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Ibrutinib inhibits free fatty acid metabolism in chronic lymphocytic leukemia

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Abstract

Unlike normal B cells, and similar to fat cells, chronic lymphocytic leukemia (CLL) cells aberrantly express lipoprotein lipase (LPL), which contributes to free fatty acids (FFAs) metabolism. Here we show that, in CLL cells, the B cell receptor (BCR) inhibitor ibrutinib reduced LPL mRNA and protein levels and inhibited FFA metabolism *in vitro*. Likewise, in CLL cells from ibrutinib-treated patients, FFA metabolism was reduced and eventually stopped. Because ibrutinib disrupts CLL cells' ability to use FFAs for energy production, and because various BCR-dependent cellular functions rely on a continuous supply of chemical energy, ibrutinib interrupts several pathways imperative for cellular function in CLL cells.

Keywords

CLL; Ibrutinib; metabolism; lipoprotein lipase

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the gradual accumulation of B lymphocytes that appear mature [1] and that aberrantly express cell surface proteins not present in normal B lymphocytes. One such protein, lipoprotein lipase (LPL), commonly detected in adipocytes and myocytes [2–4], is essential for CLL cells' metabolism of free fatty acids (FFAs) [5,6] and provides CLL cells with energy and a survival advantage [5]. Because activation of the B cell receptor (BCR) signaling pathway increases LPL levels [7,8], and Ibrutinib, an inhibitor of the BCR signaling, is highly effective in patients with

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Disclosure statement

Alessandra Ferrajoli, Jan Burger, Phillip Thompson, Nitin Jain,William Wierda, Michael J. Keating and Zeev Estrov received honoraria from pharmacyclics.

Uri Rozovski, David M. Harris, Ping Li, Zhiming Liu, Preetesh Jain report no conflicts of interest.

CLL, including those with high-risk or relapsed/refractory disease [9,10], we sought to determine whether Ibrutinib inhibits FFA metabolism in CLL cells.

Materials and Methods

Cell fractionation

After obtaining approval from The University of Texas MD Anderson Cancer Center's Intitutional Review Board, we obtained peripheral blood (PB) cells from 18 patients with CLL treated at the Leukemia Clinic. These cells were fractionated using Ficoll-Hypaque 1077 (Sigma-Aldrich, St. Louis, MO). At first sampling, more than 90% of the peripheral blood fractionated lymphocytes obtained from these patients were CD19+/CD5+, as assessed by flow cytometry.

Measurement of cellular O_2 consumption

Because fatty acid metabolism increases O_2 consumption, we assessed the cells' palmitic acid and oleic acid utilization by measuring dissolved O_2 (dO_2) levels as previously described [6]. This assay was performed only if at least 70% of the fractionated cells co-expressed CD5 and CD19. In each experiment, we used CLL cells at a concentration of 2 to 3×10^6 cells/mL. The cells were incubated at 37°C for 48 hours in minimum essential medium with Hank's salts and L-glutamine (Life-Technologies, Carlsbad, CA). Cells were cultured in tightly sealed T25 tissue culture flasks (Corning, Tewksbury, MA) in the presence or absence of 80 mM palmitic acid, 2 mM oleic acid, or 80 mM palmitic acid and 80 mM oleic acid, with or without 0.5 or 1.0 μM of ibrutinib. The dO_2 levels were measured in all flasks at 48 h of incubation. Measurements of dO_2 were repeated at least 3 times for every data point using a SevenGo pro dissolved oxygen meter (Mettler Toledo, Columbus, OH). Results were analyzed by a one-way repeated-measure ANOVA using the GraphPad version 7 (San Diego, California) and SPSS (version 22, SPSS Inc., Chicago, IL).

Viability assay

To determine cell viability of CLL cells that were incubated for 48 h we used the trypan blue exclusion assay. Briefly, after cell density was assessed by using a hemocytometer, 0.1 ml of trypan blue (0.4% in 0.81% sodium chloride and 0.06% potassium phosphate dibasic solution; Gibco, Grand Island, NY) to 0.1 ml of cultured CLL cells. A total of 200 cells of each sample were counted and the percentage of non-viable (blue) and viable cells was calculated.

Western blot analys

Western blot analysis was performed as previously described [11]. Briefly, a lysates of CLL cell extract was mixed with 4x Laemmli sample buffer and denatured by boiling for 5 minutes. Forty micrograms of lysates were dissolved and separated using 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with the following primary antibodies: monoclonal mouse anti-human STAT3 1 (BD Biosciences, San Jose, CA), monoclonal mouse anti-human LPL (Abcam, Cambridge, MA) and mouse anti-human β-actin (Sigma-Aldrich, St. Louis, MO).

After incubation with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare, Buckinghamshire, UK) for 1 hour, blots were visualized with an enhanced chemiluminescence detection system (GE Healthcare).

Densitometry analysis was performed using an Epson Expression 1680 scanner (Epson America, Inc., Long Beach, CA). Densitometry values were normalized by dividing the numerical value of each sample signal by the density of the corresponding β actin protein, used as a loading control.

RNA purification and quantitative reverse transcription–polymerase chain reaction

RNA was isolated using an RNeasy purification procedure (QIAGEN Inc., Valencia, CA). RNA quality and concentration were analyzed with a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE), and 500 ng of total RNA were used for one-step quantitative reverse transcription–polymerase chain reaction procedures that were performed according to the manufacturer's instructions using TaqMan gene expression assays for *BCL2, Cyclin D1, MCL1, c-Myc, p21, RELA, STAT3* and *LPL* (Applied Biosystems, Grand Island, NY).

Results

Ibrutinib disrupts FFA metabolism in vitro

To study the effect of ibrutinib on lipid metabolism, we obtained PB CLL cells from three ibrutinib-naïve CLL patients, cultured the cells in sealed flasks in the presence or absence of palmitic acid and measured the dO_2 concentration after 48 hours. As previously reported, dO_2 levels decreased when palmitic acid was added to cultured CLL cells [5], suggesting that the CLL cells consumed more oxygen because they metabolized FFA. However, when ibrutinib was added to the cell cultures, the delta dO_2 values (the difference between dO_2 with FFA and dO_2 without FFA) were unchanged, suggesting that ibrutinib blocked FFA metabolism (Figure 1).

Ibrutinib disrupts FFA metabolism in vivo

To determine whether ibrutinib impairs CLL cells' lipid metabolism, we obtained 2 to 5 (median: 4) samples of PB CLL cells from each of 14 CLL patients prior to and during ibrutinib treatment (median number of treatment days = 140; range, 9 – 165 days). Most patients (N=12, 80%) had received at least one previous treatment with ibrutinib (median, 1.5; range, 1 to 3). Patient and disease characteristics are shown in Table 1.

As was the case with CLL cells from ibrutinib-naïve patients, adding FFA to the CLL cells from these patients prior to ibrutinib treatment induced a reduction (12%) in delta dO_2, suggesting that the cells metabolized FFA. After a median of 4 days (range: 2 to 7 days) of ibrutinib treatment, the delta dO_2 value was only 6%, and after a median of 147 days (range: 85 to 165) of ibrutinib treatment, there was no longer a change in the dO_2 value (Figure 2, A to C). Remarkably, the number of viable cells at time of first sampling and after 48 hours of incubation was similar across all culture conditions, with and without FFA (Figure 2D). The diminished capacity of CLL cells from ibrutinib-treated patients to metabolize FFA was not

associated with patient age, sex, white blood cell count, *IgHv* mutation status, cytogenetic abnormalities or clinical characteristics.

Ibrutinib inhibits STAT3 transcription and reduces LPL protein levels in CLL cells

We have previously reported that STAT3 induces the transcription of LPL and inhibition of STAT3 significantly reduces LPL levels and decreases FFA metabolism in cells. Because FFA metabolism in CLL cells correlated with STAT3-induced aberrant expression of LPL and ibrutinib inhibits FFA metabolism [5] we asked whether ibrutinib inhibits the transcription of STAT3. We incubated CLL cells with 0.5 μM ibrutinib for 48 hours and found that ibrutinib downregulated mRNA levels in STAT3-regulated genes, including *STAT3* and *LPL* (Figure 2E). Furthermore, by using Western immunoblotting, we found that ibrutinib downregulated STAT3 and LPL protein levels in a dose- and time-dependent manner (Figure 2F).

Discussion

Unlike normal B cells, CLL cells utilize FFA for their metabolic needs [5]. Here we show that ibrutinib inhibits this metabolic pathway both *in vitro* and in PB CLL cells from ibrutinib-treated patients. Lipid metabolism in CLL cells is maintained by the transcriptional activity of the nuclear receptor peroxisome proliferator-activated receptor-α [12] and the nuclear factor (NF)-κB/STAT3 pathways [6]. A previous report demonstrated that ibrutinib inhibits NF-κB by blocking the BCR signaling pathway [13], which depends on a continuous supply of chemical energy in the form of ATP. Here we show that *in-vitro*, that ibrutinib inhibited STAT3 and, as a result, the transcription of LPL. Whether ibrutinib reduced STAT3 mRNA levels in CLL cells of ibrutinib-treated patients was not tested. However given that more than 93% of the drug is bound to plasma protein [14] and 4 hours after administration ibrutinib is covalently bound to more than 95% of the BTK [15], it is reasonable to assume that ibrutinib reduces STAT3 mRNA levels in CLL cells of ibrutinib-treated patients.

It is likely that both effects result in the inhibition of FFA metabolism in CLL cells. Whether or how inhibition of LPL transcription contributes to ibrutinib-induced inhibition of lipid metabolism is currently unknown. LPL has catalytic and non-catalytic functions. It catalyzes the hydrolysis of triglycerides into FFA and, in addition, induces a non-catalytic effect [16]. A previous report suggested that LPL is catalytically inactive in CLL cells [17]. We found that the metabolic activity of CLL cells is LPL-dependent even in the presence of FFA, circumventing the need for triglyceride hydrolysis [5], suggesting that LPL affects the lipid metabolism CLL cells through its non-catalytic activity.

Consistent with our previous report [5] we found that lipid metabolism was active in CLL cells from all patients regardless of stage, *IgHv* mutation status or cytogenetic abnormalities, suggesting that the cells utilize this metabolic pathway from early stages of the disease. Similarly, in line with a recent study, LPL protein levels were found to be increased in all CLL patients regardless of the severity of their disease [18]. Yet, whether LPL-dependent metabolism contributes to CLL cells' survival is still a matter of debate. We and others have shown that inhibition of LPL activity or LPL knock-down increased cellular apoptotic rate

[5,19]. Conversely, another group reported that LPL-siRNA did not increase CLL cell apoptosis rates likely because low levels of transfection efficacy (a median knock-down efficiency of only 33%) [18].

Ibrutinib inhibits a diverse plethora of cellular functions such adhesion [20], migration [21] and proliferation [22]. By inhibiting the capacity of CLL cells to utilize FFA, likely through downregulation of LPL levels, ibrutinib disrupts a metabolic pathway that that is imperative for cellular function.

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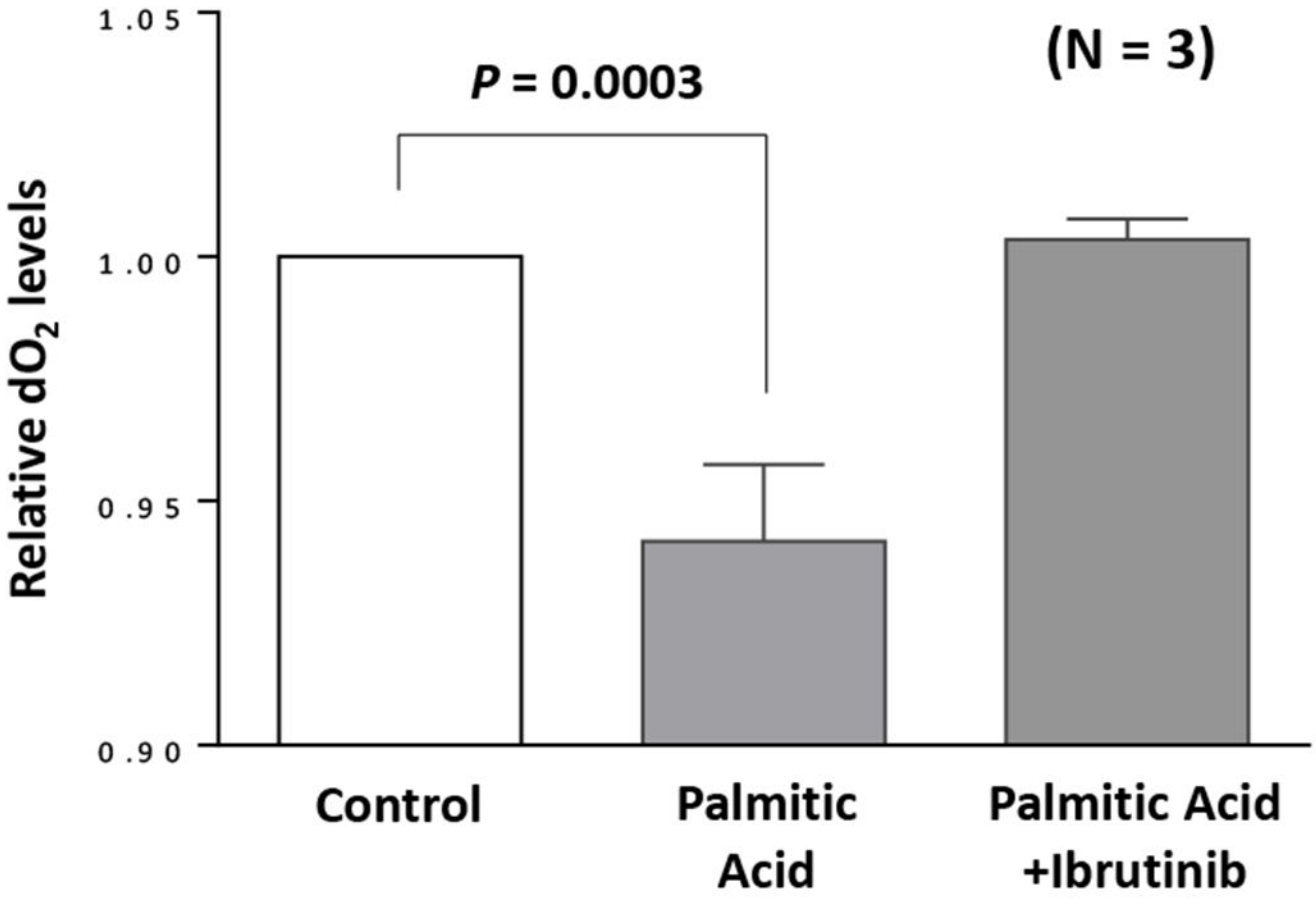
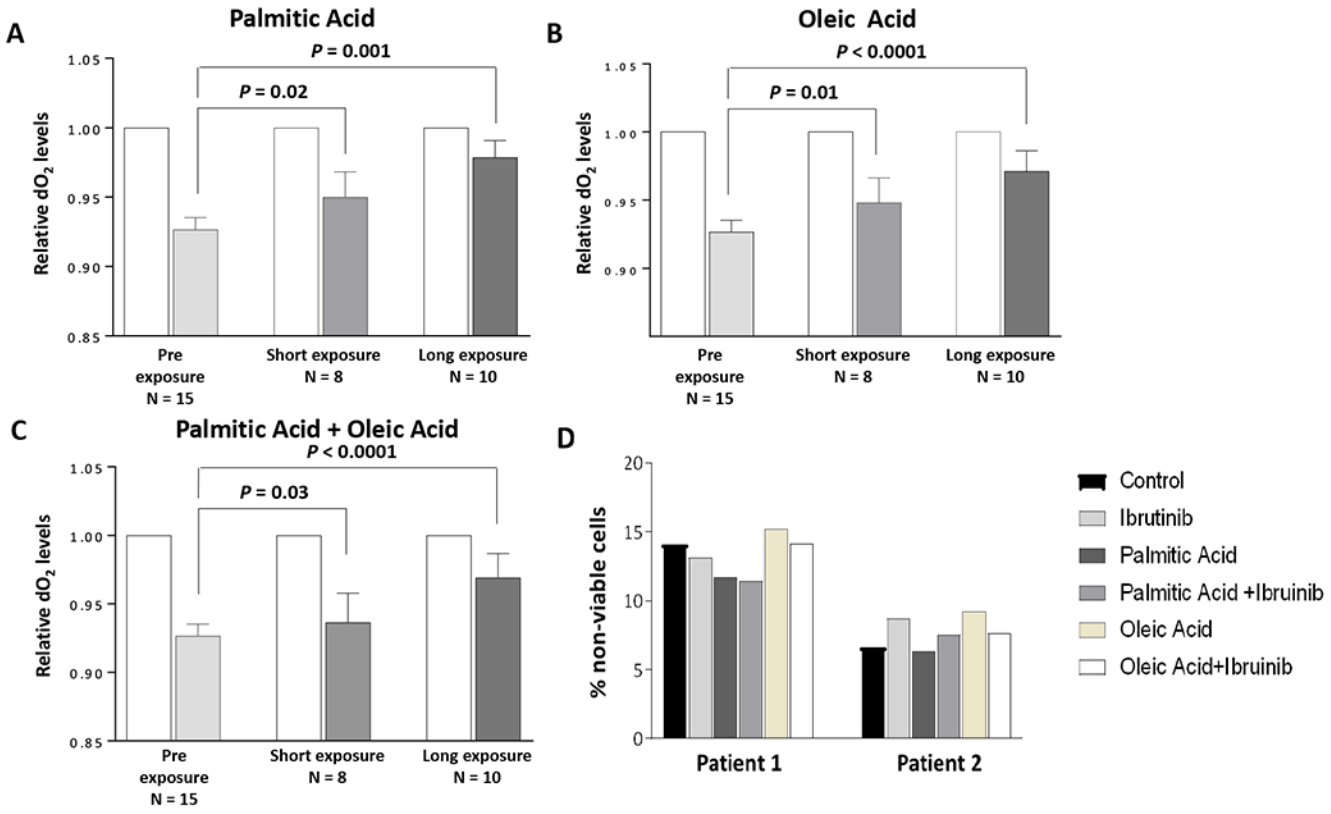


Fig 1.

Ibrutinib inhibits palmitic acid-dependent metabolism of chronic lymphocytic leukemia (CLL) cells *in vitro*. CLL cells were incubated for 48 hours in sealed tissue culture flasks in the presence or absence of 80 mM palmitic acid with or without 1.0 μM ibrutinib. The dissolved oxygen concentration (dO_2) was initially set at 1 (left bar), and the relative dO_2 after 48 hours is depicted in the middle and right bars.



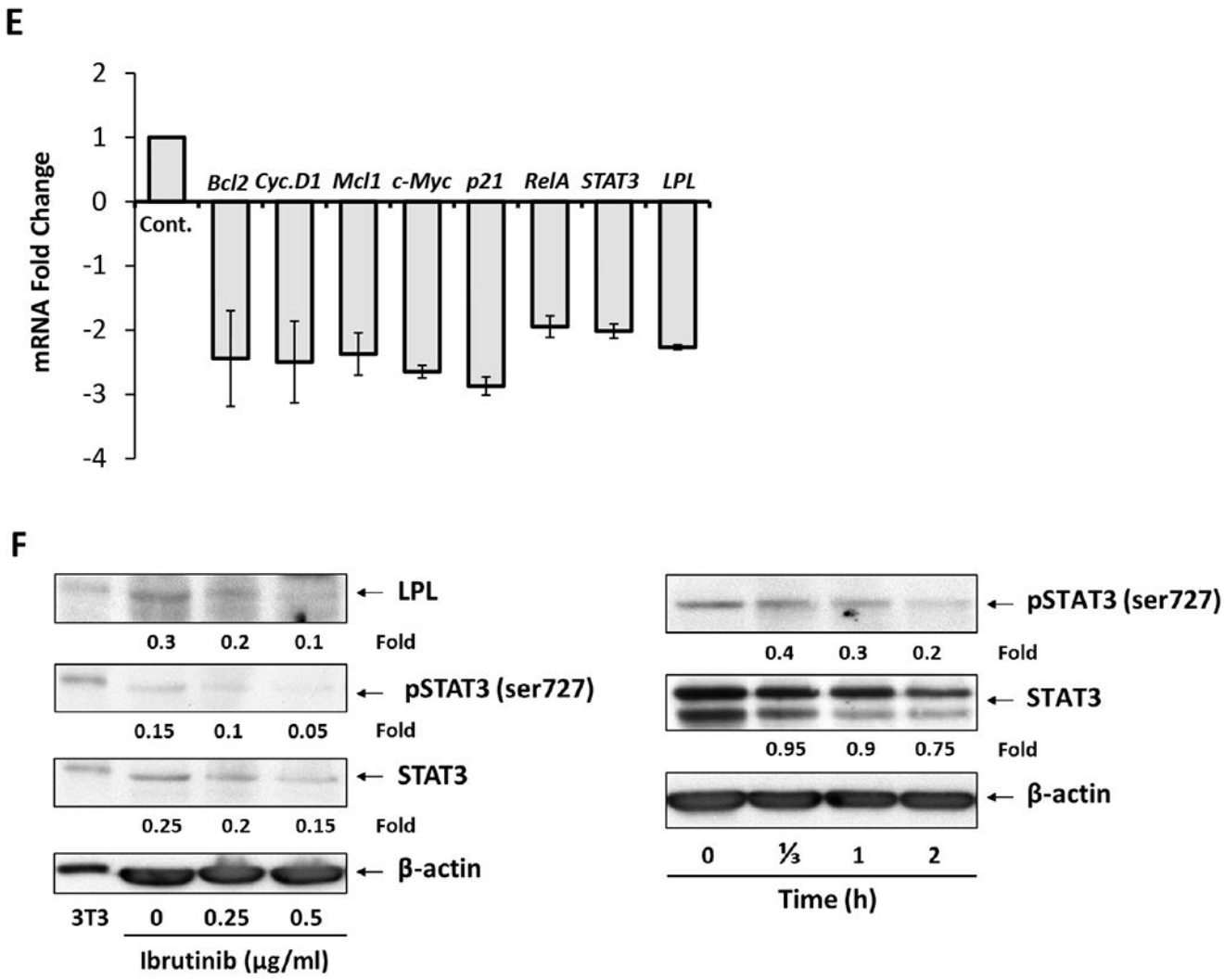


Fig 2.

Ibrutinib inhibits free fatty acid metabolism of CLL cells *in vivo*. After a median of 4 days (short exposure range: 2 to 7) and 147 days (long exposure range: 85 to 165) of treatment with ibrutinib, peripheral blood (PB) CLL cells were obtained from CLL patients prior to treatment and cultured in the presence or absence of 80 mM (A) palmitic acid, (B) oleic acid or (C) palmitic acid plus oleic acid. The dO_2 was initially set at 1 (white bars), and the relative concentration of dO_2 after 48 hours is depicted after short (middle bars) and long (right bars) durations of ibrutinib treatment. (D) Viability of CLL cells incubated with palmitic or oleic acid with or without ibrutinib. (E) Ibrutinib downregulates LPL's mRNA levels. CLL cells were incubated with 0.5 μM ibrutinib for 48 hours. Cellular RNA was extracted and subjected to quantitative reverse transcription–polymerase chain reaction testing using primers directed to detect the STAT3-regulated genes *Bcl2, Cyclin D1*, *MCL1*, *c-Myc*, *p21*, *RelA*, *STAT3* and *LPL*. The experiment was repeated using PB CLL cells from 3 different patients. The means and standard errors of the means are depicted as fold change (fold reduction in the genes' mRNA levels). As shown, ibrutinib reduced mRNA levels of STAT3-target genes, including *LPL*. (F) Ibrutinib downregulates STAT3, serine pSTAT3 and

LPL levels in a time- and dose-dependent manner. Left panel: CLL cells were incubated without or with 0.25 μM or 0.5 μM ibrutinib for 24 hours. Western immunoblotting shows a dose-dependent decrease in levels of serine pSTAT3, STAT3 and LPL. For positive controls, we used 3T3 cells. Right panel: CLL cells were incubated with 0.5 μM ibrutinib and harvested at 4 time points (0 - 2h). Western immunoblotting shows a time-dependent decrease in serine pSTAT3 and STAT3 protein levels.

Table 1.

Patient and Disease Characteristics at Time of Diagnosis

Pt. #	Age (years)	SEX	RAI staging	B2M	IgHv mutational status	FISH	ALC	% of leukemia cells
1	60	Mal	4	9.6	UM	Negative	15.13	73
2	64	F	2	4.4	M	Negative	19.6	70
3	47	F	2	4.5	UM	17p	182	93
5	77	M	2	2.7	UM	Negative	14.43	82
6	60	M	4	5.3	UM	11q,13q	26.28	93
7	43	M	3	2.2	UM	Negative	38.5	89
8	60	M	3	3.8	ND	11q,13q	79	95
9	49	M	3	2.6	UM	17p,11q,13q	83.8	94
10	68	M	4	6.4	M	11q,13q	53	91
12	44	F	3	4	UM	Negative	124	95
13	62	M	1	1.6	M	13q	8.81	52
14	65	M	0	2.3	M	del13q	6.34	42
15	64	M	0	1.4	M	T12	17.07	85
16	51	M	2	3.3	UM	11q,13q	24	80
17	66	M	2	4.1	UM	11q	158.3	93
18	54	M	2	2.2	UM	13q	124	92
19	60	M	4	9.6	UM	Negative	15.13	72

Pt, patient; B2M, beta 2 microglobulin; IgHv, immunoglobulin heavy chain; FISH, Florescence in-situ hybridization; ALC absoloute lymphocyte counts; UM, unmutated; ND, not done; M, mutated;