells were left to adhere for 1 hour at 37°C. Nonadhered cells were removed by washing three times with PBS.
(Lonza). Wells were imaged using the EVOS fl microscope (Advanced Microscopy Group). Quantification of the number of adhered CLL PBMCs was done using the ImageJ software (NIH, Bethesda, MD). Experiments were performed in duplicate plates with 4 to 6 images collected per plate; results are reported as the median cell count per visual field.

**Stromal cell adhesion assay**

HS5 bone marrow stromal cells (ATCC, CRL-11882; passages 5–10) were cultured for two days until 95% confluency in 25 cm² culture flasks. HS5 cells were routinely evaluated for changes in cell viability and morphology. CFSE-labeled PBMCs were added at 8 × 10⁶ cells/mL in 3 mL to the flasks and allowed to adhere for 2 hours at 37°C. Where indicated, cells were incubated with 1 μmol/L ibrutinib for 1 hour. Flasks were washed three times and imaged using EVOS fl microscope and the number of adhered CLL PBMCs was quantified using the ImageJ software.

**Release assay**

CFSE-labeled PBMCs obtained pretreatment were adhered to 24-well fibronectin-coated plates for 16 hours at 37°C. Nonadherent cells were removed and the plates were washed three times with PBS. Ibrutinib (1 μmol/L) was added for 1 hour at 37°C. The plates were washed three times with PBS with cells released into the media collected and counted using the Vi-Cell XR (Beckman Coulter). Wells were imaged using the EVOS microscope and the number of adhered CLL PBMCs was quantified using the ImageJ software.

**Results**

**Ibrutinib inhibits migration of CLL cells**

To determine the effect of ibrutinib on cellular migration in CLL PBMCs ex vivo, we used standard Transwell migration assays. To validate our method respective to previously published in vitro data, we first evaluated the effect of in vitro treatment of CLL cells on migration toward two important chemokines, CXCL12 and CCL19, using, as in the previous reports, a dose of 1 μmol/L ibrutinib (18, 19). As expected, there was a modest but significant reduction in the migration of ibrutinib-treated CLL cells toward the chemokine mixture (median change −33%, IQR: −2 to −59, n = 12, P = 0.04, Supplementary Fig. S1A), although 3 of 12 patients did not show a reduction in cellular migration after ibrutinib treatment. In contrast, we did not observe a significant inhibition of T-cell migration (median change −8%, IQR: −24 to −21, n = 12, P = 0.28, Supplementary Fig. S1A). We next evaluated the migratory ability of cells collected pretreatment compared with those collected from patients treated for 28 days with ibrutinib, the time point at which more than 75% of patients reached their peak ALC. This was done in all 12 patients evaluated in vitro, plus an additional three patients where samples were available (Supplementary Table S1). There was a significant reduction in migration of CLL cells obtained during treatment with ibrutinib compared with pretreatment (median change −65%, IQR: −33 to −97, n = 12, P = 0.007, Fig. 1A). Key
prognostic markers, such as del(17p), IGHV mutational status and prior treatment history could not account for the observed interpatient variability (n = 15, Supplementary Fig. S1B). Notably, a majority of the evaluated patients demonstrated a superior inhibition of migration after in vitro treatment compared with what was observed in vivo. To determine whether the superior in vivo effect could be attributed to a reduction in chemokine receptors overtime on drug, we evaluated the expression of CXCRI4 and CCR7 (the receptors for CXCL12 and CCL19, respectively). Baseline expression of these chemokine receptors is summarized in Supplementary Table S3. We found that there was a significant reduction in the expression of CCR7 on ibrutinib (median change –38%, IQR: −21 to −44, n = 13, P < 0.001; Fig. 1B). In contrast, expression of CXCRI4 was not significantly reduced (median change –14%, IQR: +46 to −30, n = 13, P = 0.89; Fig. 1B). In contrast to what was observed with CLL cells, there was no consistent effect on T-cell migration (median change +41%, IQR: −9 to +271, n = 15, P = 0.08; Fig. 1A). In fact, we found that 75% of evaluated patients demonstrated an increase in T-cell migration on ibrutinib.

Because the tumor microenvironment contains a mix of many different factors in addition to CXCL12 and CCL19, we used the plasma supernatant from bone marrow aspirates collected pre-treatment, that observed with CXCL12/CCL19 (data not shown). Compared with a mixture of equal amounts of bone marrow supernatant and plasma supernatant from bone marrow aspirates collected pre- and posttreatment, cells collected after 28 days on ibrutinib demonstrated a reduction in migration toward patient matched bone marrow supernatant (median change −44%, IQR: −22 to −96, n = 7, P = 0.02; Fig. 1C). Together, these data show that ibrutinib reduces the migration of CLL cells toward a variety of chemoattractants; however, the effect on migration is moderate and not apparent in all patients, despite a rise in ALC on treatment in all patients contributing cells for analysis.

Ibrutinib inhibits CLL cell adhesion to fibronectin and stromal cells

To determine the effect of ibrutinib treatment on cell adhesion we chose a functional assay, utilizing fibronectin-coated plates to model tumor–stromal interactions in the tissue microenvironment. We validated the performance of the assay using CFSE-labeled PBMCs (CLL cell content 88%–99% and Supplementary Table S4), either treated with 1 μmol/L ibrutinib or left untreated, and allowed them to adhere to a fibronectin-coated plate. After extensive washing, we quantified the number of adhered cells and found that there was a modest, but significant reduction in cell adhesion to fibronectin after ibrutinib treatment in vitro, confirming what has been previously reported (ref. 18; median change −35%, IQR: −18 to −63, n = 11, P = 0.002, Supplementary Fig. S2A–S2B).

Next, we determined the effect of in vivo ibrutinib treatment using PBMCs collected from patients pretreatment and after 28 days on ibrutinib. The percentage of CLL cells at all evaluated time points ranged from 88% to 99% (Supplementary Table S4). Figure 2A depicts representative microscopic images...
demonstrating the inability of CLL cells from patients treated with ibrutinib to adhere to fibronectin. This dramatic effect was seen in all patients evaluated (median change –98%, IQR: –91 to –100, n = 13, P < 0.001; Fig. 2B). Because the onset of ibrutinib-induced lympho cytosis is virtually immediate and in many patients peaked within days (16), we also analyzed cells obtained after just one dose of ibrutinib (day 2). Even at this early timepoint, adhesion was significantly reduced (median change –68%, IQR: –40 to –85, n = 10, P = 0.002; Fig. 2C) and was further reduced with continued treatment (n = 8; Fig. 2D). We again found that key prognostic markers could not account for the observed interpatient variability (n = 10, Supplementary Fig. S2C). As fibronectin is only one of the many molecules that can facilitate adhesion, we repeated our analyses using the Hs5 stromal cell line. We again was signifi cantly reduced (median change –68%, IQR: –40 to –85, n = 10, P = 0.002; Fig. 2B) and was further reduced with continued treatment (n = 8; Fig. 2D). We again found that key prognostic markers could not account for the observed interpatient variability (n = 10, Supplementary Fig. S2C). As fibronectin is only one of the many molecules that can facilitate adhesion, we repeated our analyses using the Hs5 stromal cell line. We again found that there was only a modest reduction after ibrutinib treatment in vitro (median change –14%, IQR: –6 to –29, n = 9, P = 0.004, Supplementary Fig. S3A); with a more substantial reduction observed in cells obtained from patients after 28 days on ibrutinib (median change –20%, n = 11, P = 0.005, IQR: –5 to –30, Supplementary Fig. S3B and S3C). In summary, treatment with ibrutinib consistently abrogated adhesion of CLL cells to fibronectin and significantly reduced their ability to adhere to stromal cells.

Ibrutinib treatment leads to a release of adhered cells

Having demonstrated that circulating CLL cells from the blood of patients being treated with ibrutinib have a defect in cell adhesion, we sought to determine whether ibrutinib could also detach CLL cells already firmly adhered to solid supports. To address this question, we evaluated CLL PBMCs collected pre-treatment and from untreated CLL patients enrolled on a natural history study (Supplementary Table S1). CLL PBMCs were allowed to adhere to fibronectin-coated plates overnight. The next day, the plates were washed extensively to remove all cells not firmly attached, and then incubated with 1 μmol/L ibrutinib for 1 hour. Micrographs in Fig. 3A show results of two represent ative experiments utilizing samples collected pre-ibrutinib. Overall, there was a moderate, but uniform decrease in the number of adherent cells in plates treated with ibrutinib compared with controls (median change –17%, IQR: –6 to –27, n = 11, P = 0.002; Fig. 3B). Conversely, treatment with ibrutinib lead to a significant increase in the number of cells released into the cell culture supernatant (median change +22%, IQR: + to +78, n = 11, P = 0.02; Fig. 3C). Collectively, these data show that ibrutinib can detach and mobilize cells that are adhered to fibronectin; however, only a minor fraction of the total cells are being released.

Ibrutinib modestly reduces expression of adhesion molecules that bind fibronectin

Next, we sought to investigate the mechanisms responsible for the observed decrease in cellular adhesion on ibrutinib in a subset of patients. We first analyzed expression of the most common adhesion molecules known to bind to fibronectin. In particular, we evaluated CD44, which binds to the COOH-terminal heparin binding domain, and VLA-4, a heterodimer of integrin η4 (CD49d) and integrin β1 (CD29), which binds to the IICIS (V) CS1 region and the EIIA domain of fibronectin (27, 28). Baseline expression of these key integrins is summarized in Supplementary

![Figure 3](image-url)
Table S3. Figure 4A summarizes the average change (day 28 compared with pretreatment) in the mean fluorescence intensity (MFI) of a given adhesion molecule and in the fraction of CLL cells expressing it above isotype control. The percentage of CLL cells expressing CD49 (median change –2%, IQR: 0 to –18) and CD29 (median change –7%, IQR: 0 to –27) decreased significantly on ibrutinib compared with controls (n = 20, \( P = 0.02 \) and \( P = 0.009 \), respectively; Fig. 4A). While the percentage of cells expressing CD44 did not change, the MFI of CD44 was significantly reduced after 28 days on ibrutinib (median change –9%, IQR: +5 to –19).

**Figure 4.**

In vivo effect of ibrutinib on the expression of adhesion molecules that bind fibronectin. A, shown is the reduction in the expression of common adhesion molecules that bind to fibronectin, both as the proportion of CLL cells, percent of cells expressing each molecule above isotype control (squares), and the respective mean fluorescence intensity (MFI; circles). Symbols represent the mean; lines represent the 95% CI. \( P \) values determined by a Wilcoxon matched pairs test. B, Waterfall plots depicting the percent change in cells expressing CD49d, only for patients expressing CD49d at baseline (n = 12) on day 28 compared with baseline. C, Waterfall plots depicting the percent change in cells expressing CD29 (n = 20) on day 28 compared with baseline. D, Waterfall plot depicting the percent change in MFI for CD44 (n = 19) on day 28 compared with baseline.
Adhesion of CLL cells is equally reduced by ibrutinib and VLA-4 blockade

As VLA-4 has been demonstrated to be a key molecule in facilitating binding to fibronectin, we tested the importance of VLA-4 for CLL cell adhesion using the small-molecule αβ1-integrin antagonist firategast. A dose titration of firategast was performed from 100 nmol/L to 10 μmol/L; 500 nmol/L was determined to be the lowest optimal concentration for inhibition of CLL cell adhesion to fibronectin (data not shown). Treatment of CLL PBMCs with firategast in vitro significantly reduced CLL cells adhesion to fibronectin (median change –32%, IQR: –13 to –44, n = 15, P = 0.001; Fig. 5A). To further delineate whether CD49d is the key α integrin important for CLL adhesion to fibronectin, we treated CLL PBMCs with blocking antibodies against integrin α4 and β1 separately. The blockade of integrin α4 (CD49d) inhibited cellular adhesion significantly (median change –23%, IQR: –10 to –60, n = 11, P < 0.001; Fig. 5B), while the anti-integrin β1 (CD29) antibody had no consistent effect (Fig. 5B). Notably, the degree of inhibition achieved with ibrutinib and anti-CD49d was highly correlated (R = 0.90, n = 11, P < 0.001; Fig. 5C). As a control, we used a blocking antibody against integrin β2 (CD18), an adhesion molecule that does not bind fibronectin, and saw no effect (n = 11, Supplementary Fig. S4). Thus, both CD49d and BTK are important for CLL cell adhesion and inhibition of either molecule inhibits adhesion of CLL cells to a similar degree.

The adhesion defect induced by ibrutinib is at least partially VLA-4 dependent

VLA-4-dependent cell adhesion of B cells is increased in response to BCR signaling through PKC activation (29, 30). We therefore tested whether activation of PKC with PMA, which is independent of BTK (31), would restore the ability of ibrutinib-treated cells to adhere through VLA-4. As shown in Fig. 2, CLL cells collected from patients on ibrutinib had an adhesion defect. Stimulation of these cells with PMA in vitro induced a significant increase, or recovery, in cell adhesion (n = 12, P < 0.001; Fig. 6A). Despite pronounced interpatient variability in the recovery of adhesion with PMA (IQR: 9 to +87% of control), there was a significant correlation between the abolishment of adhesion by ibrutinib and the recovery by PMA (R = 0.76, n = 12, P = 0.004, data not shown). Blocking VLA-4 with firategast abolished the PMA-mediated recovery in the ability of ibrutinib-treated cells to adhere to fibronectin (median change –65%, IQR: –38 to –94, n = 11, P < 0.001; Fig. 6B).

Notably, firategast did not further reduce the ability of CLL cells collected from patients on ibrutinib to adhere to fibronectin, indicating maximal inhibition of VLA-dependent adhesion of CLL cells is achieved with ibrutinib (n = 11, P = 0.34; Fig. 6B). Therefore, ibrutinib, through inhibition of BTK,
Ibrutinib Inhibits VLA-4-Dependent Adhesion in CLL

Figure 6. Reversibility of the ibrutinib-induced adhesion defect. A, cells obtained from patients pretreatment (Pre) or on ibrutinib were left unstimulated or stimulated with PMA for 1 hour. Shown is the mean (±SEM) percent reduction in adhesion normalized to pretreatment samples (n = 12). B, adhesion of CLL PBMCs sampled during ibrutinib treatment analyzed under four different treatment conditions; cells were left unstimulated or stimulated with PMA, and in each of these two conditions cells were incubated with or without the addition of foperator (FIR; VLA-4 antagonist). Data are normalized to cells without PMA and without FIR, representing the effect of ibrutinib (IB) in vivo. Shown is the mean (±SEM) percent change in cell adhesion (n = 11). All P values were determined by a Wilcoxon matched pairs test.

Discussion

Treatment-induced lymphocytosis, the transient increase in circulating tumor cells at the initiation of treatment of CLL patients with kinase inhibitors, is a hallmark of these targeted agents (13, 16, 32). As we previously reported, the onset of ibrutinib-induced lymphocytosis is virtually immediate and, at least initially, due to an efflux of cells from the lymph node into the peripheral blood (16). In vitro ibrutinib can disrupt several survival pathways, including cell migration and adhesion that are important for tumor-microenvironment interactions (18, 19, 33). Here, we investigated the effects of ibrutinib in vivo by analyzing CLL cells obtained from patients treated with single-agent ibrutinib. The most striking finding was that circulating CLL cells from patients having received ibrutinib for 28 days showed a near complete loss in their ability to adhere to fibronectin. Notably, this effect was ubiquitous, occurring in all samples studied, and already apparent after just one dose of drug, consistent with the timing of the lymphocytosis. Inhibition of cell adhesion appears to be a direct effect of ibrutinib as it manifested within hours of the first dose and was readily reversible in cells stimulated with PMA, which activates PKC independent of BTK (29, 31). While we also observed some reduction in the expression of the adhesion molecules CD29, CD44, and CD49d, these changes were minimal, did not occur in all patients and were only apparent at later time points, suggesting that downmodulation of adhesion molecules is not the primary reason for the observed adhesion defect. In addition, we also demonstrated a reduction in cell migration in samples obtained after 28 days of ibrutinib treatment; however, this effect was more variable and not seen in all patients.

BCR signaling is known to activate integrin-dependent cell adhesion referred to as inside-out activation (34, 35). In response to BCR activation, VLA-4 is released from a constrained position allowing the formation of VLA-4 clusters with enhanced avidity (29). Two sets of experiments indicate that ibrutinib abrogates VLA-4–dependent adhesion of CLL cells, consistent with inhibition of inside-out activation. First, pretreatment CLL cell adhesion to fibronectin was inhibited both by the small-molecule VLA-4 antagonist foperator as well as by a blocking antibody against integrin α4 (CD49d), one of the heterodimer components of VLA-4. However, in cells treated with ibrutinib there was no further reduction in cell adhesion using these inhibitors, suggesting that ibrutinib already completely blocked VLA-4 dependent adhesion. Second, while PMA rapidly restored adhesion of ibrutinib treated CLL cells, this effect was completely inhibited by foperator, indicating that reactivation of inside-out activation downstream of BTK restores cell adhesion in a VLA-4–dependent manner.

CD49d has been referred to as the master regulator of tumor microenvironmental interactions in CLL by virtue of its regulation of many signaling pathways important to CLL cell proliferation and survival (5). CD49d may be important for keeping CLL cells localized to proliferation centers, as CD49d was found to be upregulated in the proliferative subset of peripheral blood CLL cells (CXCR4dimCD5bright, ref. 36). In addition, CD49d has been demonstrated to be a critical regulator of lymphocyte migration (37). The survival effect provided to CLL cells after ligation of CD49d has been shown to be due to BCR-dependent and -independent mechanisms, which include upregulation in ant apoptotic proteins such as BCL-2 (with subsequent decreases in BAX), increases in MMP-9 leading to activation of LYN, activation of SYK and PI3K signaling and downstream activation of NFκB transcription factors (5, 38–40). Through inhibition of VLA-4 activation, ibrutinib may also interfere with the protective effects mediated by CD49d-dependent tumor-microenvironment interactions, including prosurvival and proliferation signals. This is supported by the fact that patients with CD49dhi CLL cells, who have a decreased median overall survival compared with those with CD49dlo CLL cells, have demonstrated increased sensitivity to ibrutinib in vitro (23). In addition, as shown previously,
the proliferation of CLL cells in lymph node and bone marrow of patients on ibrutinib is virtually completely inhibited (41).

While the ability of ibrutinib-treated CLL cells to adhere to stromal cells was also reduced this was not as pronounced as for fribrocinet, suggesting that interactions with the extracellular matrix are more sensitive to the inhibitory effect of ibrutinib than cell–cell interactions. Also an important distinction is that while CLL cells in the blood of patients on ibrutinib almost completely lost their ability to adhere to fribrocinet, ibrutinib was only modestly effective in releasing cells already adhered. These observations are consistent with the clinical impression that only a fraction of CLL cells in secondary lymphoid tissues are mobilized into circulation by ibrutinib, and persistent lymphadenopathy and residual disease in the bone marrow is apparent even after many months of treatment in a subset of patients (14, 16, 42). Therefore, the cells circulating in the peripheral blood during treatment with ibrutinib may be enriched for cells that have strong inhibition in adhesion, while the cells retained in the lymph node may be less affected. Whether the retention of cells in the micro-environment is due to CD49d- or BTK-independent mechanisms still remains to be determined.

Therapeutics that inhibit chemokine signaling and migration (such as plerixafor) and integrin-dependent adhesion and cell trafficking (such as natalizumab and firatregar) mobilize B cells from the secondary lymphoid tissues into the peripheral blood (43, 44). It has been hypothesized that these agents, by displacing tumor cells out of protective microenvironmental niches, could synergize with chemotherapeutic agents and monoclonal antibodies (43, 45, 46). Notably, ibrutinib not only combines inhibition of migration and adhesion but also directly inhibits the prosurvival effects of BCR and possibly CD49d-mediated signaling in CLL. Thus, ibrutinib may potently sensitize CLL cells to existing treatment modalities, which could lead to better overall responses. This hypothesis is currently being tested in ongoing clinical trials (47–50).

Disclosure of Potential Conflicts of Interest
A. Wiester reports receiving other commercial research grants from and is a consultant/advisory board member for Pharmacyclics. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: S.E.M. Herman, A. Wiester
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References


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