Targeting the LYN/HS1 signaling axis in chronic lymphocytic leukemia

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LYN kinase, a major player in BCR signaling initiation, was shown to be overexpressed and active in leukemic B cells. Following BCR engagement, LYN is activated by phosphorylation on tyrosine(Y)396 and serves as a positive regulator of the immunoreceptor tyrosine-based activation motifs within the BCR Igα/CD79a and Igβ/CD79b subunits. This step is crucial for signal transduction to proximal partners (eg, SYK kinase), cytoskeletal activators (eg, VAV1), calcium signaling modulators (eg, PLCγ2), and downstream targets (eg, ERK kinase). LYN may also negatively regulate BCR signaling by phosphorylating inhibitory cell surface receptors (eg, CD22 and CD5) and by recruiting phosphatases such as SHP1/2. Therefore, LYN fine-tunes positive and negative signaling pathways upon BCR engagement.

HS1 is a downstream kinase HS1 (hematopoietic cell-specific Lyn substrate-1) is a downstream kinase whose phosphorylation correlates with prognosis in Chronic Lymphocytic Leukemia (CLL). The differently phosphorylated sites and the kinases that regulate HS1 activity in CLL remain poorly understood. We demonstrate that HS1 kinase is differentially regulated by LYN kinase that, in a subset of patients, phosphorylates HS1 on Tyrosine (Y)397, resulting in its activation. This correlates with increased cytoskeletal functionality in terms of migration, adhesion and F-actin polymerization. In these patients, LYN is also activated on Y396 residue and its inhibition with the tyrosine kinase inhibitor Dasatinib abrogates HS1-Y397 phosphorylation. This results in the reduction of HS1 activation along with that of cytoskeletal effector VAV1 and the downstream kinase ERK also in the presence of BCR and CXC chemokine receptor CXCR4 stimulation. Interestingly, targeting the LYN/HS1 axis in vitro leads to the concomitant reduction of cytoskeletal activity, BCR signaling and cell survival in the subset of patients with activated LYN/HS1.

In a transplantable mouse model based on the Eμ-TCL1 transgenic mouse, LYN/HS1 signaling inhibition interferes with CLL progression and lymphoid organ infiltration. Thus LYN/HS1 axis marks distinct signaling profiles and cytoskeletal-related features that may represent valuable targets for cytoskeleton-targeted therapeutic intervention in CLL. (Blood. 2013;121(12):2264-2273)

Introduction

Chronic lymphocytic leukemia (CLL) monoclonal CD5+ B cells accumulate in peripheral lymphoid organs and bone marrow (BM) and flow into the peripheral blood (PB). Central to CLL pathogenesis is the activation of B-cell receptor (BCR) signaling, though the nature of the implicated antigen and the mechanisms of BCR stimulation are still unclear. In addition, the most prominent pathway activated in lymph nodes of CLL patients is that originating from BCR signaling, and BCR responsiveness of CLL cells segregates with known prognostic factors. CLL cases with mutated immunoglobulin heavy chain variable (IGHV) genes, low CD38 expression, and good prognosis show high constitutive phosphorylation of BCR signaling molecules and are unresponsive to BCR cross-linking, thus resembling B cells anergized in vivo after chronic antigenic stimulation. In contrast, IGHV unmutated/CD38-positive cases show basally reduced constitutive phosphorylation of the signaling machinery, which can be activated upon surface immunoglobulin M (IgM) triggering.

LYN kinase, a major player in BCR signaling initiation, was described to be overexpressed and active in leukemia B cells. Following BCR engagement, LYN is activated by phosphorylation on tyrosine(Y)396 and serves as a positive regulator of the downstream kinase Y397 and Y378 activatory residues that mediate phosphorylation of BCR signaling molecules and are unresponsive to BCR cross-linking, thus resembling B cells anergized in vivo after chronic antigenic stimulation. In contrast, IGHV unmutated/CD38-positive cases show basally reduced constitutive phosphorylation of the signaling machinery, which can be activated upon surface immunoglobulin M (IgM) triggering.

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2264 BLOOD, 21 MARCH 2013 • VOLUME 121, NUMBER 12

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(HS\textsuperscript{hyper-p}) with poor outcome.\textsuperscript{21} Though the nature of the differently phosphorylated sites and the kinases responsible for the regulation of HS1 activity still remain to be understood, mouse models of human CLL\textsuperscript{22} provided evidence that HS1\textsuperscript{hyper-p} may lead to protein inactivation, as HS1 knockout/knockdown CLL cells show aggressive functional features similar to those in patients with HS1\textsuperscript{hyper-p} cells. Collectively these findings suggest that HS1 phosphorylation has multiple layers of regulation, although the fine-tuning of HS1 function in CLL remains to be understood.

The aim of the present study is twofold: to functionally characterize HS1 phosphorylation and its signaling partners and to exploit the molecule as a therapeutic target in CLL. We show that HS1 phosphorylation is associated with a LYN kinase-centered BCR signaling signature and a distinct cytoskeletal functionality. We also show that targeting the LYN/HS1 signaling axis with the tyrosine kinase inhibitor dasatinib in vitro affects both survival and cytoskeletal activity in patients carrying a distinct LYN/HS1 phospho-signature and, in vivo, it delays CLL progression in the HS1\textsuperscript{hyper-p} cell line.

Taken into account the densitometric analysis of LYN-Y396 and HS1-Y397 phosphorylations and 2DE-detected HS1 status, we grouped patients in “active-HS1” and “inactive-HS1.” The cutoff for LYN-Y396 and HS1-Y397 positivity was set to OD 0.31 and OD 0.49, respectively, based on median OD values. The patients carrying at least 2 of the biochemical features (LYN-Y396/HS1-Y397/2DE-detected HS1\textsuperscript{hyper-p}) were arbitraly classified as active-HS1, while those carrying at least 2 of the biochemical features (LYN-Y396/HS1-Y397/2DE-detected HS1\textsuperscript{hyper-p}) were classified as inactive-HS1 (supplemental Table 2).

**Materials and methods**

Primary CLL samples were obtained after informed consent as approved by the Institutional Ethical Committee (protocol number 360) of San Raffaele Scientific Institute (Milan, Italy) in accordance with the Declaration of Helsinki.

EpTCL1 mice on a C57BL/6 background and C57BL/6 wild-type mice were maintained in a specific pathogen–free animal facility and treated in accordance with the European Union guidelines and with the approval of the Institutional Ethical Committee (protocol number 536) of the San Raffaele Scientific Institute (Milan, Italy).

For additional information on experimental procedures, see supplemental Methods.

**Human primary samples**

Leukemic CD19 cells were negatively selected from fresh PB using the RosetteSep B-lymphocyte enrichment kit (StemCell Technologies). Details are found in supplemental Methods and supplemental Table 1.

**Cell viability testing reagents**

Cell viability was assessed by CellTiter-Glo chemoluminescence assay (Promega) and by annexinV–fluorescein isothiocyanate and propidium iodide staining (BD Biosciences) followed by cytofluorimetric analysis (FCS500; Beckman Coulter). Dasatinib (LC Laboratories) was resuspended in dimethylsulfoxide (Sigma-Aldrich) and stored at −20°C.

**Biochemical studies**

Cells were lysed with RIPA buffer (Sigma-Aldrich) with fresh protease and phosphatase inhibitors cocktail (Roche). Immunoprecipitation (IP) and western blot (WB) were performed as described in Scielzo et al.\textsuperscript{22} For IP, 500 μg of proteins were incubated overnight at 4°C with primary antibody, then immobilized on Protein G MicroBeads (Miltenyi Biotec) for 1 hour at 4°C. Immunoprecipitated proteins were purified by 20 μm MACS cell separation columns (Miltenyi Biotec) and eluted in Laemmli buffer (50 mM TrisHCl, pH 6.8; 1% wt/vol sodium dodecyl sulfate [SDS]; 0.005% wt/vol bromophenol blue; 10% vol/vol glycerol; and 50 mM fresh dithiothreitol [DTT]). After SDS polyacrylamide gel electrophoresis, detection was performed with primary antibodies (a full list is provided in supplemental Methods) and TrueBlot secondary antibodies (eBioscience).

For WB, 30 μg proteins were used, and horse-radish-peroxidase–linked secondary antibodies (Santa Cruz Biotechnology) were used for detection.

Bidimensional electrophoresis (2DE) was performed as described in Scielzo et al.\textsuperscript{21}

**Densitometric analysis**

Densitometric analyses of WB data were performed by Personal SI Densitometer laser (GE Healthcare) and ImageQuant software (GE Healthcare). Optical density (OD) values were calculated as the ratio between total phosphorylation and total protein levels.

The distribution of the 2DE-detected HS1 phosphorylated spots before and after dasatinib treatment was evaluated by Progenesis PG240 software (Nonlinear Dynamics) analysis of 2D spots OD, which in turn was normalized as a percentage of the total HS1 signal.

Migration and F-actin polymerization assays

Chemotaxis assays were performed as described in Scielzo et al.\textsuperscript{22} Briefly, 5 × 10⁵ CLL cells were seeded on the upper chamber of a transwell (6.5-mm diameter, 0.5-μm pore size, Corning), in the presence or absence of 100 nM dasatinib. Next, 200 ng/mL CXC chemokine ligand CXCL12 (PeproTech) was added to the lower chamber. After 4 hours of incubation at 37°C, the cells migrated to the lower part of the chamber, were flow cytometer counted for 1 minute. The migration index was calculated as (number of cells migrated toward CXCL12 − number of cells migrated spontaneously) × 100/(total number of cells).

F-actin polymerization was measured as described in Ponader.\textsuperscript{23} Briefly, 2 × 10⁶ CLL cells were serum starved for 2 hours, treated or not treated with 100 nM dasatinib for 1 hour, and then transferred to 5-mL polystyrene tubes. Next, 200 ng/mL CXCL12 (PeproTech) was added to each tube for 15 seconds, and then a solution containing 20 μM fluorescein isothiocyanate–phalloidin (Sigma-Aldrich), 0.5mg/mL 1-α-lysophosphatidylcholine (Sigma-Aldrich), and 1% vol/vol paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline (PBS, Euroclone) was added to stop the reaction and fix the cells. Cells were additionally incubated for 20 minutes at 37°C, then analyzed with a flow cytometer. The percentage of F-actin polymerization was evaluated as the ratio between the mean fluorescence intensity (MFI) of the CXCL12-stimulated sample and the control MFI. The migration index and the F-actin polymerization after dasatinib treatment were calculated as the percentage of the untreated control.

**Adhesion assays**

Spontaneous adhesion assays were performed as described in Scielzo et al.\textsuperscript{22} Briefly, 5 × 10⁵ cells were labeled with 1 μM CellTracker Green CMFDA (5-chloromethylfluorescein diacetate; Invitrogen) at 37°C for 30 minutes. A 1-hour pretreatment with 100 nM dasatinib was performed, when indicated. Cells were added to a flat-bottomed, 96-well plate precoated with 2% wt/vol bovine serum albumin (BSA, Sigma-Aldrich) in PBS. After 1 hour of incubation at 37°C, adhesion was quantified using a VICTOR \textsuperscript{3} (PerkinElmer) plate reader. Total input was read first, and then plates were read once after repeated washes with 0.5% wt/vol BSA in PBS. The percentage of adhesion was calculated as the ratio of the MFI after 2 washes and the initial total input MFI.

To assess stromal cell adhesion, 1.5 × 10⁵ HS5 or OP9 stromal cells per well were seeded the day before on 48-well plates. Next, 5 × 10⁵ CLL cells were incubated at 37°C, adhesion was quantified after 1 hour, and then plates were read once after repeated washes with 0.5% wt/vol BSA in PBS. The percentage of adhesion was calculated as the ratio of the MFI after 2 washes and the initial total input MFI.
were labeled for 30 minutes at 37°C with 1 μM CMFDA, then added to the stroma monolayer. After overnight culture with or without 100 nM dasatinib, cells were subdivided into 3 different fractions and counted by flow cytometry for 1-minute: fraction 1, culture supernatant; fraction 2, cells washed off with 3 × 1 mL PBS; and fraction 3, cells recovered after 1 minute of trypsinization. The percentage of adhesion was evaluated as the number of cells in fraction 3/the number of cells (fraction 2 + fraction 3). Adhesion values to BSA- or HS5-coated plates after dasatinib treatment were calculated as the percentage of the untreated control.

### TCL1 transplantable model and in vivo dasatinib administration

Eight-week-old C57BL/6 female mice (Charles River Laboratories) were injected intraperitoneally with 10⁵ cells purified from the spleen (SP) of a 12-month-old EμTCL1 leukemic mouse (on a C57BL/6 background) by the EasySep B-cell enrichment kit (Voden). The purity of transplanted CD19⁺ CD5⁻ Igκ⁺ cells, checked by flow cytometry, was 99%. Mice were monitored weekly for leukemia development by flow cytometric analysis of the PB samples. Treatment was initiated 6 weeks after transplant (day 0) when a mean of 25% (range: 10%-60%) of CD19⁺ CD5⁻ Igκ⁺ cells was detected in the PB of transplanted mice. Dasatinib dissolved in propylene glycol/water (1:1) was administered at a concentration of 25 mg/kg per day by oral gavage on a 5-days-on/2-days-off schedule for 4 weeks. The vehicle control group was given propylene glycol/water with the same schedule. During treatment, blood was drawn weekly to monitor disease progression (day 6, day 13, day 20). The ratio between the percentage of CD19⁺ CD5⁻ Igκ⁺ at each time point after treatment started (day 6, day 13, day 20, day of euthanasia) and the percentage of CD19⁺ CD5⁻ Igκ⁺ at day 0 were evaluated to represent leukemia progression in the PB during treatment. At the time of euthanasia, PB, SPs, BM, mesenteric lymph nodes (MESLN), and peritoneal exudates were analyzed by flow cytometry. Full counts of white blood cells in each lymphoid compartment were measured by Coulter Ac-T Diff (Beckman Coulter). Log-transformed absolute counts of CD19⁺ CD5⁺ Igκ⁺ cells were calculated to represent leukemic-cell organ infiltration.

### Statistical analysis

Data were compared using either the paired or unpaired Student t tests or the nonparametric Mann-Whitney U test. In the mouse transplantation experiments, a repeated measures analysis of variance (RM-ANOVA) compared PB values at each time point following the start of treatment. A Bonferroni adjustment was used for pairwise comparisons within the ANOVA model. All statistical analyses were performed using GraphPad Prism (version 5.0d).

### Results

**HS1 activity is differentially regulated by LYN kinase in CLL subsets**

We first aimed at defining the nature of the differentially phosphorylated sites and of the still unknown kinases responsible for HS1 phosphorylation in CLL primary cells. We originally detected HS1 differential phosphorylation by 2DE analysis, which allows only a semiquantitative evaluation of the amount of total HS1 phosphorylation (either HS1hypo-p or HS1hyper-p), but provides no specific information on the activity of the protein or on the phosphorylation sites. To this end, we previously demonstrated that silencing the protein by knockdown (KD) in the MEC1 cell line leads to an aggressive functional behavior similar to that of cells from HS1hyper-p patients, thus suggesting an inactivating role for HS1 hyperphosphorylation.

Starting from this evidence, we took advantage of the HS1-KD cell line as a tool to study BCR signaling molecules in association with HS1 status in CLL, and we showed by IP that, in the absence of HS1 (and of its activity), total LYN tyrosine phosphorylation is reduced as compared with wild-type control transfected (CNTR) MEC1 cells (Figure 1A). Other signaling molecules tested (ie, SYK and PLCγ2) were not affected (data not shown).

Given the functional similarity between HS1hypo-p and HS1-KD cells, we tested whether, in primary CLL samples, the different HS1 status as detected by 2DE gels also might be associated with total LYN phosphorylation. The analysis of CLL cells from 63 patients...
(supplemental Tables 1 and 2) showed a very significant differential expression of LYN kinase phosphorylation in the 2 subsets of patients, with high LYN phosphorylation in HS1hyp-p as compared with low levels in HS1hyper-p patients ($P = .008$) (Figure 1B-C). In line with this, it was not unexpected that, similar to HS1hyp-p,21 high LYN phosphorylation is associated with favorable clinical/biological parameters and correlates with a more benign clinical course ($P = .03$), mutated IGHV genes ($P = .02$), and increased progression-free survival ($P = .0001$) and treatment-free survival ($P = .0003$) (supplemental Figure 1).

The concomitant presence of high levels of phosphorylated LYN and low levels of 2DE-detected HS1 phosphorylation appeared counterintuitive. For this reason, we studied LYN and HS1 activation status by WB using specific antibodies against the activatory residues, and we observed, in 40 primary samples, an association between LYN phosphorylation on the activatory residue Y396 and HS1 phosphorylation on the activatory residue Y397 ($P = .03$, Spearman rank correlation: 0.63).

Interestingly, the presence of HS1 phosphorylation on the Y397 activatory site inversely correlated with the total phosphorylation of the molecule as detected by 2DE; that is, HS1hyp-p cells showed increased Y397 phosphorylation ($P = .01$) (Figure 2C), which also correlated to a consistent increase in LYN-Y396 activation ($P = .0007$) (Figure 2D). On the other hand, 2DE-detected HS1hyper-p cells show low activation of both LYN and HS1 proteins (Figure 2C-D). Differential activation of LYN kinase was also confirmed and quantified through enzyme-linked immunosorbent assay (supplemental Figure 2).

These results demonstrate that, in HS1hyp-p cells, HS1 molecule is likely inactive not only because of the inactivatory phosphorylation on an unknown residue as shown in 2DE gels,22 but also because of a lack in the classic activation on residue Y397 by LYN kinase. For this reason, we will refer to the patients carrying at least 2 of these biochemical features (LYN-Y396/HS1-Y397/2DE-detected HS1hyper-p) as inactive-HS1 (supplemental Table 2).

Conversely, in HS1hyp-p cells, the molecule is more active because of the low inactivatory phosphorylation (shown by 2DE gels) as well as because of the high level of phosphorylation on LYN-targeted Y397 residue. Similarly, we will refer to the subsets...
inhibitor dasatinib, which targets LYN, among other kinases. We performed OD analysis of HS1 protein spots before and after treatment in 3 active-HS1 and 3 inactive-HS1 samples (Figure 3). The OD of the hyperphosphorylated protein spot (right spot) only in active-HS1 control samples (A), with a relative increase in the OD of the less phosphorylated protein spot (left spot) in inactive-HS1 control samples (B) after treatment, with a relative increase in the OD of the less phosphorylated spot (P = .03) (left spot) after treatment, with a relative increase in the OD of the less phosphorylated spot (P = .03) (left spot) in inactive-HS1 cells was not significantly affected by LYN inhibition (Figure 3B-D). This analysis demonstrates that LYN is responsible for total HS1 phosphorylation exclusively in the subset of active-HS1 patients, further confirming that 2DE-detected protein hyperphosphorylation in inactive-HS1 cells is dependent on other kinases.

Total HS1 phosphorylation is reduced following LYN inhibition in a subset of CLL patients

To test in the 2 subsets of patients (inactive and active-HS1) whether LYN inhibition would modify the phosphorylation status of HS1, as detected by 2DE, we took advantage of the dual SRC/BCR-ABL inhibitor dasatinib, which targets LYN, among other kinases. We performed OD analysis of HS1 protein spots before and after treatment in 3 active-HS1 and 3 inactive-HS1 samples (Figure 3) and observed that LYN inhibition by 100 nM dasatinib reduced the OD of the more phosphorylated spot (P = .03) (left spot) after treatment, with a relative increase in the OD of the less phosphorylated spot (P = .03) (right spot) only in active-HS1 samples (Figure 3A-C). The OD of the hyperphosphorylated protein spot (left spot) in inactive-HS1 cells was not significantly affected by LYN inhibition (Figure 3B-D). This analysis demonstrates that LYN is responsible for total HS1 phosphorylation exclusively in the subset of active-HS1 patients, further confirming that 2DE-detected protein hyperphosphorylation in inactive-HS1 cells is dependent on other kinases.

LYN inhibition abrogates HS1-Y397 phosphorylation and reduces VAV1 and ERK activation

To further explore the effect of LYN inhibition, not only on HS1 but also on other BCR signaling molecules, we analyzed the activation of HS1-Y397, of the cytoskeletal effector VAV1 and of the downstream kinase ERK, along with upstream positive (ie, SYK, PLCγ2) and negative (ie, SHP1, CSK) regulators of BCR signaling in the absence or the presence of 100 nM dasatinib on 6 primary CLL samples. In order to test the effect of microenvironental-like stimuli, we used anti-IgM to activate the BCR or CXCL12 to mimic CLL cell stimulation by stromal cells via CXCR4. LYN kinase inhibition completely blocked LYN-Y396, HS1-Y397, and VAV1 activation (Figure 4), both in the absence or the presence of BCR or CXCR4 stimulation. The complete dephosphorylation of LYN, HS1, and VAV1 activatory residues by dasatinib was consistently observed in all cases, regardless the basal level of phosphorylation or the levels reached following IgM or CXCR4 stimulation. Of interest, ERK kinase phosphorylation (Figure 4) was reduced by LYN inhibition, also in the presence of IgM stimulation, but partially rescued by CXCL12, suggesting that CXCR4 signaling may contribute to ERK activation in a BCR-independent way. Little, if any, effect on SYK, PLCγ2, SHP1, and the phosphorylation of the inactivatory residue LYN-Y507 by CSK kinase was observed (supplemental Figure 3) (supplemental Figure 3) was observed.

Thus, LYN inhibition may be regarded as a valuable tool for affecting the cytoskeletal functionality of CLL cells, through the blocking of HS1 and VAV1 and it is also effective in the presence of microenvironental stimuli.

Targeting the LYN/HS1 axis affects the cytoskeletal functionality and cell viability of a sizable portion of CLL patients

Active-HS1 cells migrate toward CXCL12 (P = .007) (supplemental Figure 4A), polymerize F-actin (P = .01) (supplemental Figure 4B), and adhere to BSA-coated plates (P = .03) (supplemental Figure 4C) and to the BM-derived stromal cell line HS5 (P = .001) (supplemental Figure 4D) significantly more than inactive-HS1 cells do. These opposite behaviors are mirrored by a differential HS1 protein localization, as revealed by confocal microscopy of 21 primary patient samples (10 active-HS1 and 11 inactive-HS1). Uniformly distributed cellular protrusions fail to form in the presence of inactive-HS1 protein (in 11/11 cases) (supplemental Figure 4E).
Dasatinib
\(\alpha\)-IgM
CXCL12
\(\alpha\) - P - LYN (Y396)
\(\alpha\) - P - HS1 (Y397)
\(\alpha\) - P - VAV1
\(\alpha\) - P - ERK
\(\alpha\) - LYN
\(\alpha\) - HS1
\(\alpha\) - VAV1
\(\alpha\) - ERK

Figure 4. LYN inhibition abrogates HS1-Y397 phosphorylation and reduces VAV1 and ERK activation.

Based on these data (supplemental Figure 4) and on the fact that HS1 activation is dramatically affected by LYN inhibition (Figure 4), we next explored if and how targeting the LYN/HS1 signaling might affect CLL cytoskeletal functionality. We found that 100 nM dasatinib significantly reduced CXCL12-induced migration \(P = 0.009\) (Figure 5A), F-actin polymerization \(P = 0.0004\) (Figure 5B), adhesion to BSA-coated plates \(P = 0.01\) (Figure 5C) and to HS5 stromal cells \(P = 0.007\) (Figure 5D) in active-HS1 as compared with inactive-HS1 cases. These results suggest that dasatinib targets the cytoskeleton and is more effective in the subset of patients with basally active cytoskeletal activity. In addition to the effects on the cytoskeleton, prolonged LYN/HS1 signaling inhibition reduces cell viability with a significant preference for active-HS1 cells \(P = 0.005\) (Figure 5E), ultimately supporting the relevance of LYN/HS1 activation in sustaining cell viability in a sizable portion of CLL patients.

Targeting the LYN/HS1 axis in vivo delays disease progression in a transplantable model of CLL

To model the effects of LYN/HS1 inhibition on circulating and tissue-resident CLL cells, dasatinib activity was tested in a transplantable mouse model of CLL based on the EμTCL1 transgenic mouse. In this model, purified splenic B cells from aged EμTCL1 mice were transplanted intraperitoneally in syngeneic recipients, leading to the development of CLL-like lymphoproliferation. We started treatment 6 weeks after transplantation, when a mean of 25% (range: 10%-60%) CD19+CD5+Igκ+ cells was detected in the PB of recipient mice. Dasatinib reduced the percentage of CD19+CD5+Igκ+ CLL cells as early as 1 week after initiation and throughout the entire treatment period. After the mice were euthanized, organs were collected and analyzed to assess leukemia burden, and a significant reduction in the absolute number of CD19+CD5+Igκ+ cells was observed in the SPs \(P = 0.05\), BM \(P = 0.03\), and MESLN \(P = 0.04\) of treated mice as compared with controls (Figure 6B-C), while no major differences were observed in peritoneal exudates (data not shown). As LYN is the most abundantly expressed SRC kinase in CLL cells, we tested SRC-Y416 phosphorylation as a surrogate marker for in vivo LYN activity and observed a significant reduction in SP leukemic B cells \(P = 0.04\) (Figure 6D) at the end of treatment. These results suggest that LYN/HS1 signaling can be efficiently targeted in vivo in a mouse model that recapitulates human CLL with a significant impact on disease progression.

Targeting LYN/HS1 in vitro induces apoptosis and affects the cytoskeletal functionality of EμTCL1 splenic B cells

To further investigate the effects of LYN/HS1 signaling inhibition in the mouse setting, we tested it in vitro on purified splenic B cells from the TCL1 leukemic donor mouse. Dasatinib treatment effectively induced in vitro apoptosis as early as 24 hours after incubation in a dose-dependent manner (supplemental Figure 5A) and inhibited SRC-Y416, HS1-Y397, VAV1, and ERK phosphorylations after 1 hour of incubation, while it did not affect SYK phosphorylation (supplemental Figure 5B). In vitro chemotaxis toward CXCL12 \(P = 0.02\), F-actin polymerization \(P = 0.02\), and adhesion to the OP9 murine stromal cell line \(P = 0.008\) were significantly inhibited by the treatment, while spontaneous adhesion \(P > 0.05\) was not altered (supplemental Figure 5C-F). These data suggest that the mechanism of action of dasatinib is similar both in human and murine B-CLL cells.

Discussion

BCR signaling modulates CLL cell proliferation, survival, and cytoskeletal functionality, thereby favoring the infiltration of CLL cells in lymphoid organs where clonal expansion is nourished by microenvironment interactions. Accordingly, the BCR signaling pathway has become a promising therapeutic target in CLL (reviewed in Woyach et al10).

HS1, a central BCR signaling and cytoskeleton component, is differentially phosphorylated in CLL, with patients carrying HS1hyper-p having a poor clinical outcome compared with HS1hypert-p patients. The semiquantitative analysis of HS1 differential phosphorylation originally carried out by 2DE left unsolved questions on the nature of the differentially phosphorylated sites and the activity of the protein. Our previous study showed that the functional behavior of an HS1-KD CLL cell line is similar to that of primary CLL cells carrying HS1hyper-p, suggesting an inactivating role for HS1 hyperphosphorylation as detected by 2DE gels. Here we took advantage of the HS1-KD CLL cell line to start studying which BCR signaling molecules are associated with HS1 status and observed that, in the absence of HS1 (and its activity), total LYN kinase phosphorylation on tyrosine residues is reduced. We then confirmed a similar pattern of modifications in CLL primary samples, finding high levels of total LYN phosphorylation in 2DE-detected HS1hyper-p but low levels in HS1hypert-p patients. It is relevant to note that the concomitant presence of high levels of phosphorylated LYN and low levels of 2DE-detected HS1 phosphorylation...
appears counterintuitive. Therefore, we further analyzed the canonical activatory site phosphorylations on LYN (Y396) and on HS1 (Y397) proteins, the latter being only recently possible due to the existence of a novel antibody specific for the activatory residue. Because of this, we were able to observe that, indeed, 2DE-detected HS1 hypo-p cells showed high LYN and HS1 activation, while HS1 hyper-p cells showed reduced activation of both molecules. Based on these results, we can conclude that, overall, in the cases with HS1 hyper-p, HS1 is indeed inactive,22 not only because of the 2DE-detected hyperphosphorylation on an unknown residue, but also because of the lack of the canonical activation on Y397 by LYN kinase. Conversely, in HS1 hypo-p samples, the molecule is active due to both the low inactivatory phosphorylation (detected by 2DE) and the presence of the activatory Y397 phosphorylation. The finding on the inactivatory nature of HS1 hyper-p is in line with the knowledge that protein functional inactivation by phosphorylation occurs among cytoskeletal molecules.35-36 Therefore, we are now exploiting new mass spectrometry–based technologies to analyze which sites other than the activatory Y397 are enriched in HS1 hyper-p cells, with preliminary evidence suggesting serines and/or threonines.

In our current study, the reported differential levels of the expression and activity of LYN phosphorylation in CLL patient subsets goes beyond previous studies, showing constitutive activation of the protein in all CLL cases.8,11,12 Moreover, taking into consideration HS1 status, LYN activity allowed us to classify patients as active-HS1 and inactive-HS1, with a particular focus on the
Figure 6. Targeting the LYN/HS1 axis in vivo delays disease progression in a transplantable model of CLL. (A) The graph shows the accumulation of CD19^+ CD5^+ Igk^+ CLL cells in the PB from mice treated with the vehicle control (n = 4) or 25 mg/kg dasatinib (n = 4), as assessed by cytfluorimetric analysis at day 0, day 6, day 13, day 20, and day 27 (euthanasia) after treatment initiation. Displayed are the means ± SEM of the fold increase of CD19^+ CD5^+ Igk^+ cells over time, showing the effect of the drug in reducing PB clonal cell accumulation during the whole treatment period. (B) The dot plot displays the absolute number of total CD19^+ CD5^+ Igk^+ CLL cells, quantified by the absolute count and analyzed by flow cytometry, present in the SPs, BM, and MESLNs from mice treated with the vehicle control (n = 4) or 25 mg/kg dasatinib (n = 4). Displayed are the means ± SEM of log-transformed values of total CD19^+ CD5^+ Igk^+ cells in each organ, showing significant reduction of organ infiltration after treatment. (C) Flow cytometry stainings of SPs (upper panels), BM (middle panels), and MESLNs (lower panels) of 1 representative mouse treated with the vehicle control (left plots) or 25 mg/kg dasatinib (right plots). The number in each dot plot indicates the percentage of total CD19^+ CD5^+ Igk^+ cells among lymphocytes. (D) The graph shows the MFI of phosphorylated SRC-Y416 ± SEM from the intracellular staining on SP preparations from mice treated with the vehicle control (n = 4) or 25 mg/kg dasatinib (n = 4), measured as a surrogate marker for LYN activity and analyzed by flow cytometry on gated CD19^+ CD5^+ cells. The results show significant reduction of SRC phosphorylation in vivo at the end of treatment. *P ≤ .05, unpaired Student t test; **P ≤ .01; RM-ANOVA.

This drug is currently being tested in CLL clinical trials either as a monotherapy37 (#NCT01441882, www.clinicaltrials.gov) or in combination with other agents including rituximab (#NCT00949988), fludarabine (#NCT01051115), or both (#NCT01173679). We observed that HS1 phosphorylation, as detected by 2DE, is selectively reduced by LYN inhibition only in active-HS1 cells, confirming that LYN contributes to total HS1 phosphorylation only in a selected subset of patients. When we tested the effects of LYN inhibition on various BCR signaling molecules of the LYN/HS1 axis, we observed that dasatinib reduced the activation of the cytoskeletal effector VAV1 and of ERK kinase, also in the presence of BCR and CXCR4 stimulation (though to a more limited extent in the case of ERK). This suggests that interfering with the LYN/HS1 axis may be a valuable strategy for affecting CLL cytoskeletal functionality, even in the presence of microenvironmental stimuli. This finding led us to demonstrate that the highly dynamic cytoskeletal activity of active-HS1 cells, in terms of CXCL12-mediated chemotaxis, F-actin polymerization, spontaneous and HS5-stroma-mediated adhesion, was very significantly reduced by LYN/HS1 signaling inhibition. Conversely, inactive-HS1 samples showed basically low cytoskeletal functionality and only minimal alteration after treatment. Interestingly, prolonged LYN/HS1 inhibition by 48 hours of treatment with 100 nM38 dasatinib selectively reduced the viability of active-HS1 cells compared with inactive-HS1 cells. Previous studies had shown differential responses of CLL cells to dasatinib treatment in vitro, but no correlation between drug sensitivity and target activity was provided.28-30 Our data may indeed offer an explanation as to why the published results of a clinical trial with dasatinib37 appear variable, by demonstrating, in a large cohort of patients, the significance of differential phosphorylation patterns of LYN and downstream targets and the related cytoskeletal remodeling features. In addition, our findings provide the proof of concept that LYN/HS1 activity predicts sensitivity to LYN inhibition by dasatinib and potentially can be considered for screening patients for the prediction of response in the context of a tailored therapeutic approach in an easily identifiable proportion of CLL patients. To mimic the effects of LYN/HS1 pathway inhibition in vivo, we further tested dasatinib in the EqTCL1 transplantable mouse model.
of CLL. A 4-week treatment significantly delayed disease progression in PB and peripheral lymphoid organs, consistent with the greater activity of this drug in lymph nodes observed in some patients of a phase 2 clinical trial of dasatinib monotherapy. The effect of LYN/HS1 inhibition in vivo may depend on different mechanisms, including the inhibition of CLL survival and/or the inhibition of migration/homing to specific tissue microenvironments: both activities are possible based on the in vitro studies on splenic Eμ-TCL1 B cells that show apoptosis induction as well as cytoskeletal targeting after treatment with dasatinib. The limited effects observed in the peritoneal exudates may simply reflect the higher tumor burden present in the original injection site of the leukemic cells. Actually, we observed a reduction of the peritoneal infiltrate when administering dasatinib at a higher dose of 70 mg/kg (data not shown), making this explanation plausible. Alternatively, this effect might also be ascribed to different drug penetration in the peritoneal cavity. Overall, LYN/HS1 targeting in vivo is feasible and significantly interferes with disease progression and tissue homing.

Taken together, these results lead us to conclude that patients showing active-HS1 features with cytoskeletal functionality favoring consistent in vivo recirculation, but not patients with inactive-HS1 and reduced cytoskeletal functionality, may benefit from treatment strategies aimed at interfering with the cytoskeleton to prevent tissue infiltration and promote mobilization into the blood. Notably, mobilization from tissues is consistently reported when using novel BCR signaling inhibitors and is now considered crucial to obtaining more-profound responses as it leads to the reduction of the infiltration in the organs where classic chemotherapy has difficulty penetrating.

Because of the major advances in understanding the biological basis of CLL (reviewed in Zenz et al, Burger et al, and Caligaris-Cappio and Ghia), we are in a new era where a whole range of nongenotoxic drugs has entered the clinical arena. In this unprecedented context, the novel challenge of clinical investigation in CLL is to define which elements allow the identification of patients with higher probability of clinical response and overall benefit from each novel compound or combination. Accordingly, it may be asked which patients might benefit from the use of dasatinib, considering that patients whose cells have active-HS1 are also expected to be those with better prognosis. Though these patients, who represent a sizable proportion of cases, may experience more indolent clinical courses, higher response rates to conventional therapies, and longer survival time, they still have a life expectancy shorter than controls, and they sooner or later require therapy during their lifetimes. The possibility of identifying up front this proportion of patients may allow the use of nongenotoxic drugs (dasatinib being one), possibly even in the context of an experimental preemptive approach, in order to improve the final outcome. On the contrary, patients with inactive-HS1, who appear to have a more aggressive disease, should be spared this likely ineffective drug and be treated with different strategies.

The implication of our findings is that the analysis of the LYN/HS1 axis is worth exploring in order to predict individual response to specific drugs, and it may set an example of how to guide personalized therapy in CLL patients based on distinct biological features.

Acknowledgments

Eμ-TCL1 transgenic mice on C57BL/6 background were generously provided by Professor Carlo Croce (The Ohio State University, Columbus, OH) and Professor John Byrd (The Ohio State University, Columbus, OH). The authors thank Massimo Alessio, Marta Muzio, Antonis Dagklis, Maria Gounari, Cesare Covicino, Pamela Ranghetti, and Katia Isaia for helpful suggestions and technical support.

This work was supported by: AIRC (Associazione Italiana per la Ricerca sul Cancro) Milan, Italy, part of Investigator Grant and Special Program Molecular Clinical Oncology, 5 per mille #9965; FIRB (Fondo per investimenti ricerca di base) Rome, Italy; PRIN (Programmi ricerca rilevante interesse nazionale) Rome, Italy; Ministero Istruzione, Università e Ricerca, Rome, Italy; Progetti Integrati Oncologia, Ministero della Salute, Rome, Italy; CLL Global Research Foundation-U.S./European Alliance for the Therapy of CLL, Houston, TX. Elisa ten Hacken conducted this study as partial fulfillment of her PhD in molecular medicine, Program in Biology and Biotherapy of Cancer, San Raffaele University, Milan, Italy. Cristina Scielzo was supported by the EHA Fellowship Program (2009/18).

Authorship

Contribution: E.T.H. and C.S. designed the study, performed in vitro and in vivo experiments, analyzed the data, and wrote the manuscript; M.T.S.B. performed in vivo experiments; L.S. provided patient samples and clinical data; B.A. and F.B. performed in vitro experiments; K.S. provided patient samples and clinical data and assisted in writing the manuscript; M.P. assisted in writing the manuscript; P.G. and F.C.-C. designed the study, analyzed the data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Targeting the LYN/HS1 signaling axis in chronic lymphocytic leukemia

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