Activities of SYK and PLCγ2 Predict Apoptotic Response of CLL Cells to SRC Tyrosine Kinase Inhibitor Dasatinib

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Abstract

Purpose: B-cell receptor signaling plays an important role in the pathogenesis of chronic lymphocytic leukemia (CLL). However, blocking B-cell receptor signaling with dasatinib, an inhibitor of SRC kinase, produced variable results in preclinical and clinical studies. We aim to define the molecular mechanisms underlying the differential dasatinib sensitivity and to uncover more effective therapeutic targets in CLL.

Experimental Design: Fresh CLL B cells were treated with dasatinib, and cell viability was followed. The CLL cases were then divided into good and poor responders. The cellular response was correlated with the activities of B-cell receptor signaling molecules, as well as with molecular and cytogenetic prognostic factors.

Results: Among 50 CLL cases, dasatinib treatment reduced cell viability by 2% to 90%, with an average reduction of 47% on day 4 of culture. The drug induced CLL cell death through the intrinsic apoptotic pathway mediated by reactive oxygen species. Unexpectedly, phosphorylation of SRC family kinases was inhibited by dasatinib in good, as well as poor, responders. As opposed to SRC family kinases, activities of two downstream molecules, SYK and phospholipase Cγ2, correlate well with the apoptotic response of CLL cells to dasatinib.

Conclusions: Thus, SYK inhibition predicts cellular response to dasatinib. SYK, together with phospholipase Cγ2, may serve as potential biomarkers to predict dasatinib therapeutic response in patients. From the pathogenic perspective, our study suggests the existence of alternative mechanisms or pathways that activate SYK, independent of SRC kinase activities. The study further implicates that SYK might serve as a more effective therapeutic target in CLL treatment. *Clin Cancer Res; 16(2); 587–99. ©2010 AACR.*

Chronic lymphocytic leukemia (CLL) is one of the most common hematologic malignancies in the Caucasian population. The disease is characterized by an absolute lymphocytosis in the peripheral blood and follows a highly variable clinical course. Approximately one third of patients present with an indolent disease that does not need treatment; another third of patients present initially with

indolent disease followed by progression requiring therapy; and the last third of patients present with aggressive disease that requires immediate therapy (1).

In the past decades, major advances have been made in identifying biomarkers in CLL that predict clinical outcomes. Among these, the mutational status of the DNA sequences of the immunoglobulin heavy chain gene (*IgH*) variable region (*IGHV*) and ZAP-70 expression status have become the most commonly used prognostic markers. IgH chain is a component of the multimeric B-cell receptor complex that is responsible for antigenic recognition. Unmutated *IGHV*, defined as >98% homology with germline *IGHV* sequences, is associated with poor clinical outcome in patients. In contrast, mutated *IGHV* is associated with a favorable prognosis in CLL (2, 3). ZAP-70 is a receptor-associated protein tyrosine kinase of the SYK family. The kinase is expressed in T and natural killer cells, but not in normal circulating B cells. In comparison to normal B cells, some CLL B cells aberrantly express ZAP-70. ZAP-70–positive CLL has an inferior prognosis, with a shorter interval between diagnosis and treatment and a shorter progression-free survival following treatment (4, 5).

Although substantial progress has been made in the prognosis of CLL, much remains to be learned about its pathogenesis. Mechanism-based and molecularly-targeted therapy has not yet become available for the treatment of

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Translational Relevance

B-cell receptor signaling plays a key role in the pathogenesis of chronic lymphocytic leukemia (CLL), and SRC kinases mediate this signal transduction. However, recent test of dasatinib, an SRC tyrosine kinase inhibitor, in CLL produced highly variable results in preclinical and clinical studies. The authors aim to dissect the underlying mechanisms for this differential sensitivity with an ultimate goal of identifying more effective therapeutic targets. The study reveals that cases with worse prognostic factors are more likely to respond to dasatinib. Moreover, phospho-SYK and phospho-phospholipase Cγ2 are quantitatively correlated with CLL dasatinib response. These two molecules might therefore serve as biomarkers to predict dasatinib response in perspective clinical trials. Moreover, the results from this study imply that direct inhibition of SYK may be more effective than direct inhibition of SRC in CLL treatment. In addition, the findings provided some explanation for the initial observation that SYK inhibitor has better clinical activity than SRC inhibitor in CLL.

this incurable disease. Although many patients treated with standard nucleoside analogue-based therapeutic regimens show an initial response, they usually relapse. New strategies based on the understanding of underlying pathogenesis are needed to expand the treatment options and prolong the duration of remission.

Antigenic stimulation and B-cell receptor signaling play an essential role in the pathogenesis of CLL (6, 7). In normal B cells, the signal is initiated at the cell membrane following B-cell receptor ligation by antigens. Members of SRC family kinases then phosphorylate the cytoplasmic tails of Igα and Igβ chains of the B-cell receptor complex, thus creating docking sites for the recruitment and phosphorylation of SYK tyrosine kinase. SYK then phosphorylates BTK, another tyrosine kinase, and BLNK, a linker protein that facilitates the phosphorylation and activation of phospholipase Cγ (PLCγ). The enzyme then cleaves membrane phospholipid phosphatidylinositol 4,5-bisphosphate into diacylglycerol and inositol triphosphate, which subsequently mobilize calcium and activate extracellular signal-regulated kinase/mitogen-activated protein kinase and NF-κB pathways. These molecular interactions eventually lead to cellular metabolic changes and, ultimately, cell activation, proliferation, differentiation, and antibody production (8).

In CLL cells, B-cell receptor ligation by anti-IgM or anti-μ F(ab)'$_2$ induces global tyrosine phosphorylation, including SYK phosphorylation. This response to B-cell receptor has a strong association with unmutated *IGHV* status (9–12). Recently, it has been shown that LYN, a member of SRC family kinases, is overexpressed, abnormally distrib-

uted, and constitutively active in CLL cells. Moreover, treatment of CLL cells with high doses of two nonspecific SRC family kinase inhibitors, PP2 and SU6656, induces apoptosis (13).

This finding prompted several groups to test the antileukemia effect of dasatinib in CLL cells (14–16). Dasatinib is a potent inhibitor of Abelson tyrosine kinase and SRC kinases that is clinically available for the treatment of chronic myelogenous leukemia (CML) patients with resistance or intolerance to previous therapy. As an SRC inhibitor, dasatinib binds to SRC kinase family members with high affinity. The K_D for *in vitro* binding is in the nanomolar range (17), and an *in vivo* serum concentration of 126 nmol/L can be reached in CML patients using FDA approved doses (18).

Investigations on dasatinib in CLL thus far have generated inconsistent results. An early study showed that dasatinib at 5,000 nmol/L induced cellular apoptosis through an AKT and mitogen-activated protein kinase–dependent pathway in many of the CLL cases (14). However, two later studies showed that dasatinib had minimal effects in CLL as a single agent at clinically attainable concentrations and was cytotoxic only when high doses of the drug were used (15, 16). At low concentrations, dasatinib was able to sensitize the killing effects of other therapeutic drugs, including fludarabine, bortezomib, and chlorambucil, through inhibition of CD40–Abelson tyrosine kinase pathway (15, 16). In addition, data from a phase II clinical trial of dasatinib in CLL patients showed only a moderate effect; 2 of the 15 (13%) achieved partial response after being treated for >9 months (19).

Given the importance of B-cell receptor signaling in CLL, we aim to understand why inhibition of SRC kinases, key components in the pathway, did not generate a desirable effect. The answer to this question may help elucidate the molecular pathogenic events in CLL and direct us toward a more effective therapeutic strategy. Using dasatinib at 128 nmol/L, a low and clinically achievable concentration, we studied a total of 50 CLL cases *ex vivo*. At this concentration, we found that dasatinib generated a highly variable apoptotic response in CLL cells ranging from 2% to 90%. We further investigated the molecular mechanisms that underlie this variable response. Although SRC family kinase members are the direct target of dasatinib, their inhibition did not seem to correlate with apoptotic response of CLL. In contrast, our studies revealed that activities of downstream signaling molecules, SYK and PLCγ2, quantitatively correlated with CLL response to dasatinib. This study suggests that SYK kinase can be activated independently of SRC in leukemic cells and highlights SYK as a potentially more effective therapeutic target. The initial promising results from a recent phase II clinical trial of fostamatinib, a SYK inhibitor, in CLL has provided clinical support for such a notion (20).

Materials and Methods

Reagents and human CLL cells. Dasatinib (BMS-354825; Sprycel) was obtained from Bristol-Myers Squibb Co. and

was prepared as a 12.8 mmol/L stock solution in DMSO and stored at −20°C. Peripheral blood samples were collected from B-CLL patients that had been diagnosed according to standard clinical and pathologic criteria, including morphology and immunophenotyping (Table 1). Informed consent was obtained from all patients according to the Declaration of Helsinki, and approval for the study was obtained from the Institutional Review Board of the Weill Cornell Medical College.

Cell isolation and **in vitro** *culture of CLL cells.* Ten to twenty milliliters of fresh heparin-anticoagulated peripheral blood samples were collected from 50 CLL patients. B lymphocytes were immediately isolated by negative selection using RosetteSep Human B Cell Enrichment Cocktail (StemCell Technologies) following the manufacturer's instructions. Cells were counted, and viability was assessed using trypan blue staining. The procedures result in >98% viability in all cases. Purity was assessed by flow cytometric analysis for CD5 and CD19–double positive cells. Those that had >95% in purity were analyzed in this study. CLL cells were then cultured at 2×10^6/mL in 96-well round bottom plates in RPMI 1640 (Mediatech, Inc.) supplemented with 20% fetal bovine serum (Mediatech, Inc.).

Analysis of cell viability and apoptosis. For analysis of cell viability, cells were stained with 2 μg/mL propidium iodide (Molecular Probe), as described previously (21, 22). Ten thousand events in a live cell gate were counted by a FACS Calibur (BD Biosciences). Ratio of live (propidium iodide low) to total events was calculated as percent viability. Apoptosis was analyzed by double staining with propidium iodide and fluorescein isothiocyanate–conjugated Annexin V (BD). Briefly, 2×10^6 cells were washed once with PBS and resuspended in 200 μL binding buffer containing 0.5 μg/mL Annexin V–fluorescein isothiocyanate and 100 μg/mL propidium iodide. After incubation at room temperature in a light-protected area, the samples were analyzed by flow cytometry.

Measurement of intracellular reactive oxygen species. Intracellular reactive oxygen species were detected with 5-(and 6-)carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF; Molecular Probes), as described previously (21, 22). Aliquots of 3×10^5 cells were resuspended in 500 μL of RPMI 1640 containing 5% FBS and loaded with 10 μmol/L DCF for 30 min at 37°C. DCF fluorescence of 10,000 events was measured by flow cytometry.

Measurement of mitochondrial membrane potential. At various time points following dasatinib addition, 3×10^5 cells were stained with 20 nmol/L tetramethylrhodamine ethyl ester (Sigma) in a 37°C incubator for 30 min. Ten thousand events were collected and analyzed in a tetramethylrhodamine ethyl ester–high (live cell) gate by flow cytometry using FL-2 channel, as described previously (23).

Measurement of mitochondrial cytochrome **c** *release.* The InnoCyte Flow Cytometric Cytochrome *c* Release kit (Calbiochem) was used to quantify mitochondria associated cytochrome *c*. Cells (1×10^6) were permeabilized and stained with anti-cytochrome *c*, according to the manufac-

turer's protocol. Ten thousand ungated events were collected and analyzed by flow cytometry using FL-1 channel.

Immunoblot assays. Analyses were conducted as described previously (22). The following antibodies were used to probe the corresponding proteins: anti–phospho-SRC (Y416), anti-SRC, anti–β-actin (Cell Signaling), anti–phospho-LYN (Y396; Epitomics), anti-LYN, anti–phospho-HCK (Y411), anti-HCK, anti-BCL2 (Santa Cruz Biotechnology), and anti–X-linked inhibitor of apoptosis (XIAP; BD).

Intracellular phosphospecific flow cytometric assays. Intracellular phosphospecific flow cytometric analyses of SYK (pY348) and PLCγ2 (pY759; BD) were done using Lyse/Fix Buffer and Perm Buffer III, according to the manufacturer's protocol. Assays were done on CLL cells treated with or without dasatinib for 30 min.

IGHV mutational analyses. IGHV mutational analysis was done by Genzyme Genetics. Mutated IGHV is defined as a sequence that differ from the germline sequences by >2%.

ZAP-70 expression. ZAP-70 analysis was done by immunostaining of paraffin sections that were prepared from cell blocks generated from patients' peripheral blood samples. Anti–ZAP-70 monoclonal antibody was purchased from Upstate Biotechnology. Immunostaining was done in a Bond autostainer with heat-induced antigen retrieval. The sample was scored positive for ZAP-70 if >20% of B cells were immunoreactive. The test was validated for clinical use at Weill Cornell Medical College/New York-Presbyterian Hospital.

Statistical analysis. Mann-Whitney test was used to analyze the difference in the percentage of inhibition of phospho-SRC, phospho-LYN, and phospho-HCK between the good and poor responders. The Spearman correlation was used to analyze the relationship among percentage of inhibition of phospho-SYK, phospho-PLCγ2, and dasatinib response of CLL cases. Fisher's exact test was used to analyze the associations between IGHV, ZAP-70, cytogenetic abnormalities, and Rai stage with dasatinib responses.

Results

Dasatinib induces cell death in CLL B cells. In this study, we first evaluated the efficacy of dasatinib in 50 CLL cases using a low dose of 128 nmol/L dasatinib. This concentration was chosen because it is equivalent to the maximum serum concentrations of dasatinib observed in CML patients taking Food and Drug Administration–approved doses (18). Characteristics and treatment status for the 50 patients are presented in Table 1. Freshly isolated leukemic cells were cultured and treated *in vitro* with or without the drug. During a time course of 7 to 10 days, we followed the cell viability of each sample using propidium iodide staining and flow cytometric analysis. Results of two cases that span the range of responses are shown in Fig. 1A. We analyzed the apoptotic response of all 50 cases on day 4 of the *ex vivo* culture by percentage of

Table 1. Patient characteristics, treatment status, prognostic information, and dasatinib response

Pt. no.	Sex	Age	Therapy	Rai stage	ZAP-70	*IGHV*	Cytogenetic abnormalities	Dasatinib response*
CLL001	M	47	T	1	+	Unmut	del(13q14.3), del(11q)	Good
CLL003	M	76	NT	1	+	Mut	Trisomy 12	Good
CLL004	F	58	NT	0	−	Mut	del(13q14.3)	Poor
CLL005	M	58	NT	1	N/A	Mut	del(13q14.3)	Poor
CLL006	F	76	NT	3	N/A	N/A	N/A	Poor
CLL007	M	60	NT	2	+	Unmut	del(13q14.3)	Good
CLL010	N/A	62	NT	N/A	+	N/A	N/A	Good
CLL011	M	42	T	1	+	Unmut	del(13q14.3)	Good
CLL014	F	63	T	1	−	Mut	del(13q14.3), del(11q)	Poor
CLL015	F	56	T	1	+	Mut	Trisomy 12	Good
CLL016	F	57	NT	0	−	Mut	del(13q14.3)	Good
CLL018	F	71	NT	1	−	Mut	Trisomy 12	Good
CLL019	M	72	NT	1	−	Mut	del(13q14.3)	Poor
CLL020	M	63	NT	2	−	Unmut	del(6q)	Poor
CLL021	F	55	NT	2	+	Mut	None	Poor
CLL022	M	81	T	1	−	Mut	None	Good
CLL024	M	70	T	1	N/A	Unmut	N/A	Good
CLL029	F	71	T	3	+	Unmut	del(13q14.3), del(11q)	Good
CLL030	M	60	NT	1	+	Unmut	Trisomy 12, del(11q)	Good
CLL031	M	60	NT	1	+	Mut	del(13q14.3), del(11q)	Good
CLL032	M	64	NT	1	−	Mut	del(13q14.3)	Poor
CLL035	M	64	NT	0	−	Mut	None	Poor
CLL037	F	72	NT	0	−	Mut	del(13q14.3)	Poor
CLL038	M	67	T	1	+	Mut	del(11q)	Good
CLL039	F	67	T	1	N/A	Mut	del(13q14.3)	Poor
CLL042	F	60	NT	2	−	Mut	del(13q14.3)	Poor
CLL044	F	67	NT	0	+	Mut	None	Poor
CLL050	M	88	T	4	+	Mut	del(13q14.3), trisomy 12	Good
CLL051	F	73	NT	0	N/A	N/A	N/A	Poor
CLL055	M	74	T	1	+	Unmut	del(17p)	Good
CLL057	M	63	NT	3	+	Mut	None	Poor
CLL062	M	74	T	4	+	Unmut	del(11q)	Good
CLL064	M	70	NT	1	−	Mut	13(14.3)	Poor
CLL067	F	59	NT	1	−	Mut	13(14.3)	Poor
CLL078	F	75	NT	0	−	Mut	N/A	Poor
CLL085	M	66	T	4	N/A	Unmut	del(13q14.3), del(11q)	Poor
CLL087	M	79	NT	0	−	Mut	del(13q14.3)	Poor
CLL091	M	73	NT	1	N/A	N/A	None	Poor
CLL092	M	39	NT	1	+	Unmut	del(13q14.3), del(11q)	Good
CLL095	F	78	NT	1	−	Unmut	del(17p), del(13q14.3)	Good
CLL097	M	65	T	1	+	Unmut	del(13q14.3)	Good

(Continued on the following page)

Table 1. Patient characteristics, treatment status, prognostic information, and dasatinib response (Cont'd)

Pt. no.	Sex	Age	Therapy	Rai stage	ZAP-70	IGHV	Cytogenetic abnormalities	Dasatinib response*
CLL098	M	55	NT	1	+	Unmut	del(17p)	Good
CLL120	F	49	NT	1	N/A	N/A	None	Good
CLL113	F	63	NT	2	+	Unmut	None	Good
CLL117	M	70	T	2	+	Unmut	N/A	Good
CLL118	M	82	NT	2	–	Unmut	Trisomy 12	Poor
CLL124	F	60	NT	0	–	Mut	N/A	Poor
CLL126	M	60	NT	0	–	Mut	del(13q14.3)	Poor
CLL125	F	57	NT	4	–	Unmut	del(13q14.3)	Good
CLL127	F	80	NT	1	–	Mut	del(13q14.3)	Poor

Abbreviations: Pt., patient; M, male; F, female; T, treated; NT, not treated; N/A, not available; Unmut, unmutated; Mut, mutated.
*Good responders were defined as those that had a >24% difference in viability between treated and untreated cells throughout the entire time course of treatment (n = 25). Poor responders were those that had <24% difference in viability (n = 25).

inhibition, which is expressed as [(untreated - treated) / untreated] × 100%. As shown in Fig. 1B, percentage of inhibition in all cases varied from 2% to 90%. Distribution of 50 cases among four quartiles of response rates are indicated to the right of the plot. On average, 128 nmol/L dasatinib induces 47% cell death on the 4th day of *ex vivo* CLL culture.

The maximal difference in viability between treated and untreated cells, defined here as the maximal death response, occurred between days 3 to 6 for individual cases, not necessarily on day 4. This response was plotted for all 50 cases in Fig. 1C. The cases were then divided into good and poor responders using the median (24%; in between case 001 and 091) as the cutoff. Thus, good responders were those that had >24% reduction in viability during the course of dasatinib treatment. Meanwhile, poor responders were those that had <24% difference in viability between treated and untreated cells. Among the 50 cases, 25 (half of the cases) fell into the category of good responders and 25 (half of the cases) fell into the category of poor responders. We conducted repeat studies using serial samples from eight CLL patients, and the maximal death responses were largely reproducible and did not change their status as good or poor responders.

Dasatinib induces CLL cell death through the intrinsic apoptotic pathway mediated by reactive oxygen species. We then determined the mode of dasatinib-induced CLL cell death. Annexin V and propidium iodide double staining revealed a population of Annexin V–positive/propidium iodide–negative cells in three CLL specimens tested (Fig. 2A). These results suggest that primary CLL cells underwent apoptosis upon dasatinib treatment.

Reactive oxygen species play a critical role in the process of cell death (24). They are generated from mitochondria by many types of apoptotic stimuli, including anticancer chemotherapeutic agents, such as etoposide and doxorubicin (24–28). Reactive oxygen species cause mitochondrial damage by inhibiting mitochondrial electron transport

that leads to mitochondrial membrane depolarization. Examination of intracellular oxidants showed that reactive oxygen species levels increased over time in leukemia cells during the course of the dasatinib treatment (Fig. 2B, *top*; n = 6). In addition, the tyrosine kinase inhibitor induces mitochondrial depolarization (Fig. 2B, *bottom*; n = 6). Comparison of these time course analyses revealed that the reactive oxygen species increase preceded mitochondria depolarization (compare 6- and 15-hour data between *top* and *bottom*). These effects were reproducible in fresh leukemic cells from six CLL patients.

Cytochrome *c* is released from the intermembrane space of mitochondria following mitochondrial depolarization (reviewed in ref. 29). We determined whether dasatinib induces cytochrome *c* release in treated CLL cells. Fludarabine or actinomycin D was included in this experiment (at 1 µmol/L) as a positive control for cytochrome *c* release. Six CLL cases were treated with 128 nmol/L of dasatinib, and cytochrome *c* release was monitored using flow cytometry over a time course of 24 hours. Representative good and poor responders are shown in Fig. 2C (n = 6). Similar to fludarabine, dasatinib induced marked cytochrome *c* release in the good responder but not in the poor responder in the 24 hours following treatment. Taking together the Annexin V, reactive oxygen species, mitochondrial polarization, and cytochrome *c* data, these results suggest that dasatinib causes mitochondrial damage and subsequent apoptosis through induction of reactive oxygen species generation.

The antiapoptotic protein BCL2 is a key player in the mitochondrial apoptotic pathway. It is overexpressed and contributes to the apoptotic defects in CLL (17, 30, 31). The levels of BCL2 and another antiapoptotic protein, XIAP, were compared between good and poor responding CLL cases. Western blot analysis showed that XIAP and/or BCL2 declined, although to a varying degree, in CLL cases that responded well to dasatinib (CLL062, CLL038, and
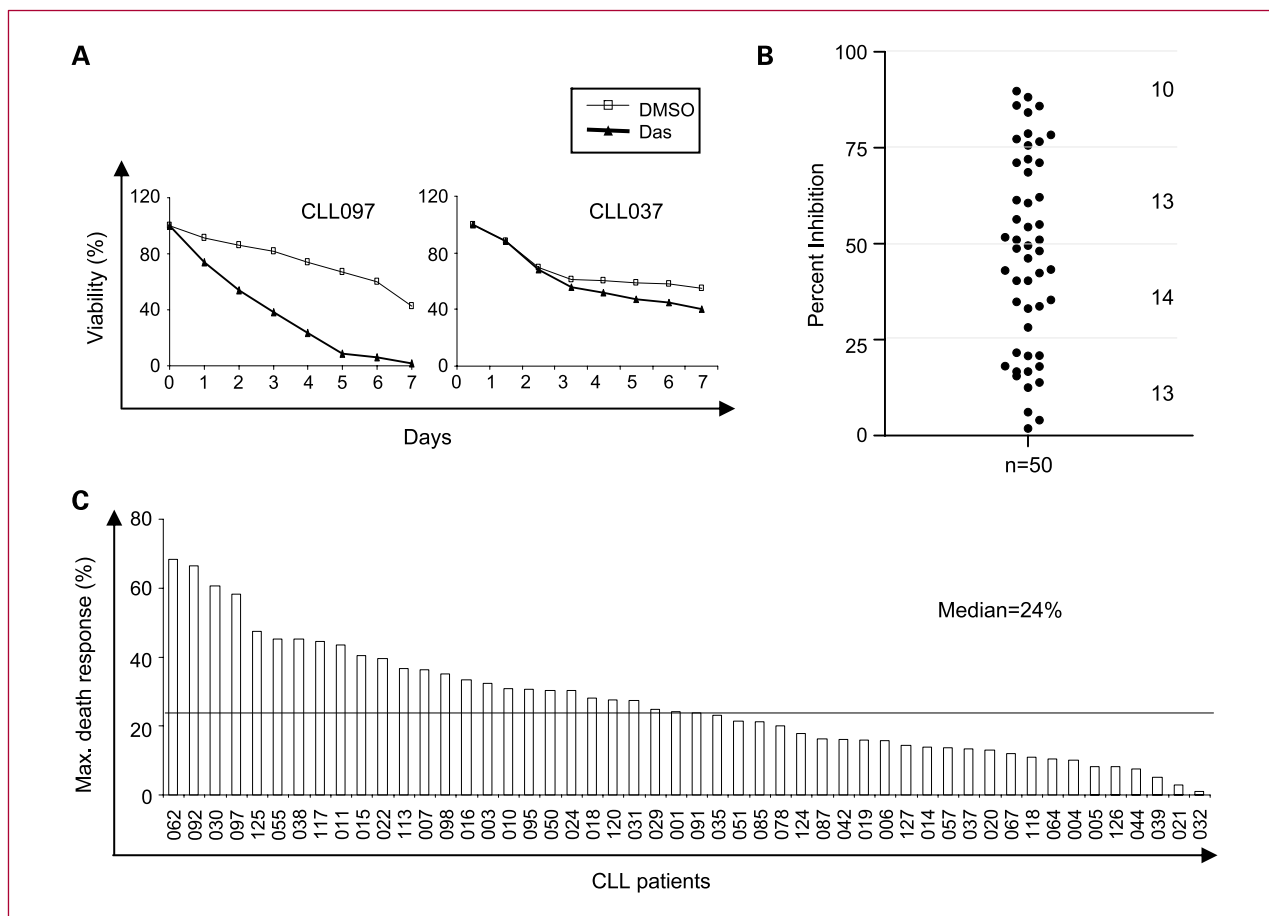
Fig. 1. Dasatinib induces cell death in CLL B cells. *A,* freshly isolated B-CLL cells (2 × 10⁶/mL) were incubated in the presence of vehicle or 128 nmol/L dasatinib. Cell viability was determined by propidium iodide staining, as described in Materials and Methods. CLL097 and CLL037 represent good or poor responders, respectively. *B,* percentage of inhibition, expressed as [(untreated - treated) / untreated] × 100% on day 4 of the *ex vivo* culture, was calculated for all 50 cases. There are numbers of cases in each quartile. *C,* maximal death response of all 44 cases. Maximal death response is defined as maximal difference in viability between treated and untreated cells occurred during the treatment time course. The horizontal line at the median (24%) divides the cases into good and poor responders (see text). Das, dasatinib; DMSO, dimethyl sulfoxide.

CLL113), but the decline did not occur for up to 72 hours in cases that had a poor response (CLL039, CLL014, and CLL78; Fig. 2D). Taken together, these data show that dasatinib induces CLL cell apoptosis through the mitochondrial pathway mediated by reactive oxygen species.

Activation of SRC kinases is inhibited in good, as well as poor, responders. As shown in Fig. 1B and C, among the 50 cases, there was a wide range of variation in terms of CLL sensitivity to dasatinib. We determined the level of SRC kinase phosphorylation in CLL cells to see if inhibition occurs and, if so, whether the degree of inhibition correlated with their apoptotic response.

Immunoblot analysis for the active phosphorylated forms of SRC, LYN, and HCK, along with the total kinase levels, was done for several CLL cases, including six good responders and four poor responders (Fig. 3A). The levels of phosphoproteins in dasatinib-treated and untreated cells were quantified, and the degree of inhibition (treated / untreated × 100%) was calculated for each of the sam-

ples (Fig. 3B). The Mann-Whitney test was used to analyze the difference between the six good and four poor responders for phospho-SRC, phospho-LYN, and phospho-HCK. Unexpectedly, the phosphorylation of all three SRC kinases was similarly inhibited in both good-responding and poor-responding cases, suggesting that the activities of these kinases were not directly responsible for the apoptotic response of CLL B cells.

Inhibition of SYK and PLCγ2 predicts a good cellular apoptotic response to dasatinib. We then determined whether the activities of downstream signaling molecules correlated with CLL apoptotic response to dasatinib. SYK and PLCγ2 are phosphorylated by SRC kinases following B-cell receptor ligation. The activities of these two molecules were measured by a sensitive and, more importantly, a quantitative phosphotyrosine-specific flow cytometric assay. Representative good and poor responders (CLL010 and CLL039) with or without 30-minute dasatinib treatment were examined (Fig. 4A). In CLL010, the sensitive

CLL case, dasatinib treatment caused marked left shifts of phospho-SYK and phospho-PLCγ2 peaks, whereas little left shifts in phospho-SYK and phospho-PLCγ occurred in CLL039 [Fig. 4A, compare treated (*open*) and untreated (*filled*) profiles]. Thus, activities of SYK and PLCγ2 in these two cases seemed to correlate well with their apoptotic response to dasatinib. To determine whether this rule applies to additional CLL cases, we used percent inhibition to quantify the degree of SYK and PLCγ2 inhibition, $[(F_{untreated} - F_{treated}) / F_{untreated}] \times 100\%$, wherein F denotes mean of fluorescence intensity of 10,000 events. We analyzed the quantitative relationship between the degree of SYK and PLCγ2 inhibition and the maximal death re-

sponse as defined in Fig. 1C. This analysis revealed that there is a statistically significant linear correlation between the degree of maximal death response and the degree of SYK inhibition (Fig. 4B; $n = 27$). For each CLL sample, phospho-PLCγ2 was determined in parallel to phospho-SYK. As expected, statistical analysis revealed a significant linear correlation between SYK and PLCγ2 activity in dasatinib-treated CLL samples (Fig. 4C; $n = 27$). Furthermore, a linear correlation was also identified between the degree of maximal death response and the degree of PLCγ2 inhibition corroborating the SYK results (Fig. 4D; $n = 27$). These data thus suggest that SYK is a key player determining the sensitivity of CLL cells to SRC inhibition
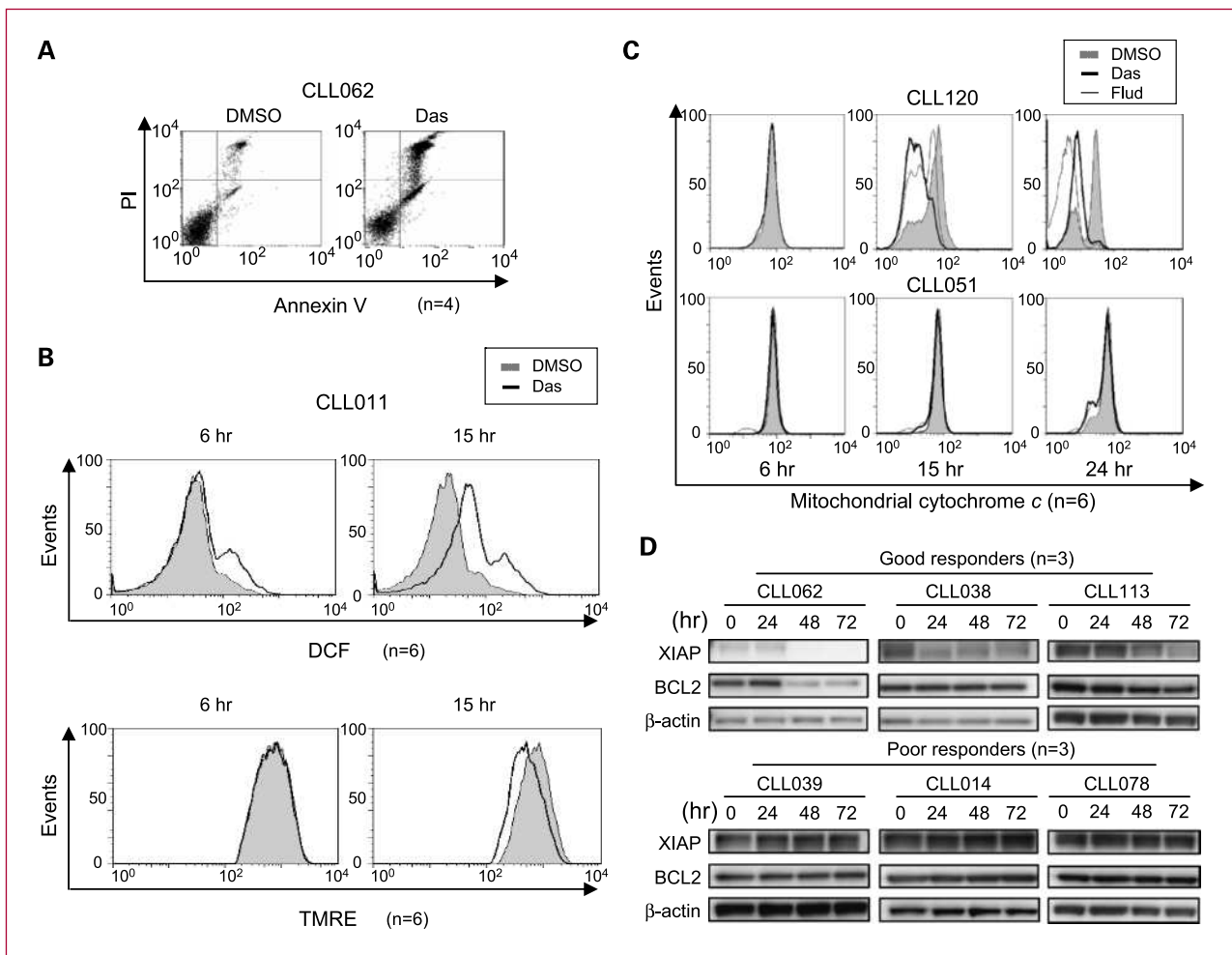

Fig. 2. Dasatinib induces CLL cell death through the intrinsic apoptotic pathway mediated by reactive oxygen species. *A,* freshly isolated CLL cells were treated with DMSO or 128 nmol/L dasatinib for 96 h. Apoptosis was measured by Annexin V and propidium iodide double staining. Plots, one of three CLL cases yielding similar results. *B,* changes in reactive oxygen species and mitochondrial membrane potential during the course of dasatinib treatment. Reactive oxygen species was assayed using DCF. Mitochondrial membrane potential was assayed using tetramethylrhodamine ethyl ester. Cells were treated with 128 nmol/L of dasatinib for the corresponding periods. Plots, one of six CLL cases yielding similar results. *C,* cytochrome *c* was released during the course of dasatinib treatment. Mitochondrial cytochrome *c* was measured using anti-cytochrome *c* monoclonal antibody in permeabilized cells. Cells from good-responding and poor-responding cases were treated for the corresponding periods. Plots, two representative cases. Fludarabine (1 μmol/L) treatment was included as the positive control. *D,* antiapoptotic proteins declined in good responders but not in poor responders. CLL cells were treated with 128 nmol/L dasatinib and followed for 72 h. The levels of XIAP and BCL2 were assayed by immunoblot analysis, as described in Materials and Methods. β-Actin levels were determined as loading controls. Das, dasatinib; DMSO, dimethyl sulfoxide.
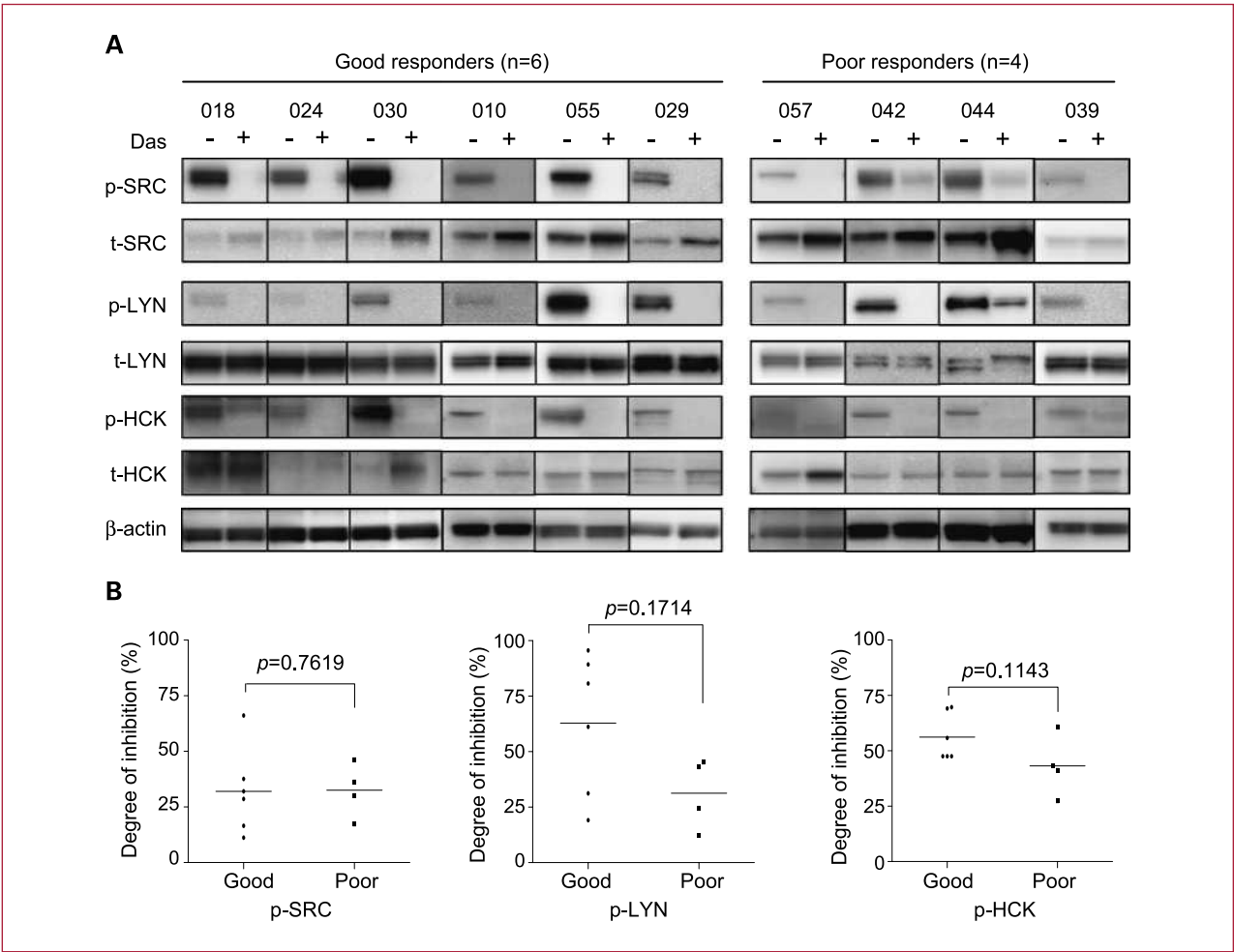
Fig. 3. SRC family kinase tyrosine phosphorylation is inhibited by dasatinib in good responders and poor responders. CLL cells were treated with vehicle or 128 nmol/L dasatinib for 30 min. *A*, immunoblot analysis for phosphorylated and total SRC, LYN, and HCK were done as described in Materials and Methods. p-SRC, pY416; p-LYN, pY396; p-HCK, pY411. β-actin levels were included as loading controls. *B*, the bands in the immunoblots shown in (*A*) were digitally captured and quantified using an Alpha Innotech FluoChem FC2 instrument. Levels of phosphoproteins were normalized to β-actin, and degree of inhibition (treated / untreated × 100%) was calculated for each sample and plotted. Differences between the good and poor responding groups were analyzed using Mann-Whitney test. *P* values are indicated above each plot. Das, dasatinib.

by dasatinib. Furthermore, the assays for phospho-SYK and phospho-PLCγ2 can be used to predict CLL response to dasatinib. These results also implicate that SRC inhibition in CLL does not always lead to SYK inhibition; there might be other mechanisms or other pathways that activate SYK independent of SRC kinases.

CLL cases with poor prognostic factors are more sensitive to dasatinib. Known prognostic factors, such as *IGHV* mutational status and ZAP-70 status, are linked to the activity of B-cell receptor signaling in CLL cells. Previous studies have found that most of *IGHV* unmutated CLL cases show a response to B-cell receptor ligation, and almost all cases that lack response to B-cell receptor ligation have mutated *IGHV* (9–11). Because B-cell receptor–initiated signaling is the process that is inhibited by dasatinib, we analyzed our cases to determine whether response of CLL to dasatinib differentiates between cases with distinct *IGHV* muta-

tional status. *IGHV* mutation data were available in 45 cases, of which 18 had unmutated *IGHV* and 27 had mutated *IGHV* (Fig. 5A and the corresponding table). Of 18 unmutated cases, 15 have good response to dasatinib. Conversely, of 22 poorly responding cases, 19 have mutated *IGHV*. Fisher's exact test revealed a significant correlation between the unmutated *IGHV* status and good dasatinib response ($P = 0.0007$).

ZAP-70 expression has been associated with poor clinical outcome in CLL patients (32, 33). In ZAP-70–positive leukemic cells, at the cellular level, the kinase is recruited to the B-cell receptor signalosome upon surface IgM ligation. The kinase is subsequently phosphorylated and activated to enhance B-cell receptor signaling (10, 34). We reasoned that increased B-cell receptor signaling in ZAP-70–positive cases would make them more sensitive to dasatinib. Among 50 cases, ZAP-70 status was

known for 42 cases. As shown in Fig. 5B, 18 of 21 ZAP-70–positive cases had good responses to dasatinib. Meanwhile, 16 of 19 poor responders did not express the kinase. Fisher's exact test revealed a statistically significant association between ZAP-70 expression and good dasatinib response, and between poor response and lack of ZAP-70 expression ($P = 0.0001$). Taken together, this correlation of dasatinib response with unmutated IGHV and ZAP-70 suggests that those cases with active B-cell receptor signaling respond better to SRC family kinase inhibition by dasatinib.

With regard to cytogenetic abnormalities, data were available for 44 patients. We noted that 9 of 44 cases had deletion in 11q (ataxia-telangiectasia mutated gene) and three had deletion in 17p (P53), and 10 of these 12 cases had good response to dasatinib. Of 22 poor responders, 20 cases did not carry these deletions (Fig. 5C; $P = 0.0157$). The data suggested that the drug is able to kill the leukemic cells even in the absence of a functional ataxia-telangiectasia mutated gene–P53 axis. Correlation was not found between dasatinib response and other chromosomal abnormalities, including del(6q), trisomy
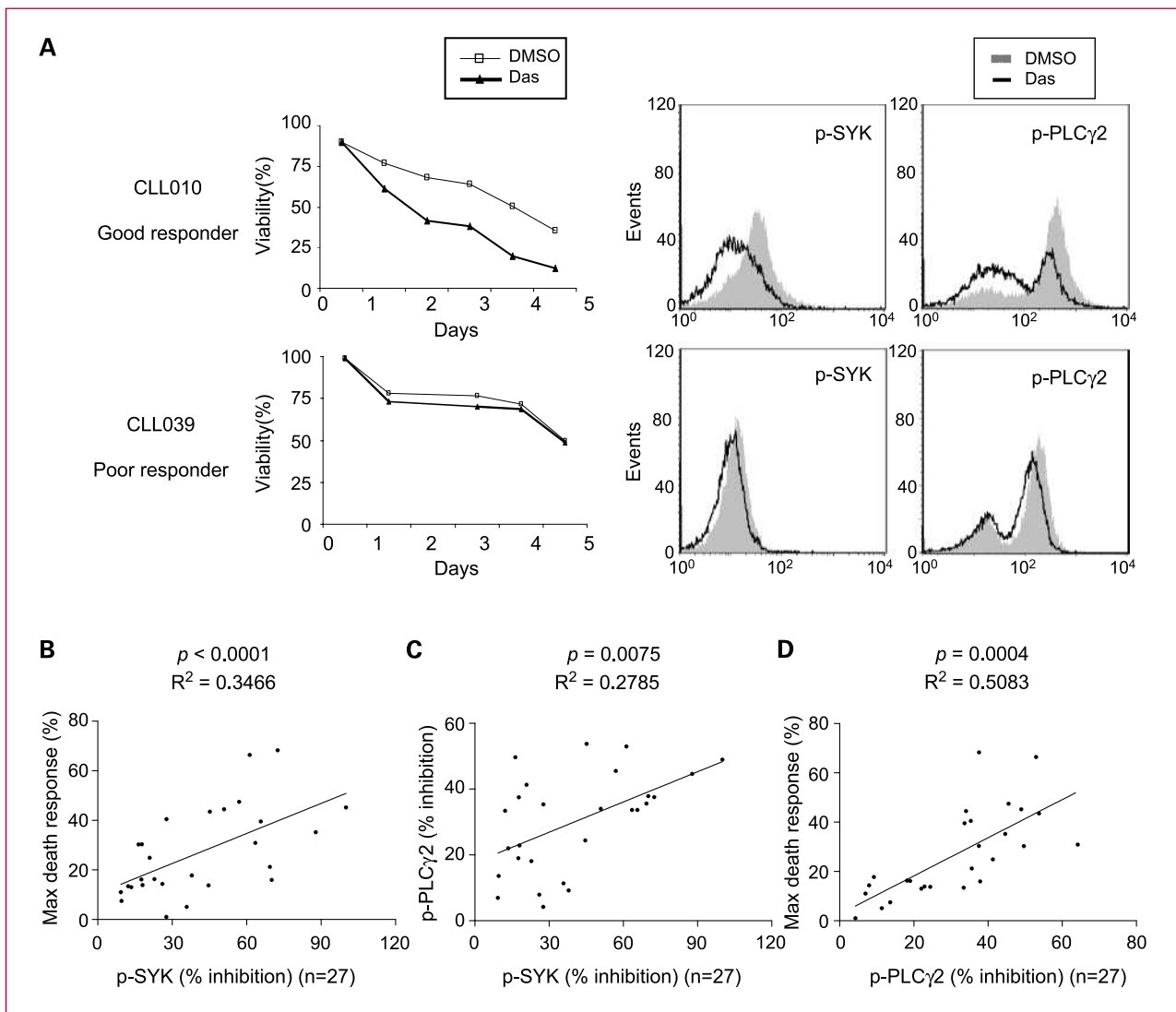

Fig. 4. Phosphorylation of SYK and PLCγ2 predicts apoptotic response of CLL cells to dasatinib. A, phosphorylation of SYK and PLCγ2 were markedly inhibited in a good responder (CLL010) and minimally in a poor responder (CLL039). Intracellular phosphospecific flow cytometry was used to assess p-SYK (Y348) and p-PLCγ2 (Y759) in cells treated with or without 128 nmol/L dasatinib (open versus filled profiles). B, inhibition of SYK phosphorylation (Y348) is linearly correlated with the maximal death response of CLL cells. C, inhibition of SYK phosphorylation (Y348) is linearly correlated with inhibition of PLCγ2 phosphorylation (Y759). D, inhibition of PLCγ2 phosphorylation is linearly correlated with the maximal death response of CLL cells. B-D, percentage of inhibition was used to define the degree of phosphorylation inhibition, $[(F_{untreated} - F_{treated})/F_{untreated}] \times 100\%$, wherein F denotes mean fluorescence intensity of 10,000 events. Maximal death response was defined as that in Fig. 1C. Data were analyzed using Spearman correlation, and P values and correlation coefficients are indicated. Das, dasatinib; DMSO, dimethyl sulfoxide.
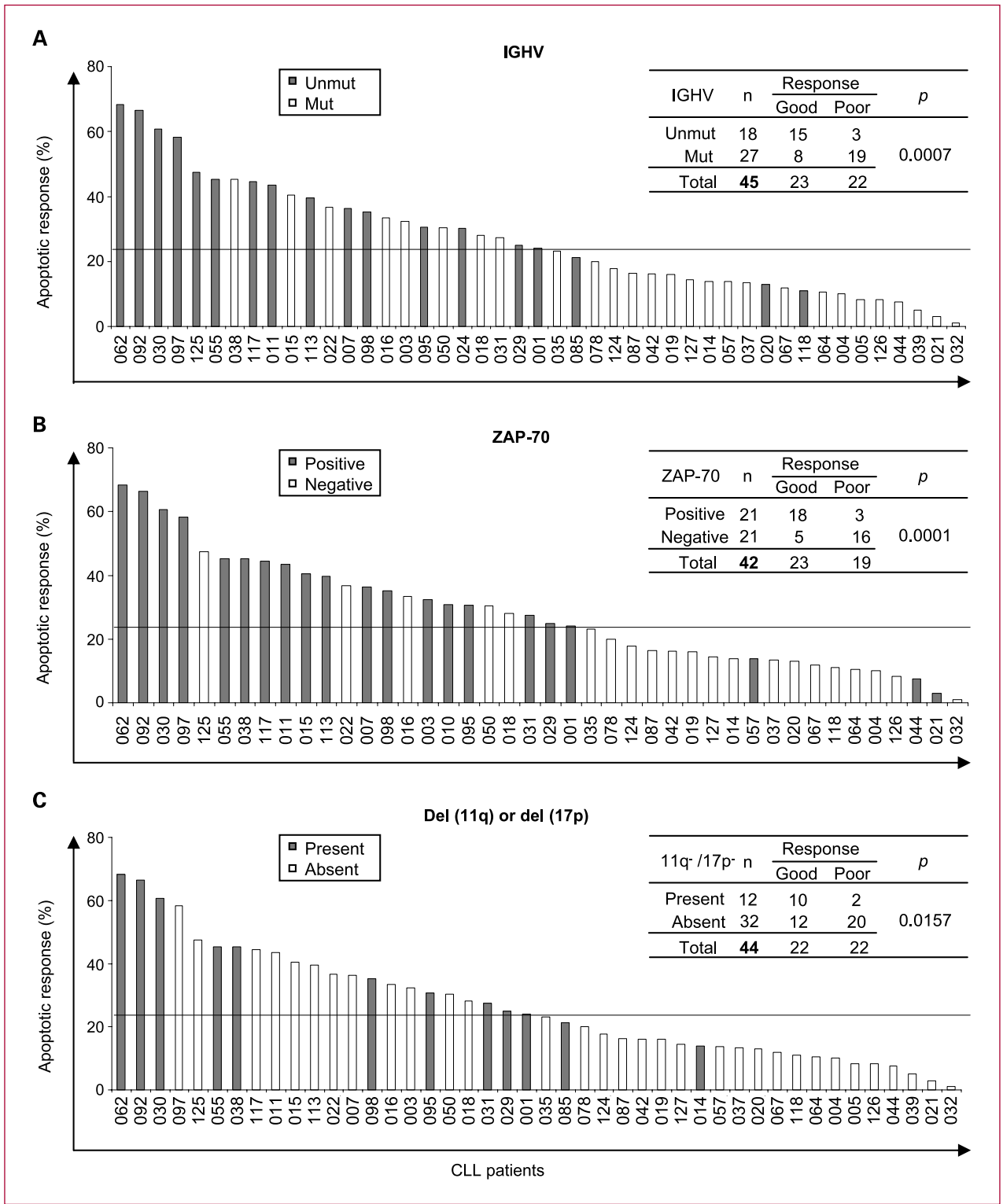
Fig. 5. CLL cases with poor prognostic factors are more sensitive to dasatinib. *A,* unmutated *IGHV* is associated with good response to dasatinib. Cases with unmutated *IGHV* are marked in gray, and mutated are in white. Horizontal line, the 24% cutoff for good and poor responders. Fisher's exact test of the 45 cases shows a *P* of 0.0007. *B,* ZAP-70 expression is associated with good response to dasatinib. Cases with positive ZAP-70 are marked in gray, and negative are in white. Horizontal line, the 24% cutoff for good and poor responders. Fisher's exact test of the 42 cases shows a *P* of 0.0001. *C,* dysfunctional P53 pathway is associated with good response to dasatinib. Cases with either del(11q) or del(17p) are marked in gray, and the rest are in white. Horizontal line, the 24% cutoff for good and poor responders. Fisher's exact test of the 44 cases shows a *P* of 0.0157.

12, del(13q14.3), and del(13q34). In addition, no correlation can be established between dasatinib response and patients' Rai stage (Table 1). Taken together, we conclude that CLL subgroups with poor prognoses by *IGHV* mutational status, ZAP-70 expression, and cytogenetic abnormalities respond better to dasatinib inhibition.

Discussion

Components of B-cell receptor signaling are promising targets for CLL treatment. However, inhibition of SRC family kinases by dasatinib only generated a modest effect in CLL patients (2 of 15 partial response) after >9 months of therapy (19). In addition, the results from three preclinical studies on dasatinib in *ex vivo* CLL cells are not entirely consistent (14–16). We designed the current investigation attempting to understand the underlying reasons with an ultimate goal of developing a more effective therapeutic strategy. We have shown that (*a*) dasatinib, at a clinically attainable concentration, causes a highly variable death response in 50 CLL cases; (*b*) mechanistically, the cells undergo apoptosis involving the induction of reactive oxygen species and mitochondrial depolarization; (*c*) although SRC kinases are uniformly inhibited in all CLL cases, regardless of their response to dasatinib, the degree of SYK and PLCγ2 inhibition quantitatively correlates with the degree of dasatinib-induced apoptotic response; and (*d*) The CLL subgroup with unmutated *IGHV*, ZAP-70 expression, or del (11q) or del(17p) responds better to dasatinib treatment.

From the clinical perspective, our data suggest that dasatinib may have antileukemic effect in a subset of CLL patients at a clinically achievable dose. The patients with more aggressive disease, by three standard prognostic factors, are more likely to benefit from dasatinib therapy. The good response of del(11q) or del(17p) cases to dasatinib is particularly interesting because CLL patients with these lesions are frequently refractory to standard chemotherapy.

As mentioned above, three other groups have already evaluated the efficacy of dasatinib against CLL cells. Veldurthy et al. (14) studied whether dasatinib works as a single agent. In contrast to our data, they found that dasatinib induces significant apoptosis in most of 35 cases examined. Different concentrations used in these studies may account for this difference in results. Dasatinib concentration used in our apoptosis experiment was 128 nmol/L versus 5,000 nmol/L in Veldurthy et al. (14). Given that clinical achievable concentration is ~126 nmol/L by pharmacokinetic and pharmacodynamic studies in CML patients (18), the lower concentration used in our study should be more clinically relevant. In our opinion, the modest effect of dasatinib observed in the CLL patients in the phase II trial is more in line with our preclinical evaluation results. In addition, two other groups found that dasatinib has a limited effect when used alone *in vitro* (15, 16).

Despite this difference, Veldurthy et al. (14) also found that *IGHV*-unmutated and ZAP-70–positive CLL cases are more sensitive to dasatinib inhibition, supporting our conclusion that patients with worse prognosis may benefit

from dasatinib therapy. Although we also showed that CLL cells with dysfunctional ataxia-telangiectasia mutated gene–P53 axis are sensitive to dasatinib, Veldurthy et al. (14) did not obtain sufficient cytogenetic data to draw any conclusions.

More interestingly, in an effort to address the differential responses of CLL to dasatinib, we have found a quantitative correlation between the degree of SYK inhibition and the degree of CLL apoptotic response to dasatinib. This observation is further corroborated by the assay for phospho-PLCγ2, a signaling molecule downstream from SYK. SYK is not a direct target of dasatinib. The K_D for *in vitro* binding is 3,000 nmol/L versus <1 nmol/L for SRC family kinase members (17). These findings have clinical and pathogenic implications. First, assay for phospho-SYK and phospho-PLCγ2 on *ex vivo* leukemic cells may help predict dasatinib therapeutic response in patients. Second, the fact that SYK activity was not inhibited by SRC inhibition in some CLL cases suggests that alternative pathways or alternative mechanisms may lead to SYK activation in the absence of SRC activity. Results from these analyses therefore highlight SYK, instead of SRC, as a key determinant in the leukemic cell survival and implicates that targeting SYK may represent a more effective strategy in the treatment of CLL. In line with this deduction, a recent clinical trial of fostamatinib, a SYK inhibitor, in CLL produced partial response in 6 (54%) of 11 patients, whereas the trial of dasatinib generated partial response in only 2 (13%) of 15 patients. Similar correlations between SYK/PLCγ2 activity and cellular response were previously identified by our group in dasatinib-treated diffuse large B-cell lymphoma cell lines (35), suggesting overly active SYK might be a mechanism used by a broader spectrum of B-cell malignancies.

Our study again reinforces the central role of B-cell receptor signaling plays in the pathogenesis and prognosis of CLL. Physical stimulation of B-cell receptor by antigens or anti-IgM is probably not required. During our prolonged *in vitro* CLL cell culture, nothing was added to activate the B-cell receptor. However, our data showed that SYK may be phosphorylated and activated even when upstream SRC kinases were inhibited, suggesting the presence of SRC-independent SYK activation (Figs. 3 and 4). Several other studies showed constitutive activation of other components of B-cell receptor signaling pathway, including LYN (13, 36–39) and AKT (38, 40). In addition, autonomous activation of SRC kinase family members and SYK was shown in diffuse large B-cell lymphoma (35, 41), suggesting antigen-independent B-cell receptor activation might be a general pathogenic mechanism. What activates SYK/PLCγ2 is unknown at this point and subject to further investigation.

Besides pathogenesis, CLL prognosis can also be linked to the B-cell receptor pathway. *IGHV* mutations and ZAP-70, the known prognostic factors, affect the activity of B-cell receptor signaling. As for *IGHV*, it has been shown by several studies that only cells with unmutated *IGHV* have the ability to respond to B-cell receptor ligation with protein phosphorylation, gene expression, and cellular functional changes (9–12). As for ZAP-70, it has been shown

that CLL B cells with this kinase have higher levels of phosphorylated SYK, BLNK, PLCγ, and Ca^{2+} flux following B-cell receptor ligation (34). Moreover, transduction of ZAP-70 into ZAP-70–negative CLL B cells increases the level of B-cell receptor signaling (34). In this context, it is not surprising for us to find that *IGHV*-unmutated and ZAP-70–positive cases, in which B-cell receptor signaling is more active, are particularly sensitive to dasatinib, an inhibitor of this exact pathway (Fig. 5).

In summary, the findings in this study add to those in the literature, and together, they highlight components or modulators of the B-cell receptor signaling pathway as potential targets for the development of therapeutic agents for CLL.

Disclosure of Potential Conflicts of Interest

F.Y. Lee is an employee of Bristol-Myers Squibb Co. (BMS). J.P. Leonard served in the past as a paid consul-tant/advisor to BMS. The other authors disclosed no potential conflicts of interest.

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