c-Abl Expression in Chronic Lymphocytic Leukemia Cells: Clinical and Therapeutic Implications

Ke Lin, Mark A. Glenn, Robert J. Harris, Andrew D. Duckworth, Sally Dennett, John C. Cawley, Mirko Zuzel, and Joseph R. Slupsy

Department of Haematology, University of Liverpool, Liverpool, United Kingdom

Abstract

c-Abl is important for normal B-cell development, but little is known about the function of this nonreceptor tyrosine kinase in chronic lymphocytic leukemia (CLL). Therefore, the aim of the present study was to examine the clinical, therapeutic, and pathogenetic importance of c-Abl in this disease. We show that the malignant cells of CLL predominantly express the type 1b splice variant of c-Abl and that the expression of c-Abl protein is higher in CLL cells than in normal peripheral blood B cells. Moreover, we show that the levels of c-Abl protein expression correlate positively with tumor burden and disease stage, and negatively with \( IgV_H \) mutation. We also show that STI-571, an inhibitor of c-Abl kinase activity, induces apoptosis of CLL cells with high c-Abl expression levels through a mechanism involving inhibition of nuclear factor \( \kappa \)B. We conclude that overexpression of c-Abl is likely to play a pathogenic role in CLL and that STI-571 may be of potential use in the treatment of this disease. (Cancer Res 2006; 66(15): 7801-9)

Introduction

Chronic lymphocytic leukemia (CLL) is characterized by clonal accumulation of mature malignant B cells expressing CD5 and CD23, together with low levels of surface immunoglobulin (1). Despite homogeneity in the malignant-cell immunophenotype, CLL is heterogeneous with respect to disease progression (1, 2). Understanding the nature of this heterogeneity is therefore an important area of intense research interest.

Several biological factors have been linked with the clinical heterogeneity of CLL. These include the extent of somatic mutation of immunoglobulin heavy chain variable region (\( IgV_H \)) genes (3), p53 mutation/dysfunction (4, 5), and expression of CD38 (6) and ZAP-70 (7). However, how these factors contribute to disease progression is still largely unknown.

It has recently emerged that there is a correlation between the degree of \( IgV_H \) mutation and cell responsiveness to B-cell receptor cross-linking (8), and that active B-cell receptor signaling may contribute to poor prognosis (1). This concept is supported by the recent demonstration that expression of the nonreceptor tyrosine kinase ZAP-70 is a feature of unmutated CLL (7) and that ZAP-70 enhances CLL cell signaling in response to B-cell receptor cross-linking (9).

Materials and Methods

Patient samples. All samples were obtained with informed consent and with the approval of the Liverpool Research Ethics Committee. Diagnosis of CLL and the other B-lymphoproliferative disorders used in this study (mantle cell lymphoma, marginal zone lymphoma, follicular lymphoma, hairy-cell leukemia, and plasma-cell leukemia, all with leukemic involvement) was based on standard morphologic, immunophenotypic, and cytogenetic criteria (14). Cryopreserved mononuclear cells were thawed at 37°C, diluted slowly in 10-mL RPMI 1640 containing 1% bovine serum albumin (Sigma, Poole, United Kingdom), and washed twice with PBS. For all the cases used in this study, cell viability after thawing was >95% and, with the exception of case 6 (see Supplementary Table S1), >90% of cells were CD19+. In case 6, CD19 positivity was 85%.

Reagents. STI-571 (Gleevec, Imatinib) was from Novartis (Basel, Switzerland). Monoclonal anti-c-Abl (clone 8E9) was from BD Biosciences (Oxford, United Kingdom). Anti-\( \kappa \)B (clone FL) and anti-DOK2 antibodies were from Cell Signaling Technology (Beverly, MA). c-Abl and control small interfering RNA (siRNA) were from Santa Cruz Biotechnology (Insight Biotechnology Ltd., Wembley, United Kingdom). Anti-ZAP-70, anti–poly(ADP-ribose) polymerase (#5942), anti-pY551-DOK2, and anti-pIKK\( \beta \) antibodies were from Cell Signaling Technology (Beverly, MA).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Ke Lin, Department of Haematology, University of Liverpool, Liverpool, 3rd Floor, Duncan Building, Daulby Street, Liverpool L69 3GA, United Kingdom. Phone: 44-151-7064326; Fax: 44-151-706-5810; E-mail: k.lin@liv.ac.uk.

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were from Dharmaco RNA Technologies (Perbio Science UK Ltd., Cramlington, United Kingdom) and Santa Cruz Biotechnology.

**Purification of CD19+ cells.** Purified CD19+ cells from peripheral blood or buffy coats (British Transfusion Service, Liverpool, United Kingdom) were obtained using a negative isolation kit according to the instructions of the manufacturer (Dynal Biotech, Wirral, United Kingdom).

**IgVH gene sequence analysis.** Analysis of IgVH gene sequences of the malignant cells from CLL cases was done as previously described (5).

**Western blotting.** Whole-cell lysates of CLL cells were separated by SDS-PAGE and electroblotted onto Immobilon membranes (Millipore, Watford, United Kingdom). Western blots were developed under standard conditions for visualization by enhanced chemiluminescence (Amersham Biosciences, Amersham, United Kingdom). Densitometric analysis of exposed radiographic film was done using Phoretix 1-D Advanced software (version 4.0, NonLinear Dynamics Ltd., Newcastle-upon-Tyne, United Kingdom). Experimental samples were always run together with the same reference sample (K562 whole-cell lysate).

**Real-time reverse transcription-PCR.** Total RNA was extracted from B cells using an RNasey mini kit (Qiagen, Crawley, United Kingdom) and reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Southport, United Kingdom) and an oligo(dT)15 primer. cDNA was mixed with DyNAse SYBR Green 1 qPCR master mix (Finnzymes, Espoo, Finland) and 1 μmol/L each of forward and reverse primers. The forward primer for c-abl was 5′-CCCAACCTTTGCTGTCAGCTGTA-3′, for c-abl isoform 1a was 5′-AATGGTGGATCCTGCTGAAAGC-3′, and for c-abl isoform 1b was 5′-AGGGAGAAGGAGGATCCTGAGG-3′. The reverse primer used for all c-abl isoforms was 5′-CCCTCCTGGGAGGAGCCTGTA-3′. We used β-actin as an internal control and employed the forward primer (5′-CTCGCCTTTGGCCGATCC-3′) and reverse primer (5′-GGATCTTCATGAGGTAGTCAGTC-3′) as amplics. All PCR reactions were done on a DNA engine Opticon 2 system (MJ Research, Waltham, MA) under optimized, identical cycling conditions consisting of a 10-minute initial denaturing step at 95°C, followed by 45 cycles of amplification (denaturation at 94°C for 20 seconds, annealing at 60°C for 20 seconds, extension at 72°C for 20 seconds, and fluorescence data collection at 80°C). Following a final 10-minute extension at 72°C, a melting curve was measured from 65°C to 98°C. The specificity of each of the PCR products was confirmed as a single band with the expected molecular size on agarose gel and as a narrow peak that appeared in the melting curve when temperatures rose higher than 80°C. c-abl expression was measured as a ratio of the c-abl transcript to that of β-actin.

**Measurement of cell viability.** Cells (2 × 10⁶/100 μL) were cultured with or without STI-571 for 1 to 3 days and viability measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (15). In other experiments, cell viability was assessed by fluorescence-activated cell sorting (FACS) using dihydroxycarbocyanine iodide (DiO<sub>64</sub> to measure mitochondrial integrity) and propidium iodide incorporation (as a measure of dead cells) according to established protocol (16).

**CLL cell transfections.** Two types of transfection technology were used to study the role of c-Abl in CLL cells. In the first method, 1 × 10⁶ CLL cells were treated with either c-Abl-specific siRNA or nonspecific control siRNA (100 pmol) for 48 hours before harvesting and analysis of protein content by Western blotting.

**Electrophoretic mobility shift assay.** Electrophoretic mobility shift assays were done as described (17). CLL cells (2 × 10⁶) were lysed with ice-cold buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl<sub>2</sub>, 10 mmol/L KCl, 0.5 mmol/L EDTA, 0.1% NP40, +10 μg/mL each of 4-[2-aminophenyl]-benzenesulfonylfluoride, pepstatin A, and apronin]. Nuclei were isolated by centrifugation (1,000 × g, 1 minute) and extracted with buffer B [20 mmol/L HEPES (pH 7.9), 25% glycerol, 1.5 mmol/L MgCl<sub>2</sub>, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT]. Insoluble material was removed by centrifugation (14,000 × g, 10 minutes). Four micrograms of nuclear extract were incubated in DNA-binding buffer [10 mmol/L Tris (pH 7.6), 50 mmol/L KCl, 1 mmol/L EDTA, 5% glycerol, 100 μg/mL poly(deoxyinosinic-deoxyctydilic acid), 0.25% NP40] with 20,000 cpm of probe that had been prepared by T4-kinase end-labeling of an NF-κB consensus binding site oligonucleotide. Samples were separated by electrophoresis using a 4% nondenaturing polyacrylamide gel. Dried gels were exposed to X-ray film at −70°C.

**Statistical analysis.** Sets of measurement data were compared for statistical significance using either a Student's t test or a Mann-Whitney U test. Sets of enumeration data were compared by χ<sup>2</sup> and Fisher's exact tests.

Pearson's linear correlation test was employed to estimate the relationship between two groups of measurement data. Two-sided tests with an α level of 0.05 were used in all analyses. The comparisons were done by computer using SPSS v13.0 software.

## Results

### c-Abl is variably expressed in the malignant cells of CLL.

Seventy CLL cases were involved in this study and were chosen at random from our Departmental tissue bank in which cases with high malignant-cell counts and advanced disease stage are overrepresented (see Supplementary Table S1).

Figure 1A shows a representative Western blot of c-Abl protein expression in the malignant cells from 13 of the above CLL cases. c-Abl levels in the malignant cells from all the CLL cases normalized to β-actin ranged from 0.01 to 1.43 (Fig. 1B). The mean and median values of the c-Abl/β-actin ratio were both 0.59.

We next examined c-Abl mRNA expression by quantitative reverse transcription-PCR (RT-PCR) in cells from eight CLL cases and observed a positive correlation between c-Abl protein and transcript levels (data not shown). These results clearly indicate that c-Abl protein expression in CLL cells is, at least in part, controlled by c-abl gene transcription.

### 1b is the predominant c-Abl isoform in CLL.

In humans, two isoforms of c-Abl, known as types 1a and 1b, are expressed. These proteins are splice variants of the c-abl gene (18) and have different functions and subcellular localizations (19). In B cells, c-Abl type 1a is involved in differentiation whereas type 1b is involved in protection from apoptosis (19).

The RT-PCR data presented in Fig. 1C show that the ratio between the 1b and 1a isoforms is much higher in CLL cells than in a range of other cell types. This predominant expression of the 1b, as compared with the 1a isoform, suggests that the overexpression of c-Abl in CLL primarily affects cell survival and is unlikely to have an influence on cell differentiation.

### c-Abl protein is overexpressed in CLL as compared with normal B cells.

Figure 1B shows that c-Abl expression in CLL cells is significantly higher than in purified normal B cells. This is consistent with the lower transcription level of c-abl in normal B cells (Fig. 1C). Only 2 of the 70 CLL cases showed a level of c-Abl expression within the range for the normal B cells. However, Fig. 1D shows that the malignant cells of hairy-cell leukemia, mantle-cell lymphoma, marginal zone lymphoma, follicular lymphoma, and plasma-cell leukemia all have quantities of c-Abl protein comparable to those observed in CLL cells expressing high levels of this protein. These data indicate that overexpression of
c-Abl protein is also a feature of other B-lymphoproliferative disorders. However, the selective overexpression of the 1b isoform was not observed in the primary cells of hairy-cell leukemia or mantle-cell lymphoma or in a number of cell lines (Raji, Jurkat, K562, and HeLa; Fig. 1C). Therefore, in contrast to the shared overall high levels of expression of c-Abl protein in chronic B-lymphoproliferative disorders, the overexpression of the antiapoptotic 1b isoform may be an important specific feature of CLL.

c-Abl expression correlates negatively with IgVH mutation and positively with ZAP-70. Given the role of c-Abl in B-cell receptor-mediated cell proliferation (13) and the probability that B-cell receptor signaling is important for CLL cell survival in vivo, particularly in those cases with less mutated IgVH genes (1), it seemed plausible that c-Abl expression might be related to the degree of IgVH mutation and possibly to other markers of CLL prognosis.

Indeed, linear regression analysis showed a negative correlation between c-Abl expression and IgVH mutation (Fig. 2A). When we divided the CLL cases into those with unmutated (≤2% IgVH mutation) and mutated (>2% IgVH mutation) phenotypes, we found that c-Abl was expressed at significantly higher levels in the unmutated CLL subgroup (Fig. 2B). Therefore, in general, high levels of c-Abl protein are associated with less IgVH mutation.

Recent evidence has suggested that ZAP-70 expression is also associated with unmutated CLL (7), suggesting a possible correlation between ZAP-70 and c-Abl. Figure 2C shows that there is indeed a positive correlation between ZAP-70 and c-Abl levels.

In addition to IgVH mutation, other important prognostic variables include CD38 expression and p53 dysfunction (5). However, in the group of patients used in this study, there was no significant correlation between c-Abl levels and either percentage CD38 positivity or p53 dysfunction (data not shown).

The association of high levels of c-Abl with less IgVH mutation suggests that c-Abl expression may have some predictive value in disease prognosis. However, this could not be pursued further in terms of patient survival because most of the patients were newly diagnosed and therefore still alive.

c-Abl expression in CLL cells correlates with disease severity. We also examined c-Abl expression in relation to WBC counts and disease stage at the time of sampling (indicators of tumor burden and disease status at that point in the evolution of the disease). Figure 3A shows that higher levels of c-Abl expression were observed in CLL cases at Binet stage B or C compared with stage A. When we pooled the results obtained from CLL cells at stage B and C and compared them with the results obtained with cells at stage A, this difference became even more significant (Fig. 3B).

When we used the mean value of c-Abl expression as an arbitrary cutoff to divide the samples into c-Ablhi (c-Abl/β-actin ratio, ≥0.59) and c-Abllow (<0.59) subgroups, 32 of the CLL cases fell into the c-Abllow subgroup whereas 38 cases were classified as c-Ablhi. Among the 61 cases where disease stage data were available (Supplementary Table S1), the majority of CLL cases at stage A fell into the c-Abllow subgroup whereas the majority of patients at stages B and C fell into the c-Ablhi subgroup. This association of advanced disease stages with the c-Ablhi subgroup and of less advanced disease with the c-Abllow subgroup was highly statistically significant using different methods of analysis as indicated in Fig. 3A and B and Table 1. These results justify the use of the mean c-Abl expression as a cutoff value to separate cases into two subgroups for studies of correlation between c-Abl levels and different, potentially important, disease variables.

We next examined the relationship between c-Abl expression and WBC count. Linear regression analysis showed a positive correlation between these two variables (Fig. 3C). Moreover, comparison of the WBC counts between the c-Ablhi and c-Abllow subgroups showed that the c-Ablhi subgroup had a significantly greater tumor burden (Fig. 3D).

Our observation that high cellular levels of c-Abl are associated with advanced stage and high tumor burden suggests that c-Abl expression may increase with disease progression. We had access to serial samples in only three cases, two of which progressed from stage A to B/C whereas the other case remained stable. In the two former cases, c-Abl increased with disease progression (mean...
c-Abl/β-actin ratio, 0.42-0.84) but remained stable in the patient with indolent disease. These limited sequential data therefore support the notion that c-Abl expression increases with disease progression.

Taken together, these data show that, in CLL, high c-Abl expression levels are a feature of advanced disease.

c-Abl hi CLL cells are more sensitive to STI-571-induced apoptosis than c-Abl low cells. We used the c-Abl-specific inhibitor STI-571 (20) to investigate the contribution of this kinase to in vitro CLL cell survival. We first established concentration- and time-dependent responses using CLL cells from three cases in the c-Abl hi subgroup. Figure 4A shows that treatment of CLL cells with STI-571 reduces the viability of these cells in a concentration- and time-dependent manner. The effect of the drug was maximal after 3 days of culture with 30 μmol/L STI-571. However, for our subsequent experiments, we chose 10 μmol/L STI-571 because this concentration was effective in killing CLL cells and corresponds to that achieved in vivo during treatment of chronic myelogenous leukemia with the routine dosage of 600 mg/d (21, 22).

We next examined the effect of 10 μmol/L STI-571 on c-Abl activity in CLL cells. Treatment of the STI-571-sensitive cell line KCL22 with this compound abolished the autophosphorylation of both Bcr-Abl and c-Abl (data not shown). This shows that STI-571 was an effective inhibitor of c-Abl in intact cells. DOK2 is a known substrate of active c-Abl in hemopoietic cells (23) and its phosphorylation on tyrosine was used as a measure of c-Abl activity in KCL22 and CLL cells. Figure 4B shows that treatment of these cells with 10 μmol/L STI-571 markedly reduced the phosphorylation of DOK2, suggesting that STI-571 also inhibits c-Abl activity in intact CLL cells.

When we extended our analysis of STI-571-induced effects to a total of 26 unselected CLL cases, we observed a highly variable effect on cell viability as measured by MTT assay (Supplementary Table S1). In some cases CLL cell survival was reduced substantially, whereas in others STI-571 had little or no effect.

To explain this heterogeneity, we used linear regression to compare the effects of STI-571 on CLL cell viability with levels of c-Abl expression. Such analysis suggested that STI-571 was more effective at inducing death of CLL cells with higher levels of c-Abl expression (Fig. 4C). This was further investigated by dividing the CLL cases into c-Abl hi and c-Abl low subgroups as defined above. Figure 4D shows that the effect of STI-571 treatment on the viability of c-Abl hi cells was greater than on c-Abl low cells (P = 0.004). Furthermore, a comparison of the effects of 10 μmol/L STI-571 on the viability of CLL cells and normal peripheral blood mononuclear cells (PBMC) showed that the former were generally more sensitive to STI-571 than were the latter cells (P = 0.007; Fig. 4D, inset). However, the statistical significance of this difference disappeared when the c-Abl hi patients were excluded from the analysis, underlying the importance of c-Abl expression levels for STI-571-induced killing. Taken together, the above results suggest that the sensitivity of malignant cells to STI-571 is directly related to the level of c-Abl expression. Moreover, sensitivity to STI-571-induced killing was independent of p53 functional status (data not shown), pointing to the potential importance of this compound for the treatment of p53 dysfunctional CLL cases known to be resistant to conventional chemotherapy (24, 25).

To investigate how STI-571 produces its effects in CLL cells, we used the cleavage of poly(ADP-ribose) polymerase (a target of caspase-3 activation; ref. 26) and mitochondrial depolarization (measured by FACS analysis of DiOC6 incorporation) as indicators of apoptosis. Figure 5A shows that incubation with 10 μmol/L STI-571 for a period of 24 hours induced poly(ADP-ribose) polymerase cleavage in CLL cells from the c-Abl hi subgroup. Figure 5B shows that, after treatment with STI-571, the proportion of DiOC6 and propidium iodide dim early apoptotic cells increased in CLL cells from the c-Abl hi subgroup. Figure 5C shows that the STI-571-induced apoptosis of CLL cells in the c-Abl hi subgroup was...
significantly more pronounced than in the c-Abl\textsuperscript{low} subgroup. Taken together, these results strongly suggest that the inhibitor was selectively inducing apoptosis of c-Abl\textsuperscript{hi} CLL cells.

**Figure 3.** c-Abl expression correlates with clinical status. A, B, and C, comparison of c-Abl protein expression in the malignant cells from CLL cases classified into Binet stages A, B, C, and B + C. C, comparison of c-Abl protein expression and WBC counts in 67 cases of CLL. D, comparison of WBC counts in c-Abl\textsuperscript{low} and c-Abl\textsuperscript{hi} cases of CLL. Tests for statistical significance in (A, B, and D) were done using Mann-Whitney U test.

STI-571 inhibits the constitutive activation of the NF-κB pathway in CLL cells expressing high levels of c-Abl. To further examine the contribution of c-Abl to CLL cell survival, we investigated the role of this kinase in relation to the NF-κB pathway because activation of NF-κB is important for CLL cell survival (27, 28) and because activation of this pathway is an important part of the prosurvival effects of active Abl in many cell types (29, 30). We found that STI-571 treatment of c-Abl\textsuperscript{hi} CLL cells resulted in an increase in IκBα protein levels (Fig. 5A). Because activation of the NF-κB pathway results in degradation of IκBα through a mechanism involving phosphorylation and ubiquitination (31), the observed increase in IκBα protein within STI-571-treated cells indicates an inhibition of NF-κB signaling by c-Abl, presumably in response to c-Abl overexpression.

We also measured the effect of STI-571 on NF-κB activity in CLL cells by electrophoretic mobility shift assay. Figure 5D shows that 24-hour treatment of CLL cells from the c-Abl\textsuperscript{hi} subgroup with 10 μmol/L STI-571 resulted in a decrease of NF-κB binding to a consensus site oligonucleotide. These results provide further support for the above assertion that STI-571 inhibits NF-κB activity in CLL cells expressing high levels of c-Abl.

To confirm the role of c-Abl in NF-κB activation in CLL cells, we used siRNA. Figures 6A and B show that transfection of CLL cells with c-Abl siRNA resulted in a reduction of both c-Abl mRNA and protein expression compared with CLL cells treated with control, nonspecific siRNA. Analysis of the phosphorylation (active) status of IκB kinases (IKK) revealed that the activation of these kinases decreased dramatically as a result of c-Abl knockdown (Fig. 6B). This suggests that c-Abl is present in the pathway that leads downstream to serine phosphorylation of IKKs. In addition, measurement of NF-κB-driven transcription in CLL cells using a luciferase reporter assay revealed that the knockdown of c-Abl resulted in an overall decrease (54 ± 24%; \( P = 0.003 \)) in NF-κB transcriptional activity (Fig. 6C). In keeping with the effect of STI-571 on cell viability and NF-κB activity in the c-Abl\textsuperscript{hi} subgroup, siRNA knockdown of c-Abl also reduced CLL cell viability (Fig. 6D). However, the siRNA-induced reduction in cell viability required 5 days of incubation instead of the 3 days required for STI-571. This is likely because of the slower kinetics of the siRNA knockdown of c-Abl expression as compared with the kinetics of inhibition by

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<th>Table 1.</th>
<th>Relationship between clinical stages and c-Abl expression</th>
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<tr>
<td></td>
<td>c-Abl\textsuperscript{low}</td>
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<td>c-Abl\textsuperscript{hi}</td>
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NOTE: \( P = 0.004 \) (χ² test). A versus B + C: \( P = 0.004 \) (Fisher’s exact test).
STI-571. The above results were achieved using different siRNA constructs, indicating that inhibition of NF-κB signaling is the result of c-Abl protein knockdown and not an off-target effect of the siRNA used (data not shown). Thus, taken together, the above results strongly suggest that STI-571 mediates its proapoptotic effects in CLL cells through an inhibition of c-Abl-driven NF-κB pathway signaling.

Discussion

The aim of the present study was to examine the pathogenetic and therapeutic importance of c-Abl expression in CLL cells. We show that this kinase is overexpressed in CLL as compared with normal B cells, and that expression levels positively correlate with tumor burden and disease stage and negatively with IgVH mutation. We also show that STI-571, an inhibitor of c-Abl kinase activity, induces apoptosis of CLL cells with high c-Abl expression levels, at least partially through a mechanism involving inhibition of NF-κB. Thus, c-Abl is likely to be important in CLL cell survival and STI-571 may be of potential use in the treatment of CLL in cases where c-Abl is highly expressed.

c-Abl expression has previously been examined in a number of B-cell malignancies including non-Hodgkin’s lymphomas, multiple myeloma, and CLL (10). In the present study, we also found high levels of c-Abl expression in mature B lymphoid malignancies. However, in contrast to the 29% of CLL cases that were designated as c-Abl positive in the previous study (10), our use of the more sensitive and quantitative methods of Western blotting and/or real-time PCR revealed expression of c-Abl in all of the CLL cell clones analyzed. Although this expression significantly varied from case to case, it was generally higher than in normal B cells.

Our data show that CLL cells predominantly express the antiapoptotic c-Abl isoform 1b, suggesting that an overexpression of this isoform could contribute to the pathogenesis of the disease by providing cytoprotection to the malignant cells. This cytoprotection is underscored by our demonstration that CLL cells from the c-Ablhi subgroup are more susceptible to STI-571-induced apoptosis than are the cells with low levels of this kinase, a finding supported by a recent report involving 6 CLL cases (32). We also show a link between high c-Abl expression and markers of poor prognosis including low IgVH mutation, advanced clinical stage, and high tumor burden. However, because of limitations in our tissue bank, the follow-up of the patients in our study was unavoidably short and assessment of the true prognostic significance of high c-Abl expression would require prolonged follow-up of these and additional patients.

Our finding of significantly higher c-Abl expression in unmutated CLL cells, which are known to respond to B-cell receptor cross-linking (8, 33), enforces the notion that in vivo antigenic stimulation could have provided a stimulus for this expression. Moreover, this role of antigenic stimulation is further underlined by the positive correlation between c-Abl and ZAP-70 expression that we observed in CLL cells. ZAP-70 expression in CLL has recently been suggested to be the result of such in vivo B-cell receptor engagement (34, 35). Because c-Abl is implicated in B-cell receptor signaling, this kinase could then, in turn, be at least partly responsible for some of the effects of this signaling. If so, our experiments...
showing that STI-571 inhibits c-Abl-induced phosphorylation of DOK2, a known c-Abl substrate (23), support the proposition that, in unmutated CLL, signals from the B-cell receptor provide cell rescue from apoptosis (1, 27). Such rescue includes an activation of NF-κB that partly depends on constitutively active c-Abl. Thus, treatment of c-Ablhi CLL cells with STI-571 resulted in an increase in IkBα protein levels and a decrease in the DNA binding ability of NF-κB. Moreover, treatment of c-Ablhi CLL cells with c-Abl siRNA resulted in inhibition of IKK and of NF-κB-driven transcription. Activation of c-Abl could be the result of in vivo antigenic stimulation leading to phosphatidylinositol bisphosphate hydrolysis, known to relieve c-Abl from inhibition by this phospholipid (36). Alternatively, c-Abl overexpression could itself lead to enzyme activation (37). c-Abl together with Src-activated protein kinase Cα is known to phosphorylate protein kinase D (38), which then leads to downstream activation of NF-κB (39). It remains to be established whether this pathway is responsible for the STI-571-sensitive NF-κB activation observed in the present study. If c-Abl indeed provides a key signal for CLL cell rescue via B-cell receptor stimulation, then the rescue of cells in mutated cases, which do not respond to B-cell receptor stimulation (8, 33) and are insensitive to STI-571, must involve some other mechanism of cytoprotection (e.g., c-Abl-independent overexpression of antiapoptotic proteins).

In any case, our work gives further support to the concept that mutated and unmutated CLL are two distinctly different variants of the disease.

Our finding that STI-571 and c-Abl siRNA reduce cell viability in c-Ablhi CLL suggests that the malignant cells in these cases have become dependent on c-Abl for their survival. The STI-571-induced killing was associated with the cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase and mitochondrial depolarization, suggesting that cells were dying at least partly by apoptosis. Although STI-571 is a relatively specific inhibitor of c-Abl, it can also inhibit the activities of Lck (40), c-Kit (41, 42), and the platelet-derived growth factor receptor (41, 43). The contribution of the two latter receptor tyrosine kinases can be excluded because neither is expressed on CLL cells (44). With respect to Lck, this Src-related kinase is variably expressed in all CLL cases (45, 46). However, c-Abl is much more sensitive to STI-571 than is Lck (40, 47). Moreover, we found that STI-571 and c-Abl siRNA treatment of CLL cells showed similar effects on NF-κB pathway signaling. Taken together, the above data strongly suggest that the proapoptotic effect of STI-571 observed in the present study was mainly due to the inhibition of c-Abl.

The heterogeneity of CLL cell sensitivity in vitro to STI-571 has also been recently observed by others (32, 44). However, Aloyz et al.
(44) did not relate this variable STI-571 sensitivity to c-Abl expression, and neither study (32, 44) related STI-571 sensitivity to any variable of disease prognosis or activity. The quantitative data linking c-Abl expression in CLL to STI-571 responsiveness and disease activity/progression in the present study indicate that STI-571 may be useful in the treatment of CLL, in particular in cases with poor prognosis and those resistant to conventional therapies. Because p53 dysfunction is associated with active disease and is a particular therapeutic problem (24, 25), our finding that STI-571-induced apoptosis of CLL cells is independent of p53 status lends further support to the potential therapeutic value of this drug.

In conclusion, we show that high levels of c-Abl expression predispose CLL cells to the killing effects of STI-571. Because c-Ablhi expressing cells mainly belong to the unmutated, poor-prognosis group of CLL, this agent might find novel therapeutic application for the treatment of some cases in this group.

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