Spleen Tyrosine Kinase Is Overexpressed and Represents a Potential Therapeutic Target in Chronic Lymphocytic Leukemia

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Abstract

B-cell receptor signaling contributes to apoptosis resistance in chronic lymphocytic leukemia (CLL), limiting the efficacy of current therapeutic approaches. In this study, we investigated the expression of spleen tyrosine kinase (SYK), a key component of the B-cell receptor signaling pathway, in CLL and its role in apoptosis. Gene expression profiling identified enhanced expression of SYK and downstream pathways in CLL compared with healthy B cells. Immunoblotting showed increased expression and phosphorylation of SYK, PLCγ_2, signal transducers and activators of transcription 3, and extracellular signal regulated kinase 1/2 in CLL compared with healthy B cells, suggesting enhanced activation of these mediators in CLL. SYK inhibitors reduced phosphorylation of SYK downstream targets and induced apoptosis in primary CLL cells. With respect to prognostic factors, SYK inhibitors exerted stronger cytotoxic effects in unmutated and ZAP70$^+$ cases. Cytotoxic effects of SYK inhibitors also associated with SYK protein expression, potentially predicting response to therapy. Combination of fludarabine with SYK Inhibitor II or R406 increased cytotoxicity compared with fludarabine therapy alone. We observed no stroma-contact–mediated drug resistance for SYK inhibitors as described for fludarabine treatment. CD40 ligation further enhanced efficacy of SYK inhibition. Our data provide mechanistic insight into the recently observed therapeutic effects of the SYK inhibitor R406 in CLL. Combination of SYK inhibitors with fludarabine might be a novel treatment option particularly for CLL patients with poor prognosis and should be further evaluated in clinical trials. [Cancer Res 2009;69(13):5424–32]

Introduction

B-cell chronic lymphocytic leukemia (CLL), the most prevalent B-cell malignancy in adults, is characterized by expansion of monoclonal mature B lymphocytes with low levels of surface membrane immunoglobulin. Despite advances in treatment, the disease remains incurable, warranting further efforts to identify novel molecular targets in CLL.

Signals propagated through the B-cell receptor (BCR) guide the maturation and survival of B cells and may promote progression of CLL. Structural and functional features of the BCR associate with the clinical course, indicating that antigen stimulation and signaling may play an important role in the pathogenesis of this disease (1, 2). Leukemic cells of patients with an aggressive clinical course typically display BCRs encoded by unmutated immunoglobulin variable heavy chain (IgV_H) genes and express the protein tyrosine kinase ZAP70. In contrast, CLL cells from most patients with stable disease express mutated IgV_H and lack ZAP70 (3–5). The two subsets also differ in their ability to transmit BCR-derived signals, as *in vitro* ligation of IgM induces stronger signaling responses and prolongs cell survival in CLL cells from patients with a poor prognosis (6–9). Moreover, biased V_H segment usage and stereotyped complementarity determining region 3 substantiate the theory of BCR involvement in the pathogenesis of CLL (10, 11), suggesting the BCR signaling pathway as new target for therapy in CLL.

The protein tyrosine kinase spleen tyrosine kinase (SYK) represents a key mediator of proximal BCR signaling, providing proliferation and survival signals in a variety of hematopoietic cells (12). After antigen stimulation, SYK is recruited to the BCR and becomes activated by sequential phosphorylation at conserved tyrosine residues. Once activated, SYK propagates the BCR signal by associating with adaptor proteins and phosphorylating important signaling intermediates, such as VAV, Phospholipase Cγ_2 (PLCγ_2), Bruton's tyrosin kinase, and B-cell linker protein. The signaling cascade then proceeds with the activation of further downstream signaling molecules including extracellular signal regulated kinase 1/2 (ERK 1/2), and p38 (13). Translocations involving SYK have recently been identified in myelodysplastic syndromes and T-cell lymphoma, indicating that SYK may also function as a proto-oncogene (14, 15). Protein tyrosine kinase inhibitors such as Curcumin or Piceatannol exert proapoptotic effects in some B-cell lymphoma entities (16, 17). Recently, more specific SYK kinase inhibitors, such as SYK Inhibitor I to IV or R406 have been developed. The oral SYK inhibitor R406 has been safely tested in humans for nonmalignant diseases (18).

Based on these considerations, we hypothesized that SYK may be a rational candidate for targeted therapy of CLL and that SYK inhibitors might eliminate the constitutive BCR signal and, therefore selectively promote cytotoxicity of CLL cells.

Materials and Methods

Patients and healthy donors. After informed consent, peripheral blood samples were obtained from patients with CLL. IgV_H mutational status (3, 4), ZAP70 expression (5), disease stage according to Binet (19) or Rai

Note: C. Dierks and K. Zirlik share senior authorship.

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criteria (20), and history of treatment were analyzed. All patients were untreated or off therapy for at least 6 mo before inclusion. B cells were isolated by Ficoll gradient centrifugation and negative selection (B-cell isolation kit II; Miltenyi Biotech).

Microarray and pathway analysis. Microarray and pathway analysis were performed by reanalysis of the baseline raw gene expression data from Vallat and colleagues (21) using Genedatas Expressionist (Genedata AG). These include 12 previously untreated patients and 6 healthy donors. After condensation with Wu's GC-RMA algorithm (22), differentially expressed genes were determined for pathways from the KEGG database[6] using Student's *t* test.[7]

Reagents and cell lines. Curcumin was obtained from Sigma-Aldrich, Piceatannol, SYK Inhibitor II (SYKII), and IV from Calbiochem. R406 was provided to H. Jumaa, Freiburg, Germany by Rigel Pharmaceuticals. All inhibitors were dissolved in DMSO, also used as negative control in the respective concentration. Arabinosyl-2-fluoroadenine (F-ara-A) was from Sigma-Aldrich, SYK antibody from Santa Cruz. Antibodies against phosphorylated and total ERK1/2, STAT3, PLCγ_2, Akt, and secondary horseradish peroxidase–conjugated antibodies were from Cell Signaling. The B-CLL–derived cell line MEC-1 and stromal cell line M2-10B4 were from DSMZ cell collection and American Type Culture Collection, respectively, and cultured in RPMI or Iscove's modified Dulbecco's medium, supplemented with 10% FCS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 2 mmol/L L-glutamine.

Real-time quantitative PCR analysis. One microgram of total RNA isolated from CLL cells and healthy B cells (Qiagen RNeasy Mini kit) was transcribed into first strand cDNA (Qiagen Omniscript RT kit), after quantification and quality control via the Agilent 2100 BioAnalyzer. Real-time PCR was carried out in duplicates using FastStart SYBR green Master Mix and the LightCycler 480 system (Roche). SYK primers were from SuperArray, β-glucoronidase primers were self-designed. C_t values were determined by the second derivative maximum method (23) and relative quantification was performed using the ΔC_t method: $\Delta C_t = C_t(\text{SYK}) - C_t(\beta\text{Glu})$ (24).

Immunoblot analysis. Total cell protein extracted from B cells of patients with CLL and healthy donors were analyzed for the expression of the indicated proteins by immunoblotting as previously described (25). Densitometric analysis of immunoblots was performed using the ImageJ software.

Immunoprecipitation. For immunoprecipitation, equal amounts (5 μg) of protein lysates were incubated with antibodies overnight. After centrifugation and at least three washing steps, the precipitated protein was resuspended in loading buffer and stored at −20°C. Samples were heated to 95°C for 2 min, centrifuged, and the supernatant was analyzed by immunoblotting.

Apoptosis assay. Cells were cultured in RPMI supplemented with 10% FCS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mmol/L L-glutamine at a concentration of 1 to 5×10^6 cells/mL in 96-well plates in the presence or absence of varying doses of the SYK inhibitors at 37°C for 48 h. For CD40 activation, cells were treated with 100 ng/mL sCD154 (Biosource). For coculture experiments, M2-10B4 cells were cultured for 24 h before CLL cells were added. For apoptosis analysis, cells were stained using Guava Viacount (Guava Technologies) or stained with the Annexin-V Apoptosis Detection kit I (BD Biosciences) and assayed by flow cytometry. Results were analyzed with FlowJo and GraphPad Prism software.

Intracellular staining of active Caspase 3 and p-Akt. For active Caspase 3, cells were cultured for 24 h with SYKII, R406, or vehicle control. For p-Akt detection, cells were cultured 72 h after siRNA transfection. Cells were stained for CD19, fixed, and permeabelized (Foxp3 staining kit; eBioscience), stained intracellularly with active Caspase 3 antibody (BD Bioscience) or p-Akt, subsequently with FITC-labeled anti-rabbit $F(ab')_2$ fragment (Jackson Immunoresearch), and analyzed by flow cytometry.

Protein tyrosine kinase activity assay. Tyrosine kinase activity assay (Sigma Aldrich) was performed in duplicates using human recombinant SYK and ZAP70 (R&D Systems). For SYK, a final concentration of 0.77 ng/mL per well was used and, for ZAP70, due to lower enzymatic activity, 1.1 ng/mL. To determine inhibitory capacity, untreated tyrosine kinase activity was defined as 1.

Transfection with SYK siRNA. CLL cells (7×10^6) were resuspended in Nucleofector solution (Nucleofector kit V; Amaxa), containing 1 μmol/L control siRNA or SYK siRNA (Dharmacon), and transfected using the Amaxa Nucleofector program U-013. After 24 h, cells were centrifuged, resuspended in 400-μL culture medium, and plated in a round-bottomed 96-well plate. Viability and SYK expression was determined 72 h after transfection by flow cytometry using a PE-labeled anti-SYK antibody (Santa Cruz) or Annexin-V/PI staining. SYK expression was calculated by ΔMFI = MFI (SYK) − MFI (unstained).

Results

Gene expression profiling identifies enhanced expression of SYK and its pathway in CLL cells versus healthy B cells. The BCR signaling pathway was highly overexpressed in CLL compared with healthy B cells as assessed by gene expression profiling (21). SYK, a central mediator of BCR signaling, was significantly overexpressed in three of four Affymetrix probe sets in CLL patients compared with healthy donors. Because mutated and unmutated CLL differs in BCR signaling, we compared the expression of SYK in mutated and unmutated CLL. Unmutated cases showed a trend for higher SYK mRNA expression compared with mutated CLL samples. In line with SYK overexpression, CLL cells showed enhanced expression of SYK downstream signaling molecules within the BCR signaling pathway including VAV, RAC, PLCγ_2, RAS, AKT, and ERK1/2 (Fig. 1*A*).

Enhanced quantitative gene expression and protein expression of SYK in CLL cells. Real-time PCR using cDNA of 19 CLL patients (14 mutated, 5 unmutated) and 17 healthy donors confirmed the enhanced SYK expression in CLL cells observed by gene expression profiling (Fig. 1*B*). Accordingly, a trend for higher SYK expression in the unmutated CLL samples was detected (data not shown).

SYK protein expression normalized to β actin was ~2-fold higher in CLL samples compared with healthy B-cell samples as assessed by immunoblotting (Fig. 1*C*). The CLL-derived cell line MEC-1 also expressed high SYK levels (Fig. 1*C, right*). CLL cells from unmutated patients revealed a significantly higher SYK protein expression than those from mutated cases (Fig. 1*D*), confirming the trend observed at mRNA level.

Enhanced phosphorylation of SYK and its downstream targets in CLL cells compared with healthy B cells. Because expression of SYK does not provide information about its activity, we analyzed the phosphorylation status of SYK in CLL cells compared with healthy B cells. Direct detection of phosphorylated SYK in whole cell lysates is hampered by the lack of suitable antibodies. We therefore performed phospho-tyrosine immunoprecipitation followed by total SYK immunoblotting. In parallel, we performed a total SYK immunoblot with equal protein amounts as used for immunoprecipitation. To rule out that any effects observed on phospho-SYK resulted from differential expression of total SYK, densitometric ratios were generated between phosphorylated and total SYK. In addition to the higher overall SYK expression levels (Fig. 1*C*), the phosphorylation of SYK in CLL was ~2-fold higher than in normal B cells (Fig. 2*A*). Finally, phosphorylation of PLCγ_2, and signal transducers and

[6] http://www.genome.ad.jp/kegg/

[7] K. Bartholome, C. Kreutz, J. Timmer. Estimating of gene induction enables a relevance-based ranking of genes sets. J Comp Biol. In press 2009.

activators of transcription 3 (STAT3), typical downstream intermediates of SYK, was significantly increased and p-ERK1/2 tended to be increased in CLL cells compared with healthy B cells (Fig. 2*B*).

SYK inhibitors reduce phosphorylation of SYK downstream mediators in CLL cells. To test whether activation of these downstream targets is SYK dependent, we analyzed their phosphorylation pattern in the presence and absence of SYK

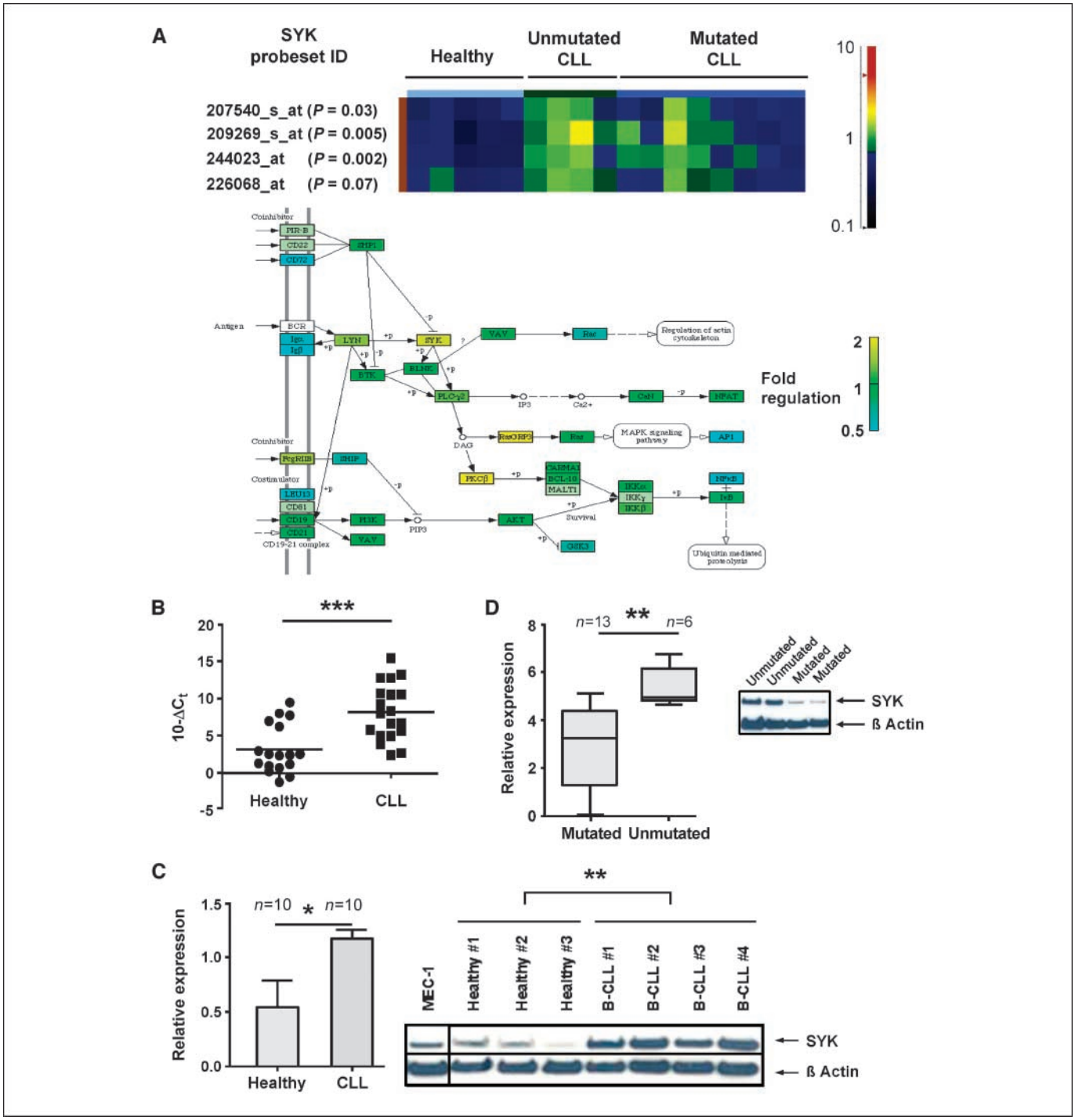

Figure 1. SYK and downstream pathway molecules are differentially expressed in CLL cells compared with healthy B cells. *A, top,* reanalysis of gene expression profiling data revealed four affymetrix probe sets corresponding to the SYK gene with increased expression in CLL cells ($n = 12$) compared with healthy B cells ($n = 6$), and a nonsignificant trend of higher SYK mRNA in unmutated ($n = 4$) CLL compared with mutated ($n = 8$). Signal values were itemwise half Z-normalized. *Bottom,* schematic diagram of SYK-dependent pathway based on the BCR from the KEGG database by averaging all CLL and healthy samples. The color bar denotes fold changes. *B,* total RNA of CLL ($n = 19$; 14 mutated, 5 unmutated) and healthy B cells ($n = 17$) was analyzed for SYK expression by real-time PCR. ***, $P < 0.001$. *C,* SYK protein expression was quantified in lysates of 10 CLL (4 unmutated, 6 mutated) and 10 healthy donors by immunoblotting. *Left,* densitometric results normalized to β actin are shown as mean ± SE. *, $P < 0.05$. *Right,* a representative blot is displayed (CLL samples all mutated). **, $P < 0.01$. *D,* SYK protein expression was quantified in lysates of CLL cells from 19 patients stratified by mutational status (13 mutated, 6 unmutated). *Left,* densitometric results normalized to β actin are shown as median ± quartiles (*box*) ± SE (*bars*); *right,* a representative blot. **, $P < 0.01$.

inhibitors. All inhibitors significantly reduced phosphorylation of PLCγ_2, STAT3, and ERK1/2 in MEC-1 cells (Fig. 2*C*). Similar effects were observed in primary CLL cells (low expression levels prevented analogous analysis of p-PLCγ_2; Fig. 2*D*). MEC-1 cells were analyzed after overnight treatment. Due to the common high rate of apoptosis in extended culture of primary CLL cells, phosphorylation was analyzed after 2 hours of SYK inhibitor treatment (4 μmol/L).

SYK inhibitors induce apoptosis in primary CLL cells. Because increased SYK kinase activity might promote survival of CLL cells, we tested the effect of SYK inhibitors on the viability of CLL cells. Quantification of viable and necrotic cells by Guava Viacount revealed significant cytotoxicity for Curcumin, Piceatannol, SYKII, and SYK Inhibitor IV in primary CLL cells. SYKII showed the highest cytotoxic effect on primary CLL cells, whereas the effect on the viability of healthy B cells was minor (Fig. 3*A*). SYKII achieved a significant decrease in viability at a concentration of as low as 1 μmol/L (Fig. 3*B*). Reduction of viability by SYK inhibition predominantly resulted from increased apoptosis rather than cell necrosis both in CLL and healthy B cells as assessed by flow cytometric quantification of Annexin-V and 7-AAD staining (Fig. 3*C*). This might explain the overall low effects observed in Fig. 3*A* (note that Guava Viacount analysis is unable to detect apoptotic/Annexin-V$^+$/7-AAD$^-$ cells). Because the concentration of 4 μmol/L has been described to be achievable *in vivo* for R406 (26), and SYKII and R406 share equal IC_{50} values regarding SYK inhibition *in vitro* (41 nmol/L), we continued with a concentration of 4 μmol/L. Intracellular staining for active Caspase 3 suggested that induction

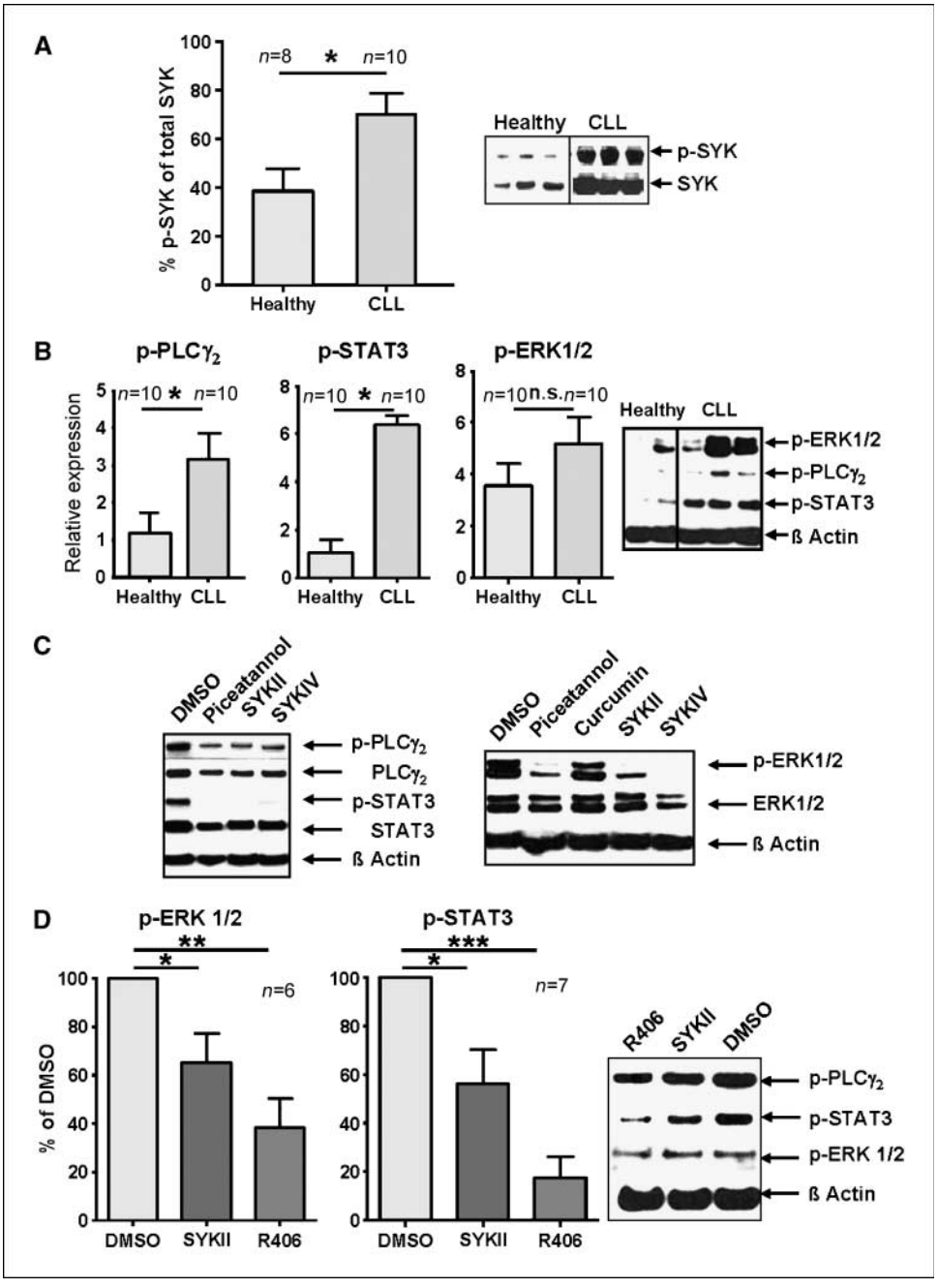

Figure 2. Phosphorylation pattern of SYK and downstream mediators in CLL cells compared with healthy B cells. *A,* phospho-tyrosine immunoprecipitation followed by SYK immunoblotting from 8 healthy donors and 10 CLL samples (6 mutated, 4 unmutated) was performed in parallel with a total SYK immunoblot loaded with equal amounts of protein. *Left,* densitometric results represent % of phosphorylated SYK to total SYK and are given as mean ± SE; *right,* a representative blot is shown. *, $P < 0.05$. *B,* immunoblot analysis of the SYK downstream pathway members phospho-PLCγ_2, phospho-STAT3, and phospho-ERK 1/2 in 10 CLL samples (4 mutated, 6 unmutated) and 10 healthy B-cell samples. *Left,* densitometric results normalized to β actin are shown as mean ± SE; *right,* a representative blot. *, $P < 0.05$; *n.s.,* not significant. *C,* MEC1 cells were incubated with 20 μmol/L Piceatannol, 20 μmol/L SYKII, 10 μmol/L SYKIV, 20 μmol/L Curcumin, or DMSO control overnight and analyzed for the expression of the indicated proteins by immunoblotting. Representative blots are shown. *D,* primary CLL cells ($n \geq 6$) were incubated with 4 μmol/L SYKII, 4 μmol/L R406, or DMSO control for 2 h and analyzed for the indicated proteins by immunoblotting. *Left,* relative expression after densitometric analysis and β actin normalization are shown as mean ± SE; *right,* a representative blot. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

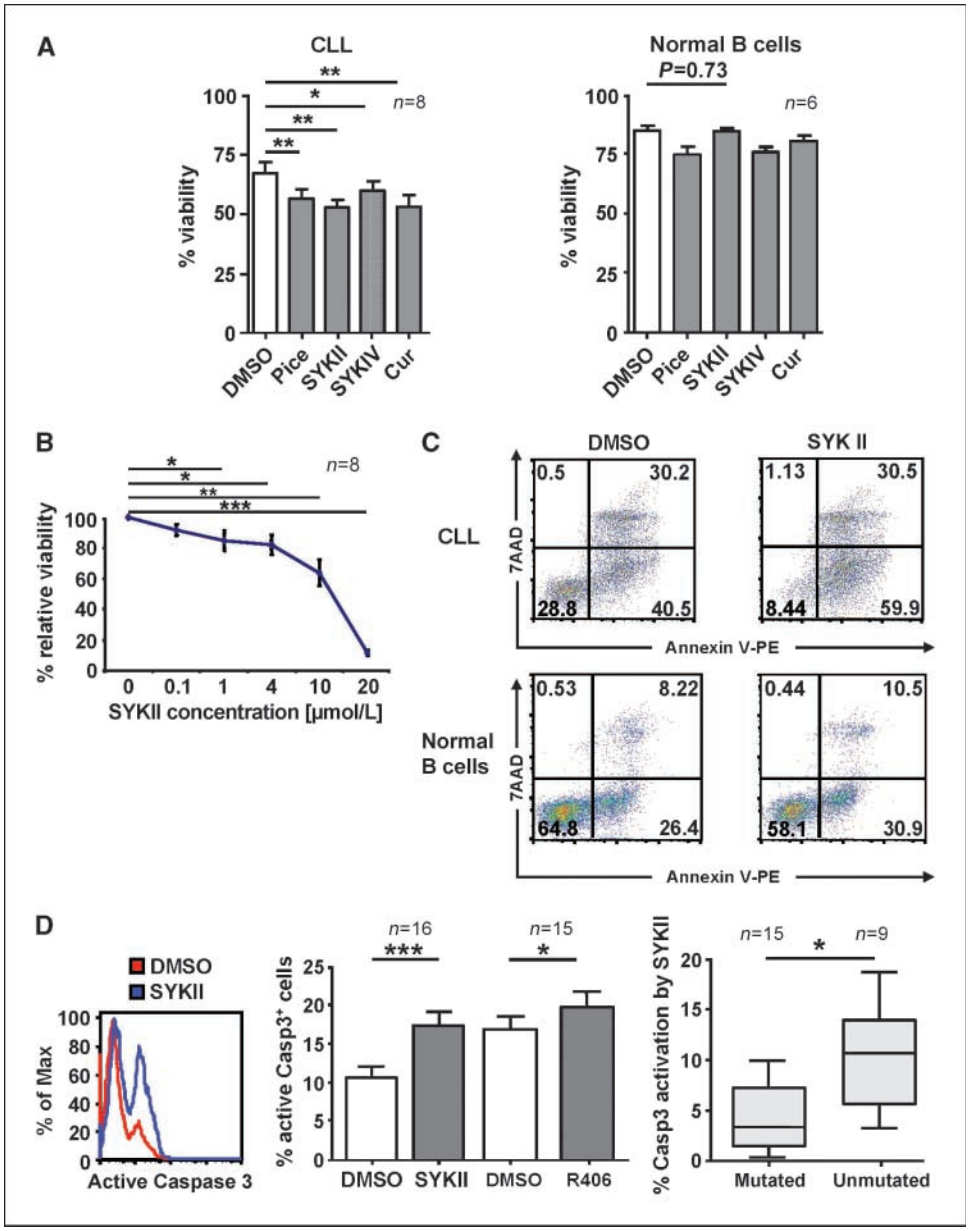

Figure 3. SYK inhibitors induce apoptosis in primary CLL cells. *A,* primary CLL cells (*left,* $n = 8$, 3 unmutated, 5 mutated) and healthy B cells (*right,* $n = 6$) were treated with DMSO control, 20 μmol/L Piceatannol, 20 μmol/L SYKII, 10 μmol/L SYKIV, or 20 μmol/L Curcumin for 48 h before quantification of viability by Guava technologies. Results represent % of viability given as mean ± SE. *, $P < 0.05$; **, $P < 0.01$. *B,* dose response of 8 CLL samples (3 unmutated, 5 mutated) toward indicated concentrations of SYKII inhibitor after 48 h. The viability assessed by Annexin-V^-/PI^- of untreated cells was defined as 100% and results are given as mean ± SE. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. *C,* CLL cells or normal B cells were treated with DMSO control or 4 μmol/L SYKII and analyzed for Annexin-V/7AAD staining by flow cytometry. Representative histograms of 1 of 40 analyzed patients and 1 of 14 analyzed healthy donors are shown. Apoptotic, necrotic, and viable populations are defined as Annexin-V^+/$7AAD^-$, Annexin-V^+/$7AAD^+$, and Annexin-V^-/$7AAD^-$. *D,* primary CLL cells were incubated with SYKII, R406, or vehicle and analyzed for intracellular active Caspase 3 by flow cytometry. *Left,* a representative histogram is shown, followed by the pooled results expressed as mean ± SE of % Caspase 3–positive cells ($n \geq 15$). *Right,* differential induction of Caspase 3 in mutated ($n = 15$) versus unmutated CLL samples ($n = 9$). *, $P < 0.05$.

of apoptosis was Caspase 3 dependent (Fig. 3*D*). Interestingly, SYKII-induced Caspase activation was more effective in unmutated CLL samples than mutated ones (Fig. 3*D, right*). Finally, we treated CLL cells from 38 CLL patients with SYKII (4 and 10 μmol/L) or R406 (4 μmol/L) for 48 h. SYK inhibitors reduced the relative viability of CLL cells to 76% and 44% for SYKII (4 and 10 μmol/L) and 66% for R406 (4 μmol/L), respectively, as assessed by Annexin-V/7AAD staining (Fig. 4*A*).

SYK inhibitor–induced apoptosis correlates with prognostic factors and SYK protein expression. Given the differences in clinical course, the differential SYK expression pattern (Fig. 1*D*), and Caspase 3 activation (Fig. 3*D*) depending on mutational status, we separately analyzed the susceptibility of CLL cells of these patient subgroups to SYK inhibition. CLL cells from unmutated patients showed a significantly higher rate of SYK inhibitor–induced apoptosis corresponding to decreased viability compared with cells of mutated patients. Similar results were obtained when cells were stratified by ZAP70 expression status. These data suggest that particularly patients with a poor prognosis might benefit from SYK inhibition (Fig. 4*B*). In addition, SYK protein expression correlated with cytotoxic effects of SYK inhibition (Fig. 4*C*). These data show that high SYK expression correlates with better response to SYK inhibitor treatment, indicating that SYK expression might predict for SYK inhibitor responsiveness.

Specificity of SYK inhibition with respect to induction of apoptosis. Because ZAP70 and SYK belong to the same protein family and share a high homology, we performed a protein tyrosine kinase activity assay demonstrating that both inhibitors only decreased the kinase activity of SYK but not ZAP70 at the concentrations used (Fig. 5*A*). Still, SYK inhibitors might act via unspecific binding of other kinases. To verify that SYK regulates apoptosis in CLL, we performed SYK gene silencing experiments in CLL cells. Seventy-two hours after transfection, we observed a significant induction of apoptosis along with down-regulation of

SYK protein expression in SYK siRNA-transfected compared with control siRNA-treated CLL cells as assessed by flow cytometry (Fig. 5*B*). In line with our previous findings, we observed a concomitant induction of Caspase 3 (Fig. 5*C*). Finally, in three of three analyzed samples, a reduction of Akt phosphorylation was observed, another SYK downstream signaling molecule (Fig. 5*D*).

SYK inhibitors increase the efficacy of fludarabine. The combination of fludarabine, the most potent cytoreductive agent in CLL, with other drugs enhances its antileukemic effects (27). Therefore, we assessed the *in vitro* effects of SYK inhibitors in combination with the active fludarabine derivate F-ara-A. Cotreatment of F-ara-A with SYKII or R406 significantly decreased viability of CLL cells at low, *in vivo* achievable doses of R406 (Fig. 6*A*). The mean viability of F-ara-A–treated cells was reduced from 47% to 34% by SYKII and to 30% by R406, respectively.

Stromal cell coculture does not influence the effect of SYK inhibitors on CLL cells, but CD40L stimulation sensitizes CLL cells toward SYK inhibition. Because the microenvironment enhances the viability of CLL cells and thereby decreases their sensitivity toward chemotherapy (28), we assayed apoptosis rates of CLL cells incubated with SYKII in the presence and absence of the stromal cell line M2-10B4. Stromal coculture protected CLL cells against spontaneous apoptosis (data not shown). However, the presence of stromal cells did not limit SYK inhibitor-induced cytotoxicity of CLL cells (Fig. 6*B*).

In proliferative centers, CD40L-expressing T cells represent an important feature of the CLL microenvironment (29). Therefore, we analyzed the effect of SYKII in combination with CD40 ligation. Stimulation with CD40L (100 ng/mL) significantly increased SYKII-mediated cytotoxicity (Fig. 6*C*), suggesting that SYK inhibitors might ensure potential targeting of both, CD40 stimulated, proliferating and resting CLL cells.

Discussion

The signal transducer SYK is a crucial factor for function and survival of B cells. In the absence of SYK, B-cell maturation is blocked at the early pro–B-cell state in murine knockout models (30). Furthermore, inducible depletion of Igα, the linker between the BCR and its signaling cascade, results in rapid death of mature B cells in mice, revealing the importance of BCR signaling for B-cell survival independent of antigen contact (31). Moreover, SYK

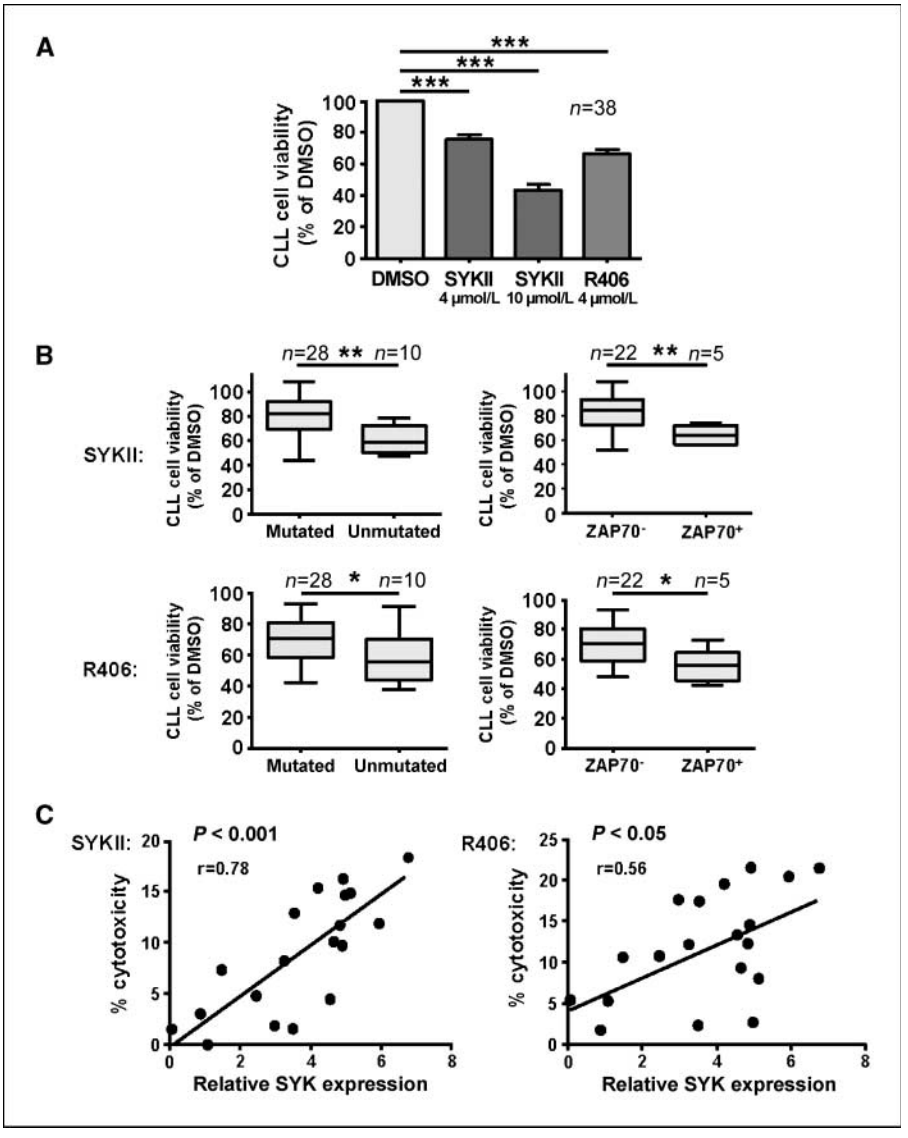

Figure 4. Apoptosis induction correlates with mutational status, ZAP70, and SYK protein expression. *A,* CLL cells ($n = 38$) were treated with SYKII (4 and 10 μmol/L) or R406 (4 μmol/L) for 48 h, and viability was assessed by flow cytometry using Annexin-V/7AAD staining. *Columns,* mean % of vehicle control; *bars,* SE. ***, $P < 0.0001$ (one-way ANOVA with Bonferroni correction). *B,* CLL cell viability (relative to DMSO) was assessed after 48 h treatment with 4 μmol/L SYKII (*top*) or with 4 μmol/L R406 (*bottom*) in mutated ($n = 28$) versus unmutated ($n = 10$) CLL samples (*left*), and in ZAP70$^+$ ($n = 22$) versus ZAP70$^-$ ($n = 5$) CLL samples as quantified under *A*. Results are given as median ± quartiles (*box*) ± SE (*bars*). *, $P < 0.05$; **, $P < 0.01$. *C,* densitometric analysis of SYK immunoblotting versus analysis of the cytotoxic effect of SYKII (*left*) and R406 as assessed under *A* revealed significant correlation with a Pearson coefficient of r = 0.78 and r = 0.56, respectively. Cytotoxicity is defined as difference of viability of control-treated cells and viability of SYKII- or R406-treated (4 μmol/L) cells.

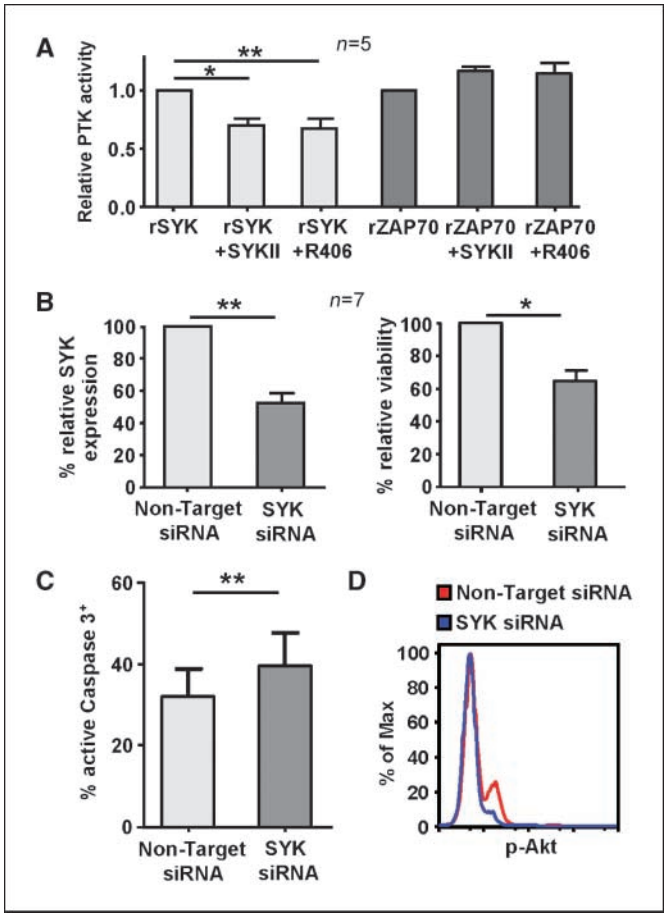

Figure 5. Validation of SYK inhibitor specificity and SYK gene silencing. *A,* protein tyrosine kinase activity assay performed with human recombinant SYK (*rSYK*) and ZAP70 (*rZAP70*) in the absence and presence of 4 μmol/L R406 or SYKII (*columns,* mean of five independent experiments; *bars,* SE). For relative activity absorption without inhibition was defined as 1. *B,* primary CLL cells (7×10^6, respectively) were transfected with control siRNA or SYK-specific siRNA. After 72 h of culture, viability and SYK expression were determined by flow cytometry ($n = 7$). SYK expression and viability of control siRNA-transfected cells was defined as 100%. *C,* after siRNA transfection and 72 h of culture, active Caspase 3 expression was determined by flow cytometry ($n = 7$). *Columns,* mean; *bars,* SE (*A–C*). *, $P < 0.05$; **, $P < 0.01$. *D,* cells treated as described under *C* were analyzed for p-Akt by flow cytometry. A representative histogram is shown ($n = 3$).

formally acts as a proto-oncogene and is suggested to be involved in tumorgenesis of diverse hematologic malignancies including mantle cell lymphoma, diffuse large B cell lymphoma (DLBCL), lymphoblastic leukemia, and follicular lymphoma (26, 32–34). Here, we hypothesized that SYK plays a crucial role in CLL by transmitting BCR-mediated signals to the dependent downstream signal transduction pathways.

In contrast to a previous report claiming normal SYK expression in CLL compared with normal B cells (35), but lacking quantitative analysis, we show SYK overexpression in CLL compared with healthy B cells. SYK protein expression was enhanced in unmutated compared with mutated CLL, potentially reflecting the increased BCR signaling in unmutated CLL reported by others (6–9). In accordance with its role for tonic BCR signaling, active SYK is crucial for DLBCL cell survival (26). In line with these data, all tested SYK inhibitors resulted in apoptosis induction and reduction of phosphorylation of downstream targets in CLL in this study. SYK inhibitor treatment also affected normal B cells. However, we observed a nearly 2-fold higher expression along with an ~2-fold higher phosphorylation status of SYK in CLL compared with healthy B cells, suggesting that SYK inhibition, although not fully CLL specific, may be feasible in CLL. In fact, the prodrug of R406, R788 (Fostamatinib disodium, FosD), an orally available SYK inhibitor under development for rheumatoid arthritis, showed significant *in vitro* activity against BCR-dependent non–Hodgkin lymphomas (26) and, very recently, even high clinical activity in patients with relapsed/refractory CLL. CLL/SLL patients showed an overall response rate of 54%, whereas other B-cell malignancies included in the study such as DLBCL (21% overall response rate), follicular lymphoma (10%), and mantle cell lymphoma (11%) showed considerably lower response rates (36), lending direct *in vivo* evidence to the concept of SYK inhibition in CLL.

Our data may contribute to the understanding of these effects. We observed a decrease of CLL cell viability along with an increase

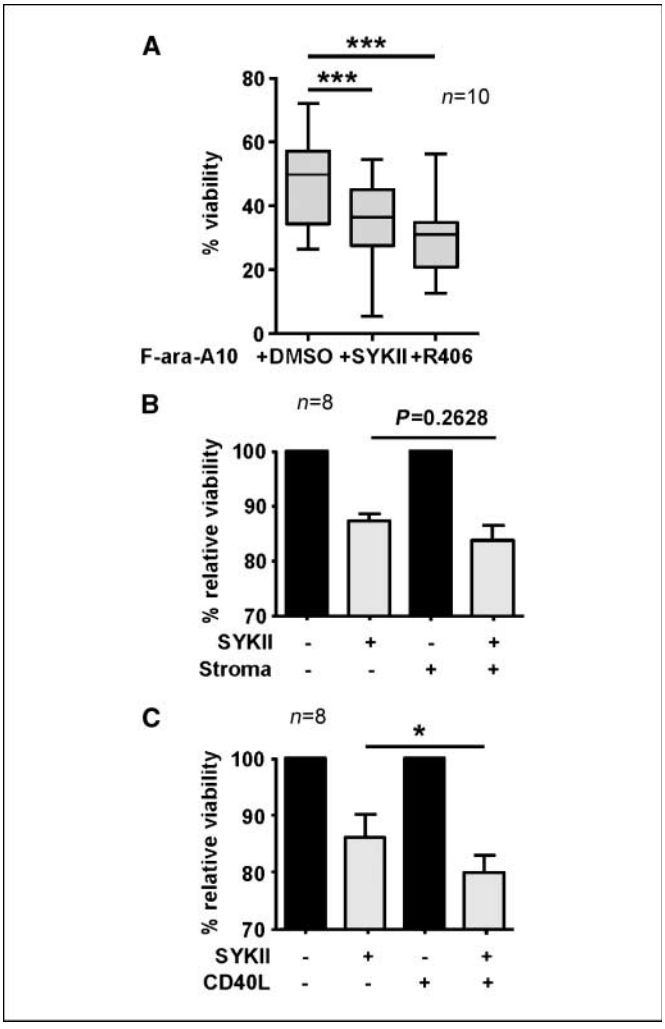

Figure 6. SYK inhibitors and the influence of chemotherapy and microenvironment. *A,* viability of primary CLL cells ($n = 10$, 5 mutated, 5 unmutated, median ± quartiles ± SE) after treatment with F-ara-A (10 μmol/L), DMSO control, or the combination of F-ara-A with SYKII (4 μmol/L) or R406 (4 μmol/L) as assessed by Annexin-V/7AAD staining. ***, $P < 0.0001$. *B,* viability of CLL cells ($n = 8$, 3 unmutated, 5 mutated; *columns,* mean; *bars,* SE) as assessed under *A*, cultured with or without stromal cells for 48 h in the presence or absence of SYKII (20 μmol/L). *C,* viability of CLL cells ($n = 8$; *columns,* mean; *bars,* SE) cultured with or without sCD40L for 48 h in the presence or absence of SYKII (20 μmol/L). *, $P < 0.05$.

of Caspase 3 activation in CLL cells upon treatment with SYKII or R406. Importantly, these events correlated with prognostic factors suggesting that SYK targeting may be most effective in patients with worst prognosis. Recently, a similar correlation was reported for the treatment of CLL cells with the SRC and ABL kinase inhibitor Dasatinib (37). Other studies showed elevated levels of SYK in cases with high ZAP70 expression, arguing for a synergistic role of ZAP70 and SYK in initial BCR signaling (38).

The enhanced sensitivity of $ZAP70^+$ CLL samples and the homology of SYK and ZAP70 raise the possibility that SYK inhibitors also interact with ZAP70 kinase activity. Such cross-inhibition is, however, highly unlikely because SYK but not ZAP70 activity was significantly reduced by both SYK inhibitors in a protein tyrosine kinase activity assay. However, the inhibitors tested may interfere with other molecules. In non–Hodgkin lymphoma cell lines, SYK but not Janus-activated kinase has been found to be the relevant target to inhibit proliferation and survival by comparing effects of specific SYK inhibitors and a bispecific SYK- and JAK-inhibitor (39). Expression of the constitutively active TEL-SYK in BaF3 cells induced Akt and ERK phosphorylation, an effect abrogated by R406 treatment (40). Finally, our gene silencing experiments provide direct evidence for SYK-dependent regulation of apoptosis in CLL cells. Taken together, although off target effects cannot be completely excluded, our data indicate that SYK inhibition is the most likely mechanism underlying apoptosis induction in CLL cells by SYKII and R406.

Fludarabine represents an efficient therapy for CLL but still bears disadvantages in terms of frequent relapses and development of fludarabine resistance (41). Fludarabine-based combination therapies offer superior therapeutic efficacy (42). With respect to molecularly targeted fludarabine combinations, the tyrosine kinase inhibitor genisteine increased induction of apoptosis in CLL compared with fludarabine alone (43). Because fludarabine treatment results in dephosphorylation of STAT1 (44), whereas SYK inhibition leads to STAT3 dephosphorylation (Fig. 2*D*), we hypothesized that SYK inhibition might represent a rational combination partner for fludarabine. Activated STAT1 and STAT3 can dimerize and modulate the expression of target genes related to prosurvival or antiapoptotic effects (45). Moreover, unmutated patients with early requirement for therapeutic intervention carry a substantially higher risk for the development of fludarabine resistance (46). Because these prognostically poor cases have higher sensitivity to SYK inhibitors, the combination of SYK inhibitors and fludarabine may have the potential to prevent development of resistance. We indeed observed that the simultaneous exposure to fludarabine and SYK inhibitor II or R406 resulted in synergistic apoptotic effects on primary CLL cells.

Interactions between CLL cells and the microenvironment exhibit prolonged cell survival, growth advantage, and drug resistance to malignant CLL cells. For fludarabine, reduced cytotoxicity on CLL cells in stromal coculture is described (28). Here, we observed no protective effect by stromal cell contact that would limit the efficacy of SYK inhibition in CLL cells. The absent protective effect for SYK inhibitors might be due to involvement of SYK in the cross-talk between stroma and CLL cells, as SYK was recently described to be involved in integrin signaling (47). CD40L-expressing $CD4^+$ T cells provide important proliferation stimuli in proliferative centers in CLL (29) and may rescue CLL cells from apoptosis (48). In contrast to fludarabine (49, 50), CD40 ligation sensitized CLL cells toward SYKII, potentially suggesting that combined treatment might efficiently target both, proliferating and resting CLL cells.

In summary, this work supports the concept of SYK inhibition as therapeutic strategy in CLL. Our data provide mechanistic understanding of the therapeutic effects of SYK inhibition recently observed in CLL, and warrant further clinical assessment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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