Association Between Imatinib-Resistant BCR-ABL Mutation-Negative Leukemia and Persistent Activation of LYN Kinase

Ji Wu, Feng Meng, Ling-Yuan Kong, Zhenghong Peng, Yunming Ying, William G. Bornmann, Bryant G. Darnay, Betty Lamothe, Hanshi Sun, Moshe Talpaz, Nicholas J. Donato

Background Imatinib is a tyrosine kinase inhibitor that is used to treat chronic myelogenous leukemia (CML). BCR-ABL mutations are associated with failure of imatinib treatment in many CML patients. LYN kinase regulates survival and responsiveness of CML cells to inhibition of BCR-ABL kinase, and differences in LYN regulation have been found between imatinib-sensitive and -resistant CML cell lines.

Methods We evaluated cells from 12 imatinib-resistant CML patients with mutation-negative BCR-ABL and from six imatinib-sensitive patients who discontinued therapy because of imatinib intolerance. Phosphorylation of BCR-ABL and LYN was assessed in patient cells and cell lines by immunoblotting with activation state–specific antibodies, co-immunoprecipitation studies, and mass spectroscopy analysis of phosphopeptides. Cell viability, caspase activation, and apoptosis were also measured. Mutations were analyzed by sequencing. The effect of silencing LYN with short interfering RNAs (siRNAs) or reducing activation by treatment with tyrosine kinase inhibitors was evaluated in cell lines and patient cells.

Results Imatinib treatment suppressed LYN phosphorylation in cells from imatinib-sensitive CML patients and imatinib-sensitive cell lines. Imatinib treatment blocked BCR-ABL signaling but did not suppress LYN phosphorylation in cells from imatinib-resistant patients, and persistent activation of LYN kinase was not associated with mutations in LYN kinase or its carboxyl-terminal regulatory domains. Unique LYN phosphorylation sites (tyrosine-193 and tyrosine-459) and associated proteins (c-Cbl and p80) were identified in cells from imatinib-resistant patients. Reducing LYN expression (siRNA) or activation (dasatinib) was associated with loss of cell survival and cytogenetic or complete hematologic responses in imatinib-resistant disease.

Conclusions LYN activation was independent of BCR-ABL in cells from imatinib-resistant patients. Thus, LYN kinase may be involved in imatinib resistance in CML patients with mutation-negative BCR-ABL and its direct inhibition is consistent with clinical responses in these patients.

J Natl Cancer Inst 2008;100:926–939

Targeted inhibition of BCR-ABL kinase with imatinib mesylate is now frontline therapy for newly diagnosed patients with chronic myelogenous leukemia (CML) and other leukemias that express BCR-ABL kinase (1,2). However, the disease of some chronic-phase patients and most patients with late-stage disease (ie, accelerated phase or blast crisis) progresses during imatinib therapy (3,4). Several mechanisms have been proposed to explain the loss of imatinib sensitivity, including physiological changes in the patients and molecular changes in BCR-ABL kinase (5–10). Initial studies (5–8) of CML patients with progressing disease concluded that BCR-ABL mutations play a major role in imatinib resistance. However, failure of imatinib treatment has also been described in patients who do not have BCR-ABL mutations or amplification (11–16). Moreover, expression profiling and in vitro studies (17–19) predict the involvement of additional genes in imatinib resistance and disease progression, but most of those genes have not been thoroughly investigated or described in clinical specimens from CML patients.

LYN and HCK are SRC family kinases that are expressed in CML cells and activated by BCR-ABL kinase (20,21). Results of gene knockout studies support a role for LYN, HCK, and FYN (another SRC family kinase) in BCR-ABL kinase–mediated transformation and leukemogenesis (22–25). However, there appears to be complex cross talk between BCR-ABL and LYN or HCK

Affiliations of authors: Departments of Experimental Therapeutics (JW, FM, LYK, BGD, BL, NJD) and Experimental Diagnostic Imaging (ZP, YY, WGB), The M. D. Anderson Cancer Center, Houston, TX; Division of Hematology/Oncology, Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, Ann Arbor, MI (HS, MT).

Correspondence to: Nicholas J. Donato, PhD, Division of Hematology/Oncology, Department of Internal Medicine, University of Michigan Comprehensive Cancer Center, 1500 E. Medical Center Dr, Rm CCGC 4306, Ann Arbor, MI 48109 (e-mail: ndonato@med.umich.edu).

See "Funding" and "Notes" following "References."

DOI: 10.1093/jnci/djn188

kinases because several studies (23–29) have shown multiple sites of kinase interaction and cross phosphorylation. Site-specific BCR-ABL kinase phosphorylation that is catalyzed by HCK and LYN kinases alters the oncogenicity of BCR-ABL kinase (28,29). Thus, the expression and activity of these SRC family kinases may be biologically important and regulate the clinical response to the inhibition of specific kinases.

BCR-ABL kinase controls the activation of LYN and HCK kinases in freshly isolated primary cells from CML patients (30). However, activation of LYN and HCK kinases may also be controlled by other mechanisms in some CML patients (30,31). For example, LYN kinase is overexpressed in some imatinib-resistant CML cell lines and its activation is not dependent on BCR-ABL kinase (26–33). In animal models of imatinib resistance, inhibition of persistently activated LYN kinase with dasatinib has been associated with suppression of tumor growth (34). Expression of a deregulated LYN kinase may interfere with the inhibition of one or more members of the BCR-ABL signaling complex, including CRKL, STAT5 (signal transducer and activator of transcription 5), and MAPK (mitogen-activated protein kinase) (26–29).

In this report, the role of LYN and HCK activation was examined in CML cells derived from imatinib-sensitive and -resistant patients and in CML cell lines. For this study, we focused on 18 CML patients who expressed either an unmutated BCR-ABL kinase or a mutated BCR-ABL kinase that had negligible impact on imatinib sensitivity to assess the role of SRC family kinases in imatinib-resistant disease.

CONTEXT AND CAVEATS

Prior knowledge

The tyrosine kinase inhibitor imatinib is used to treat chronic myelogenous leukemia (CML). Failure of imatinib treatment in many but not all CML patients is associated with BCR-ABL mutations. LYN kinase regulates survival and responsiveness of CML cells to inhibition of BCR-ABL kinase, and differences in LYN regulation have been found between imatinib-sensitive and -resistant CML cell lines.

Study design

In vitro study of imatinib-sensitive and -resistant CML cell lines and of cells isolated from imatinib-sensitive CML patients and from imatinib-resistant patients without BCR-ABL mutations.

Contribution

Imatinib treatment suppressed LYN phosphorylation in cells from imatinib-sensitive CML patients and cell lines but not in cells from imatinib-resistant patients who were BCR-ABL mutation negative. Unique LYN phosphorylation sites and associated proteins were identified in cells from imatinib-resistant patients. Reducing LYN expression with short interfering RNAs or activation with tyrosine kinase inhibitors was associated with loss of cell survival and cytogenetic or complete hematologic responses in imatinib-resistant disease.

Implication

LYN kinase appears to be involved in imatinib-resistant CML.

Limitations

Sample availability and access to patient material were limited, and repetitive analyses were not usually possible. Although the concentration of tyrosine kinase inhibitors used was in line with pharmacologically achievable levels, the cellular concentration of each inhibitor may vary widely between patients and may only partially reflect the concentrations used. Therefore, the effects described for kinase inhibitor activities may only partially reflect their clinical activity.

Patients, Materials, and Methods

Cell Lines

Imatinib-sensitive K562 and imatinib-resistant K562R cells originally derived from a CML patient were grown and maintained in RPMI 1640 medium with 10% fetal bovine serum (Hyclone, Logan, UT). Characteristics of the K562R cell line were previously described (31). Briefly, K562R cells resist imatinib-mediated apoptosis, overexpress LYN kinase, and retain a mutation-negative BCR-ABL. WDT-1, WDT-2, and WDT-3 are newly isolated Philadelphia chromosome–positive cell lines established from CML patients (30). WDT-1 cells are deficient in BCR-ABL protein and resistant to imatinib-mediated cell death, whereas both WDT-2 and WDT-3 cell lines express BCR-ABL protein and are highly sensitive to imatinib. These cells were also grown in RPMI 1640 medium with 10% fetal bovine serum.

Cell Lysate Preparation

Lysates were prepared from CML cell lines as follows. Cells were washed in phosphate-buffered saline and then solubilized in lysis buffer consisting of 50 mM Hepes (pH 7.0), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, leupeptin (10 μg/mL), and aprotinin (10 μg/mL) for 20 minutes on ice. Lysates were clarified by centrifugation (12 000 × *g* for 15 minutes at 4°C), and the supernatant fraction was used as a source of protein for immunoblotting. Lysates were also prepared from mononuclear cells isolated from CML patients as described above except that the lysis buffer also contained phosphatase and protease inhibitor mixtures (products P5726 and P8340, respectively; Sigma, St Louis, MO).

Expression of LYN in K562 Cells

Full-length LYN cDNA (*Eco*RI digest of the pcDNA3.1-LYN expression vector) was subcloned into the *Eco*RI site of the pMX-IRES-GFP vector, which was modified to include a puromycin selection marker (35,36) (where IRES is internal ribosome entry site and GFP is green fluorescent protein). The pMX-LYN-IRES-GFP construct (pMX-LYN) (5 μg) or an empty vector (pMX) (5 μg) was electroporated into 2.5×10^6 freshly washed K562 cells in solution T (AMAXA, Gaithersburg, MD) by use of a Nucleofector II on a setting of 017, according to the manufacturer's (AMAXA) instructions. Two days after electroporation, cells were cultured in puromycin (10 μg/mL) and sorted for GFP expression by flow cytometry. Empty vector–derived GFP-positive cells and GFP-positive cells isolated from pMX-LYN transfectants were continually cultured in puromycin (10 μg/mL). To measure drug sensitivity and LYN expression, cells were grown in normal growth medium in the absence of puromycin for 2 days before examination.

Kinase Inhibitors and Antibodies

Imatinib was synthesized as previously described (37) by our study's medicinal chemist (W. G. Bornmann). Synthesized product was chemically and biologically identical to the material previously provided by Novartis AG, Basel, Switzerland (38). Dasatinib (39,40) was kindly provided by Bristol-Myers Squibb Co. (Princeton, NJ). Antibodies used in this study were as follows: anti-pY411-HCK (polyclonal) that is specific for the HCK phosphorylation site, anti-pY396-LYN (polyclonal) that is specific for the LYN phosphorylation site, anti-HCK (polyclonal), and anti-LYN (polyclonal) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-c-ABL monoclonal antibodies (8E9 clone; Pharmingen, San Diego, CA); anti-phosphorylated tyrosine monoclonal antibodies (clone 4G10) and anti-CRKL (polyclonal) (UBI, Lake Placid, NY); anti-phosphorylated CRKL (polyclonal), anti-pY177-BCR-ABL (polyclonal) that is specific for the BCR-ABL phosphorylation site, and anti-poly(ADP-ribose) polymerase (PARP; polyclonal) (Cell Signaling, Beverley, MA); and β-actin (Sigma). All primary antibodies were used at a 1 : 1000 dilution. Secondary antibodies included goat anti-mouse horseradish peroxidase and goat anti-rabbit horseradish peroxidase (Sigma).

Immunoblot Analysis

Immunoblot analysis was performed as previously described (31). Briefly, equal amounts of protein lysate (30 μg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and the bands were electrophoretically transferred to nitrocellulose membranes (0.2-mm pore size; Whatman, Florham Park, NJ). The blots were blocked with 5% nonfat dry milk (or 3% bovine serum albumin, for phosphorylated tyrosine blotting) in Tris-buffered saline with 0.05% Tween-20 (TBS-Tween) for 2 hours. The membrane was incubated with primary antibody for 16 hours at 4°C, washed with TBS-Tween, and incubated with secondary antibody for 30 minutes at room temperature. After additional washing in TBS-Tween, chemiluminescent reagent (ECL; GE Healthcare, Piscataway, NJ) was added, and the image was developed on x-ray film.

Cell Survival Analyses

Cell survival was estimated by use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, as previously described (31). Briefly, K562 or K562R cells (2×10^4 cells per well in a 96-well plate) were incubated with tyrosine kinase inhibitor in a total volume of 100 μL of cell culture medium. MTT (Sigma) reagent (20 μL of a solution of 10 mg/mL in phosphate-buffered saline) was added to each well, and the mixture was incubated for an additional 2 hours at 37°C. Lysis buffer (100 μL of 20% SDS in 50% dimethylformamide and 30% water at pH 5.8) was added to each well, and incubation continued for 6–18 hours at 37°C. Absorbance (at 570 nm) was determined with a microplate reader (Spectra max M2, Molecular Devices, Sunnyvale, CA). Viability of cells from clinical specimens was determined by assessing trypan blue exclusion. Briefly, cell suspensions (20–200 μL) in culture medium were gently mixed with an equal volume of 0.4% trypan blue (in phosphate-buffered saline). After 5 minutes at room temperature, 10 μL of stained cells was placed in a hemocytometer and the number of viable (unstained) and dead (stained) cells was determined microscopically.

Apoptosis Analyses

Apoptosis was assessed by use of MTT assays and trypan blue exclusion, as described above. Caspase-associated apoptosis was assessed by determining the extent of PARP cleavage in protein lysates (prepared as described above) by immunoblotting with anti-PARP antibodies, as previously described (31).

Patients With CML and Cells Isolated From These Patients

Sixty-eight CML patients who were treated with imatinib at M. D. Anderson Cancer Center during the period from January 1, 2004, through October 31, 2005, contributed blood samples to an institutionally approved tissue sample collection protocol. Samples were collected from imatinib-treated patients who had been off therapy for at least 2 weeks. All 59 patients whose disease progressed while they were being treated with imatinib were screened for mutations in the kinase domain of their BCR-ABL gene and then entered a phase 1 clinical trial of dasatinib, a tyrosine kinase inhibitor with BCR-ABL and SRC kinase inhibitory activity (39). Of the 59 progressing patients, we found that 33 (56%) had mutations in the BCR-ABL kinase domain and 26 (44%) had mutation-negative BCR-ABL genes. For this analysis, we selected 18 CML patients who were imatinib intolerant or had imatinib-resistant disease, who had mononuclear cells that carried BCR-ABL genes with no mutations in the kinase domain, and for whom a sufficient blood sample was available for our analyses. Mononuclear cells from four newly diagnosed CML patients (ND1, a 44-year-old male in chronic phase; ND2, a 66-year-old female in chronic phase; ND3, a 34-year-old female in chronic phase; and ND4, a 51-year-old male in blastic phase) who had undergone leukophoresis at the University of Michigan Cancer Center were also used in this study. None had received previous therapy, and three of the four began imatinib therapy after leukophoresis.

Patient characteristics and treatment histories are presented in Table 1. The clinical definition of acquired resistance has been described previously (40). Briefly, in chronic phase, the patient achieved a complete hematologic response and subsequently developed higher white blood cell counts of 10 000 white blood cells per mm^3 or greater and two consecutive white blood cell counts at least 14 days apart with at least one of greater than 15 000 white blood cells per mm^3. In advanced phase, patients must achieve a major or minor hematologic response with imatinib and then progress.

The phase 1 trial of dasatinib and this analysis were reviewed and approved by The M. D. Anderson Cancer Center Institutional Review Board, and all patients signed informed consent documents before entry on study. Therapeutic responses in CML patients have been described previously (40). Briefly, the clinical definition of a complete hematologic response for chronic-phase CML is a white blood cell count of 11 000 cells per mm^3 or less, a platelet count of less than 450 000 platelets per mm^3, no blasts or promyelocytes in peripheral blood, less than 5% myelocytes plus metamyelocytes in peripheral blood, less than 20% basophils in peripheral blood, and no extramedullary involvement (including

Table 1. Characteristics of patients with chronic myelogenous leukemia*

Patient	Age, y	Sex	Previous therapy	Disease stage at imatinib start	Duration of imatinib response, mo	Reason to stop imatinib	BCR-ABL mutational status	Best imatinib response	Disease stage at dasatinib start	Dasatinib response
S1	48	F	IFN, Ara-C, Peg-IFN, HU, HHT	CP	42	Intolerant	ND	MCyR	CP	CHR
S2	52	M	None	CP	4	Intolerant	ND	CHR	CP	CCyR
S3	75	F	IFN, Peg-IFN, HU, HHT	CP	2	Intolerant	ND	CHR	CP	CHR
S4	69	F	HU, IFN	CP	4	Intolerant	ND	CHR	CP	CHR
S5	48	F	Peg-IFN	CP	14	Intolerant	ND	CHR	CP	CCyR
S6	70	F	IFN, ATRA, Ara-C, HU	BC	10	Intolerant	ND	CHR	AP	CHR
R1	49	M	HU, Ara-C	CP	12	HRes	ND	CHR	BC	CHR
R2	68	F	IFN, Ara-C, HU, FTI	CP	18	HRes	ND	CHR	CP	CCyR
R3	68	M	IFN, HHT	CP	14	HRes	ND	CHR	CP	CHR, MCyR
R4	51	M	IFN, Ara-C, HHT	CP	16	HRes	V299L (30%)	CHR	BC	CHR
R5	55	M	IFN, Ara-C	CP	12	HRes	ND	CHR	BC	CHR
R6	57	F	IFN, HU	CP	36	HRes	ND	CHR	CP	CHR
R7	55	F	IFN, Ara-C, ATRA, Peg-IFN, HU	CP	24	HRes	ND	PCyR	CP	CCyR
R8	69	F	IFN, HU	AP	12	HRes	ND	CHR	BC	CCyR
R9	34	F	IFN, Ara-C, HU, Nilotinib	BC	3	HRes	I360T (20%)	CHR	BC	CCyR
R10	50	M	HyperCVAD	BC (lymphoid)	5	HRes	V299L (70%)	CHR	BC	CCyR
R11	67	F	IFN, HHT, FTI	CP	36	HRes	ND	Stable disease	CP	CCyR
R12	48	M	HU	CP	21	HRes	ND	CHR	CP	CCyR

* IFN = interferon; Ara-C = cytosine arabinoside; Peg-IFN = pegylated interferon-α 2b; HU = hydroxyurea; HHT = homoharringtonine; CP = chronic phase; ND = none detected; MCyR = minor cytogenetic response; CHR = complete hematologic response; M = male; CCyR = complete cytogenetic response; ATRA = all *trans*-retinoic acid; BC = blast crisis; AP = accelerated phase; HRes = hematologic resistance; FTI = farnesyltransferase inhibitor; PCyR = partial cytogenetic response; HyperCVAD = cyclophosphamide, vincristine, dexamethasone, and doxorubicin. Disease and response criteria were as defined (40).

no hepatomegaly or splenomegaly). Cytogenetic responses were defined on the basis of the percentage of Philadelphia chromosome–positive cells in metaphase in the bone marrow as follows: complete cytogenetic response, 0%; partial cytogenetic response, 1%–35%; minor cytogenetic response, 36%–65%.

Mononuclear cells were isolated from patient blood samples by density centrifugation (Ficoll–Hypaque, 400 × *g*, 40 minutes, and 18°C) and washed with phosphate-buffered saline; a minimum of 4×10^6 cells were either lysed immediately (2 minutes at room temperature; for RNA extraction, as described below for mutation analysis) or resuspended in cell culture medium (RPMI 1640 medium and 10% fetal bovine serum) and incubated overnight at 37°C in a 5% CO_2–95% air incubator before treatment with tyrosine kinase inhibitors.

Analysis of Mutations in BCR-ABL, *LYN,* and *HCK*

BCR-ABL mutations were analyzed in mononuclear cells from CML patient blood or bone marrow specimens as previously described (5,7). In brief, total RNA was extracted from a minimum of 4×10^6 cells by use of RNeasy Protect Mini Kit (Qiagen, Valencia, CA) and used in a one-step reverse transcription–polymerase chain reaction (RT-PCR; Invitrogen, Carlsbad, CA) with the following primers: forward primer, CM10 (5′-GAAGCTTCTCCCTGACATCCGT-3′ (BCR bases 2609-2630) and reverse primer, 3′ ABL KD (5′-GCCAGGCTCTCGGGT GCAGTCC-3′ (ABL bases 1292-1271). This reaction produced a 1.3-kb fragment that corresponds to the BCR-ABL fusion junction and ABL kinase domain. This 1.3-kb fragment was purified by use of the QIAuick gel extraction kit (product 28704, Qiagen) and then used as a template to prime a second PCR with the primers 5′ABL KD (5′-GCGCAACAAGCCCACTGTCTATGG-3′) and 3′ ABL KD (PCR SuperMix High Fidelity, product 10790-020; Invitrogen). This reaction amplified the ABL kinase domain as a 0.6-kb fragment, which was isolated, as described above, and subcloned into the pGEM-T vector (product A3600; Promega, Madison, WI). At least 10 clones containing the ABL kinase domain were directly sequenced with an ABI377 automated sequencer (Applied Biosystems, Foster City, CA). All mutations are reported in the *Abl-1a* gene orientation (41).

Both the kinase domain and carboxyl-terminal regions of *LYN* and *HCK* genes were also sequenced by use of an RT-PCR product derived from the total RNA isolate described above. Primers for the *HCK* analysis include 5′HCK (5′-CCTTCATCGAGCAGA GGAAC-3′; HCK bases 1214–1223) and 3′HCK (5′-GTGGGT GTCTGGGAGTAGGA-3′; HCK bases 1791-1772). Primers for the *LYN* analysis include 5′LYN (5′-TCCTGAAGAGCGA TGAAGGT-3′; LYN bases 1286-1305) and 3′LYN (5′-CCCG CACATGAAATCATAAGT-3′; LYN bases 1931-1922). After the one-step RT-PCR, the resultant approximately 0.6-kb fragments were isolated, subcloned, and sequenced as described for the BCR-ABL products. At least eight clones were sequenced for each SRC family kinase gene in 12 CML patients with imatinib-resistant disease.

Animal Studies

We resuspended 1×10^6 K562 cells or 1×10^7 K562R cells in equal volumes of ice-cold Matrigel (BD Biosciences, Bedford, MA). Two hundred microliters of cell suspension was injected subcutaneously into the right rear flank of 6- to 7-week-old female Swiss nude mice (Department of Experimental Radiology,

M. D. Anderson Cancer Center, Houston, TX). Five mice were used in each experimental group and the control group. Ten days after inoculation, when the tumor volume was approximately 0.3 cm^3, treatment with imatinib or dasatinib began. Imatinib (50 mg/kg) was injected intraperitoneally in a 0.1-mL suspension of dimethyl sulfoxide/polyethylene glycol 300 (1 : 1 vol : vol) every day for 9 days. Dasatinib (15 mg/kg) was injected intraperitoneally in a 0.1-mL suspension of H_2O and propylene glycol (1 : 1 vol : vol) every day for 5 days, followed by a 2-day rest, and then dasatinib injections continued for the next 2 days, for a total of 9 days. Control mice were treated daily with equal volumes of vehicle (dimethyl sulfoxide and polyethylene glycol 300). Each mouse received a total of nine injections. Mice were maintained in accordance with the Institutional Animal Care and Use Committee procedures and guidelines. Animals were weighed every other day and observed daily for signs of toxicity. Tumors were measured three times a week with a digital caliper (Cel Associates, Inc., Pearland, TX). Tumor volumes were calculated in cubic millimeters according to the formula $(L \times W \times D) \times \pi/6$, where L is the length, W is the width, and D is the depth. All animals were euthanized (with CO_2) when control animal tumor volumes approached the maximal allowable burden (1.5 cm^3). All tumors were excised from animals 4 hours after the final injection of kinase inhibitor and frozen immediately in liquid nitrogen. Tumor homogenates were prepared by use of a tissue microdisruptor (Brinkmann Instruments, Westbury, NY) at a final protein concentration of 20 mg/mL of lysis buffer (described above) and centrifuged (12 000 × *g*, for 30 minutes at 4°C). The supernatant fraction was subjected to immunoblot analysis to determine levels of total and phosphorylated CRKL and total and phosphorylated LYN, as described above.

LYN and HCK Knockdown With siRNA

K562 and K562R cells were electroporated with short interfering RNAs (siRNAs; 20–100 nM) against LYN or HCK or with a scrambled control sequence (Dharmacon, Lafayette, CO) by use of an AMAXA nucleoporation system. All siRNA was purchased from Dharmacon. Electroporation was as described above. For cells isolated from patient blood samples, we used the CD34 cell nucleoporation reagent (AMAXA) and a setting of U08 for the AMAXA nucleoporation system, as described previously (42). Forty-eight (for K562 and K562R cells) or 96 (cells isolated from patients R3, R5, R10, R12, ND1, ND2, ND3, and ND4) hours after electroporation, lysates were prepared as described above and analyzed for the expression of LYN, HCK, and phosphorylated LYN and for PARP cleavage by immunoblotting and for cell survival by MTT assays. Survival of cells from patient samples was determined by trypan blue exclusion.

Immunoprecipitation and Mass Spectrometry Analysis of LYN Kinase Tyrosine Phosphorylation in Cells Isolated From Patients

LYN kinase was immunoprecipitated with anti-LYN (Santa Cruz Biotechnology) and protein A-immunobeads (GE Healthcare) from lysates prepared from mononuclear cells from two imatinib-resistant CML patients (patients R10 and R12; starting with at least 500 μg of total protein from a lysate). Immune complexes were released from the immunobeads by heating (5 minutes at 95°C) in 50 μL of SDS sample buffer (1% SDS, 5% glycerol, 30 mM Tris-HCl at pH 6.8, 0.005% bromophenyl blue, and 2.5% 2-mercaptoethanol) and resolved by SDS-PAGE. The band corresponding to LYN kinase (as determined by immunoblotting companion lanes from the same gel) was excised from the gel, and protein was extracted and cleaved with modified porcine trypsin (sequencing grade, Roche Applied Science, Indianapolis, IN). Resulting peptides were analyzed by nano-scale liquid chromatograpy (Nano-LC) tandem mass spectroscopy with online desalting on a system consisting of a Famos autosample, an Ultimate Nano-LC module, and a Switchos precolumn switching device (LC-Packings/Dionex Corp., Sunnyvale, CA). Electrospray ion-trap mass spectrometry was performed on a linear ion-trap mass spectrometer to detect the mass of tryptic peptides (LTQ, ThermoFinnegan, San Jose, CA). Proteins were identified by searching for the fragment spectra in the National Center for Biotechnology Information nonredundant protein database by use of Mascot (Matrix Science, London, UK) and Sequest (ThermoFinnegan). Modification analysis (to define potential sites for tyrosine phosphorylation) was then performed by use of combinations of error-tolerant searching with both search tools as well as manual inspection of spectra.

Statistical Methods

Statistical significance of differences in tumor volumes between control and treated mice was evaluated by using Student *t* test. All statistical tests were two-sided. Five replicates and two independent animal tumor experiments were evaluated.

Results

Analyses in Cell Lines

Imatinib-resistant K562R cells have mutation-negative BCR-ABL but overexpress LYN kinase (31). We investigated the association between LYN kinase and imatinib resistance by inserting a LYN expression vector into imatinib-sensitive K562 cells by electroporation and measuring LYN levels in lysates from LYN-transfected K562 cells and K562R cells. LYN-transfected K562 cells had higher LYN expression and phosphorylation than untransfected K562 cells (Figure 1, A) and lower sensitivity to imatinib (for K562 cells, imatinib IC_{50} = 0.6 μM; for LYN-transfected K562 cells, IC_{50} = 2.1 μM) (Figure 1, B). The SRC and BCR-ABL kinase inhibitor dasatinib (40) induced a similar pattern of reduced survival in K562, K562R, and LYN-transfected K562 cells (Figure 1, B). Thus, decreased imatinib sensitivity that is associated with the overexpression of LYN kinase appears to be overcome by inhibiting LYN and BCR-ABL kinases with dasatinib.

To further investigate this association, LYN kinase expression in both K562 and K562R cells was reduced by use of an siRNA against LYN. Although LYN expression was markedly reduced in both cell lines after treatment with LYN siRNA (Figure 1, C), apoptosis was detected only in LYN siRNA–transfected K562R cells, indicating that elevated LYN expression is involved in the survival of some imatinib-resistant CML cells (Figure 1, C and D).

LYN kinase phosphorylation and caspase activation were also examined in imatinib- or dasatinib-treated K562 and K562R cells.

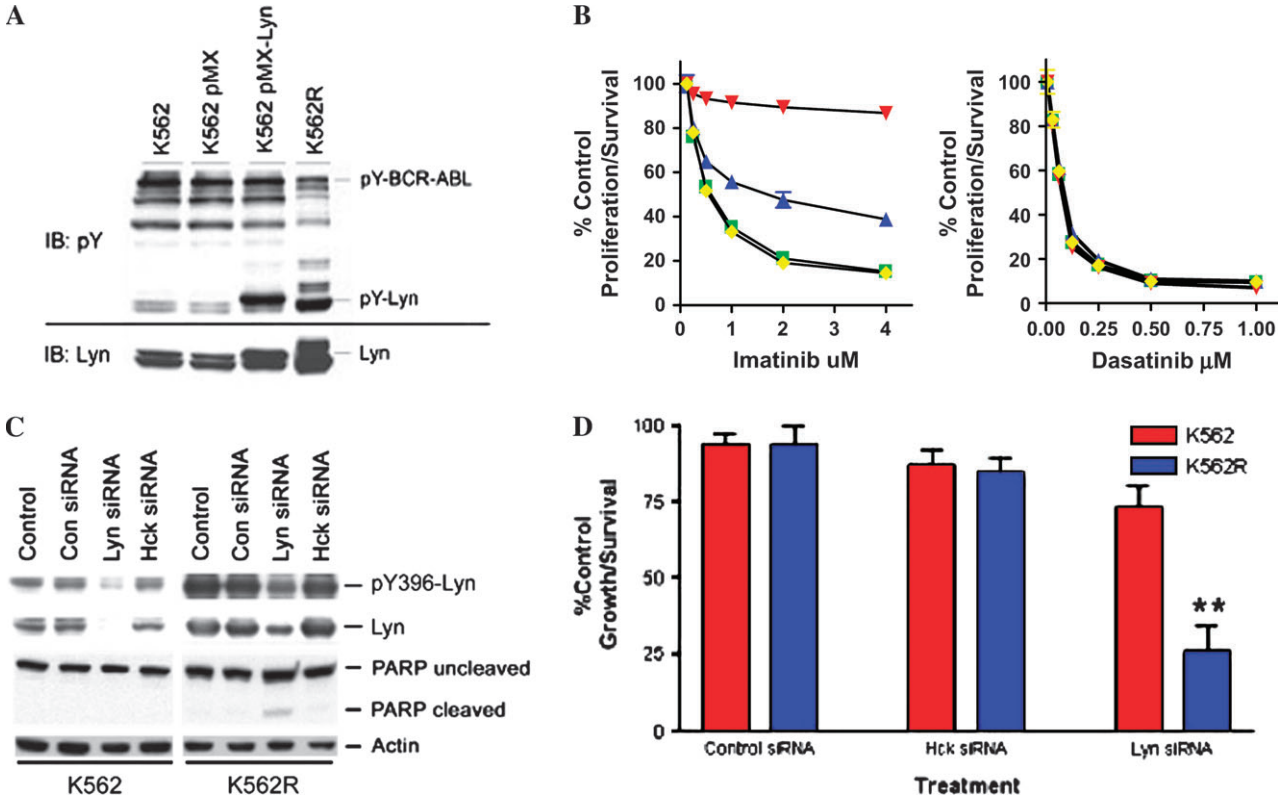

Figure 1. LYN kinase, imatinib sensitivity, and cell survival in vitro. **A**) Overexpression of LYN in chronic myelogenous leukemia patient–derived K562 cells. Proteins (30 µg) from lysates of K562 cells, control vector (pMX)–electroporated or LYN vector (pMX-LYN)–electroporated K562 cells, and imatinib-resistant K562R cells were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed for phosphotyrosine (pY) content in BCR-ABL and LYN kinases and total LYN protein expression by immunoblotting (IB). **B**) Imatinib and dasatinib treatment and survival of K562 (**squares**), LYN-overexpressing (pMX-LYN–transfected) K562 (**triangles**), K562R (**inverted triangles**), and control K562 cells transfected with empty vector (pMX) (**diamonds**). Cells were treated with the indicated concentration of imatinib or dasatinib for 48 hours. Survival was then estimated by use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. Because the MTT assay does not fully discriminate between survival and proliferation, both endpoints were included in the axis label for accuracy. Each data point is the average of four determinations; **error bars** are 95% confidence intervals (CIs) (some of which are within the symbols). For the comparison of K562-pMX vs K562-pMX-LYN cells, *P* values are as follows: at 0.5 µM imatinib, $P = .045$; at 1 µM imatinib, $P = .017$; at 2 µM imatinib, $P = .015$; and at 4 µM imatinib, $P = .005$. **C**) The effect of LYN silencing by short interfering RNA (siRNA) on LYN activation and expression in K562 and K562R cells. K562 and K562R cells were electroporated with 100 nM LYN siRNA or 100 nM HCK siRNA or with 100 nM scrambled control sequence (Con siRNA). Control cells were left untreated (Control) for 48 hours. Cell lysates were prepared and probed for phosphorylated LYN (pY396-LYN) and total LYN protein levels by immunoblotting. HCK is not expressed in K562 or K562R cells and so serves as an additional negative control. Proteins from the same lysates were also subjected to immunoblotting for poly(ADP-ribose) polymerase (PARP), as an indicator of the activation of the caspase cascade. Actin was used as the loading control. **D**) The effect of LYN silencing with LYN siRNA on the survival of K562 and K562R cells. K562 and K562R cells were electroporated with 100 nM siRNA against HCK or LYN or 100 nM of control siRNA as indicated, and cell proliferation and survival were measured collectively by use of MTT assays 72 hours later. Data are the average of four determinations; **error bars** are 95% CIs. Similar results were obtained in two additional experiments. $^{**}P < .002$, by a two-tailed Student *t* test, for LYN siRNA in K562 vs K562R cells.

Imatinib or dasatinib treatment of K562 cells rapidly reduced the level of tyrosine phosphorylation of BCR-ABL and CRKL (a BCR-ABL substrate, whose phosphorylation reflects the level of BCR-ABL kinase activity) (Figure 2, A). In contrast, suppression of the phosphorylation of both BCR-ABL and CRKL was lower in imatinib-treated K562R cells than in imatinib-treated K562 cells. Although phosphorylation of LYN was not affected by short-term treatment of K562R cells with imatinib, phosphorylation of LYN, CRKL, and BCR-ABL in both K562 and K562R cells was rapidly suppressed by dasatinib treatment. Dasatinib treatment activated caspase cascades in both K562 and K562R cells, as shown by cleavage of PARP (Figure 2, B), whereas imatinib treatment activated PARP cleavage only in K562 cells.

Because the suppression of LYN phosphorylation was associated with dasatinib-induced activation of PARP cleavage in K562R cells, we examined LYN phosphorylation further in three recently established and characterized CML cell lines, WDT-1, -2, and -3 (30). All cell lines carry the Philadelphia chromosome, a genetic marker of CML. WDT-2 and WDT-3 cells express BCR-ABL protein and are sensitive to imatinib, whereas WDT-1 cells are deficient in BCR-ABL protein and are resistant to imatinib. In WDT-2 and -3 cells, imatinib treatment reduced the level of tyrosine phosphorylation of BCR-ABL, increased the mobility of CRKL after SDS-PAGE (indicative of its dephosphorylation), and reduced the levels of phosphorylated LYN and HCK (Figure 2, C). In WDT-1 cells, imatinib treatment did not alter the level of phosphorylated LYN kinase, supporting a role for BCR-ABL–mediated activation of LYN kinase in CML cells (20,21). Thus, LYN activation in imatinib-sensitive cells can be suppressed by the imatinib-associated inhibition of BCR-ABL.

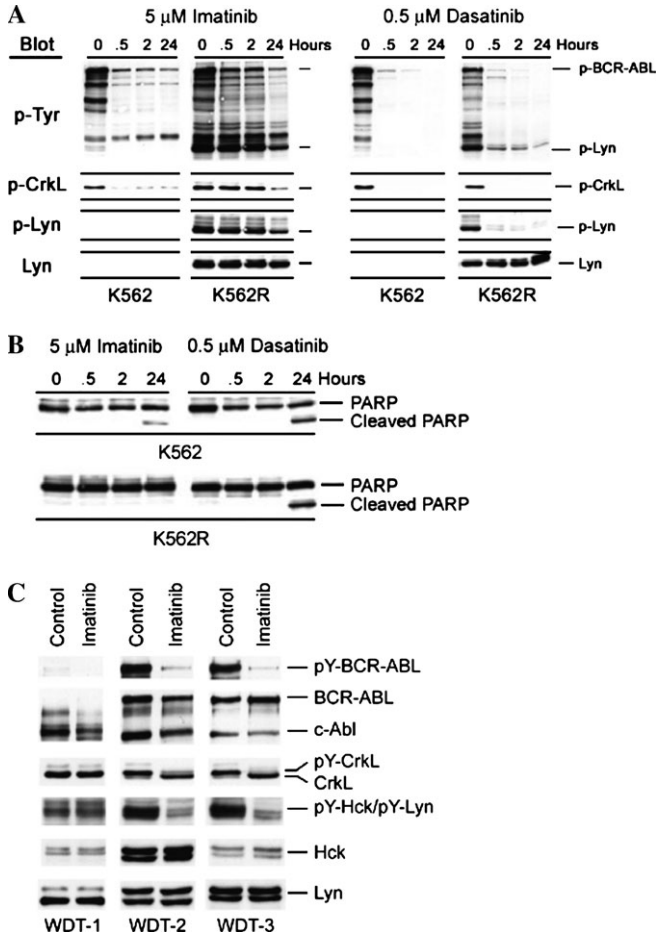

Figure 2. Control of LYN activation in chronic myelogenous leukemia (CML) cells. **A**) Kinase inhibitors and tyrosine phosphorylation in total protein, CRKL, and LYN in K562 and imatinib-resistant K562R cells. K562 and K562R cells were treated with 5 μM imatinib or 0.5 μM dasatinib as indicated. Total phosphotyrosine (p-Tyr), phosphorylated CRKL (p-CRKL), phosphorylated LYN (p-LYN), and total LYN levels were examined in cell lysates by immunoblotting. **B**) Kinase inhibitors and activation of poly(ADP-ribose) polymerase (PARP) cleavage in K562 and K562R cells. K562 and K562R cells were treated with imatinib or dasatinib as indicated. Cell lysates were examined by immunoblotting for caspase cascade activation as assessed by PARP cleavage. **C**) BCR-ABL kinase and the phosphorylation of LYN and HCK in imatinib-sensitive human CML cell lines WDT-2 and WDT-3 and the imatinib-resistant human CML cell line WDT-1. Phosphorylation of BCR-ABL, HCK, and LYN from lysates of control or imatinib-treated (2.5 μM; 60 minutes) WDT-1, WDT-2, and WDT-3 CML cell lines was assessed by immunoblotting with phosphorylation-site specific antibodies.

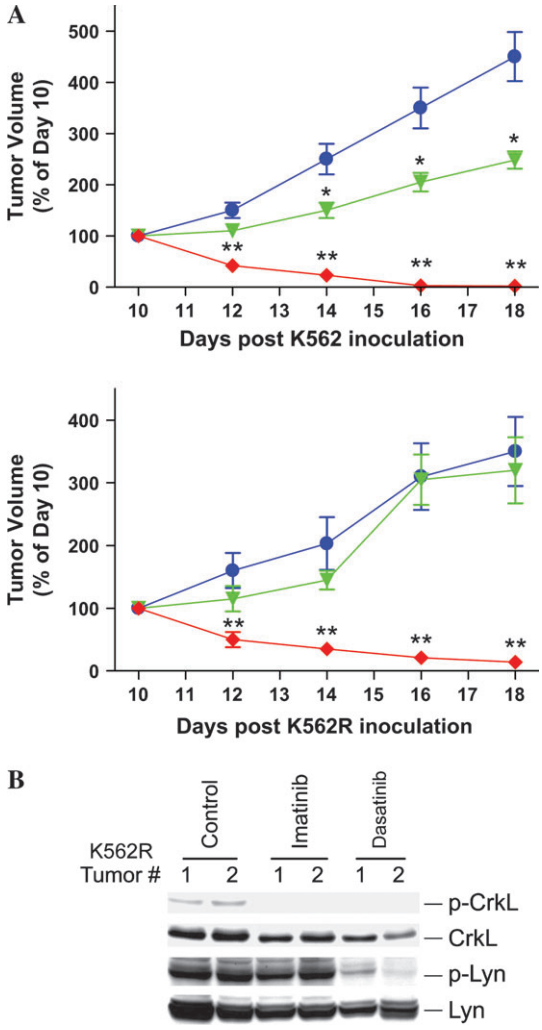

Figure 3. Tyrosine kinase inhibitors; in vivo antitumor activity and effects on CRKL and LYN tyrosine phosphorylation. **A**) Tyrosine kinase inhibitor treatment and K562 and K562R tumor growth in nude mice. Female Swiss nude mice were injected subcutaneously with 1×10^6 K562 cells (upper) or 1×10^7 K562R cells (lower) in Matrigel (day 0). On day 10, when tumor volumes were approximately 0.3 cm^3, mice were randomly assigned to one of three treatment groups—imatinib, dasatinib, and control. Each group contained five mice. Vehicle alone was injected into mice in the control group (**blue circles**). Imatinib (50 mg/kg, **green inverted triangles**) was administered every day for 1.5 weeks. Dasatinib (15 mg/kg, **red diamonds)** was administered for 5 days followed by a 2-day rest, and then daily dasatinib injections resumed for 2 more days (for a total of 9 days). Tumor dimensions were measured every other day, and tumor volumes were calculated and compared with those at the initiation of therapy (day 10). Each data point is the average of measurements from five mice; **error bars** are 95% confidence intervals. **P*<.05; ***P*<.005, compared with vehicle control (for control vs imatinib-treated K562 tumors, day 12, *P* = .014; day 14, *P* = .018; day 16, *P* = .011; day 18, *P* = .004; for control vs dasatinib-treated K562 and K562R tumors, all *P*<.001; for control vs imatinib-treated K562R tumors, all *P*>.2.) **B**) The effect of kinase inhibitors on phosphorylation of BCR-ABL and LYN kinase in K562R tumors. Tumors were excised from two control, two imatinib-treated, and two dasatinib-treated mice bearing K562R tumors 4 hours after the last treatment. Lysates were subjected to immunoblotting (each lane contains lysate from one tumor) for phosphorylated CRKL (p-CRKL), total CRKL, phosphorylated LYN (p-LYN), or total LYN protein.

Analyses in Mice

Nude mice bearing subcutaneous K562 and K562R tumors were treated with imatinib or dasatinib (n = 5 mice per group; each mouse received a total of nine injections). Growth of K562 tumors in imatinib-treated mice was statistically significantly reduced (more than twofold) after 4 days of treatment, and, after 6 days of treatment, no measurable K562 tumors remained in dasatinib-treated mice (Figure 3, A). The mean tumor volume at day 18 was 1.35 cm^3 in control mice, 0.74 cm^3 in imatinib-treated mice (difference = −0.61 cm^3, 95% CI = −0.96 to −0.25 cm^3; *P* = .004), and 0.006 cm^3 in dasatinib-treated mice (difference = −1.34 cm^3, 95% CI = −1.68 to −1.02 cm^3; *P* < .001). Mice bearing K562R tumors were initially responsive to imatinib treatment (2–4 days of treatment), but after continual therapy (6–8 days), tumors in imatinib-treated mice could not be distinguished from tumors in control

mice. Dasatinib treatment statistically significantly reduced K562R tumor burden, with only two of the five mice having measurable tumor after therapy. The mean tumor volume at day 18 was 1.23 cm^3 in control mice, 1.12 cm^3 in imatinib-treated mice (difference = −0.105 cm^3, 95% CI = −0.72 to −0.51 cm^3; $P = .70$), and 0.049 cm^3 in dasatinib-treated mice (difference = −1.18 cm^3, 95% CI = −1.62 to −0.73 cm^3; $P < .001$). Both phosphorylated CRKL and phosphorylated LYN were detected in K562R tumors from control mice (Figure 3, B), only phosphorylated LYN was detected in K562R tumors from imatinib-treated mice, and levels of both phosphorylated LYN and CRKL were reduced in K562R tumors from dasatinib-treated mice. Thus, the phosphorylation levels of LYN and CRKL are associated with the K562R tumor burden, and the enhanced antitumor effects of dasatinib were associated with suppression of both BCR-ABL kinase activity, as shown by the level of phosphorylated CRKL, and LYN activation in K562R tumors. Thus, direct inhibition of the phosphorylation of both CRKL and LYN may be associated with reduced CML cell survival or proliferation.

Analyses of Patient Samples

Phosphorylation of the LYN and CRKL kinases was examined in mononuclear cells isolated from six CML patients who responded to imatinib (patients S1–S6) and from 12 CML patients who developed resistance to imatinib (patients R1–R12). The clinical characteristics of these patients are summarized in Table 1. Fifteen of the 18 patients expressed mutation-negative BCR-ABL kinase, one chronic-phase patient had 30% clonal expression of the BCR-ABL mutation V299L, one blast-crisis patient had 20% clonal expression of the BCR-ABL mutation I360T, and another blast-crisis patient had 70% of the BCR-ABL mutation V299L. From in vitro studies (43), expression of V299L does not appear to influence imatinib sensitivity. After receiving imatinib therapy, all 18 patients entered a phase 1 clinical protocol that evaluated dasatinib, and all patient blood samples were collected before entering that trial (blood samples were not obtained or available from these patients before imatinib therapy). Imatinib-intolerant patients were defined as patients who had one or more nonhematologic toxic effects (eg, skin rash or elevated liver enzymes) that prevented their continued imatinib therapy. Seventeen of the 18 patients, the exception being patient S2, received other types of therapy before receiving imatinib treatment. Fifteen achieved a complete hematologic response to imatinib, one achieved a partial cytogenetic response, one achieved a minor cytogenetic response, and one had stable disease. Most CML patients were in chronic phase when they began imatinib therapy, and most had progressed to more advanced disease when they began dasatinib therapy. During dasatinib therapy, eight patients achieved at least a complete hematologic response to dasatinib, one achieved a minor cytogenetic response and a complete hematologic response, and nine achieved a complete cytogenetic response.

We investigated whether LYN or HCK kinase activation in imatinib-sensitive patients is dependent on BCR-ABL kinase by use of the mononuclear cells from four imatinib-sensitive CML patients (patients S1–S4). These cells were treated with imatinib or dasatinib; control cells were untreated. Cell lysates were prepared, and the levels of phosphorylated LYN and HCK were measured. Phosphorylation of LYN and HCK, which was detected in all four cell lysates, was inhibited by 5 μM imatinib (to >90%) and 0.5 μM dasatinib (100%), both of which are clinically achievable concentrations (Figure 4, A). Next, we immunoprecipitated LYN and HCK kinases from lysates of cells isolated from two other imatinib-sensitive CML patients (patients S5 and S6) and assessed each immunoprecipitate for phosphorylated LYN and HCK. Dasatinib treatment fully suppressed phosphorylation of both LYN and HCK, whereas imatinib treatment reduced the tyrosine phosphorylation of LYN by 100% and of HCK by 74%–79% (Figure 4, B). On the basis of the specificity of imatinib for BCR-ABL kinase, we conclude that the activation of LYN and HCK kinases by phosphorylation appears to be regulated by BCR-ABL in imatinib-responsive CML patients, as noted in imatinib-treated cell lines (Figures 1 and 2).

We next examined LYN or HCK activation in mononuclear cells isolated from four imatinib-resistant CML patients (patients R1–R4) who retained a mutation-negative BCR-ABL (Table 1). Cells were treated with clinically achievable concentrations of imatinib (1 or 5 μM) or dasatinib (0.5 μM), and lysates were examined for the phosphorylation of LYN and HCK kinases. Imatinib (1 μM) suppressed the phosphorylation of LYN and HCK by 32% or less (mean = 14.3%, 95% CI = −8.9% to 37.4%) in lysates from patients R1, R2, and R4, whereas 5 μM imatinib was required to inhibit phosphorylation by 55% in the lysate from patient R3 (Figure 4, C) and had less inhibitory activity in the other three patient (R1, R2, and R4) samples (mean = 29.2%, 95% CI = −8.9% to 67.4%). These values were obtained from a single experiment (due to sample availability) and averaged across four patients. We failed to detect consistent distinctions in the level of LYN and HCK protein expression between imatinib-sensitive and -resistant patients but commonly detected distinctions in control of their phosphorylation after treatment with imatinib.

Mononuclear cells isolated from five additional imatinib-resistant patients (patients R5–R9) were then treated with a high concentration of imatinib (5 μM) or with dasatinib (0.5 μM), and the maximal suppression of kinase activation was assessed in cell lysates. Because cells were isolated from imatinib-resistant patients (or nilotinib resistant for patient R9; nilotinib is a higher-affinity ABL kinase inhibitor than imatinib, and it is currently undergoing clinical studies) who carried mutation-negative BCR-ABL genes, we anticipated that imatinib would suppress essentially all BCR-ABL kinase signaling, as assessed by CRKL phosphorylation. After treatment with imatinib, phosphorylation of CRKL was inhibited 78%–100% in each lysate (mean = 92%, 95% CI = 77% to 100%), but phosphorylation of LYN and HCK was inhibited by less than 15% in lysates R5–R7 (mean = 9%, 95% CI = −10% to 28%) and by approximately 50% in lysates R8 and R9 (mean = 50%, 95% CI = 25% to 75%) (Figure 4, D). In contrast, dasatinib treatment completely blocked the phosphorylation of CRKL, LYN, and HCK in all five lysates (R5–R9). Again, these values were obtained from a single experiment (due to sample availability) and averaged across five patients. Thus, LYN and HCK kinases appear to be phosphorylated by a mechanism that is independent of BCR-ABL kinase in some CML patients because imatinib-mediated inhibition of BCR-ABL substrate phosphorylation (CRKL) had little effect on LYN or HCK phosphorylation.

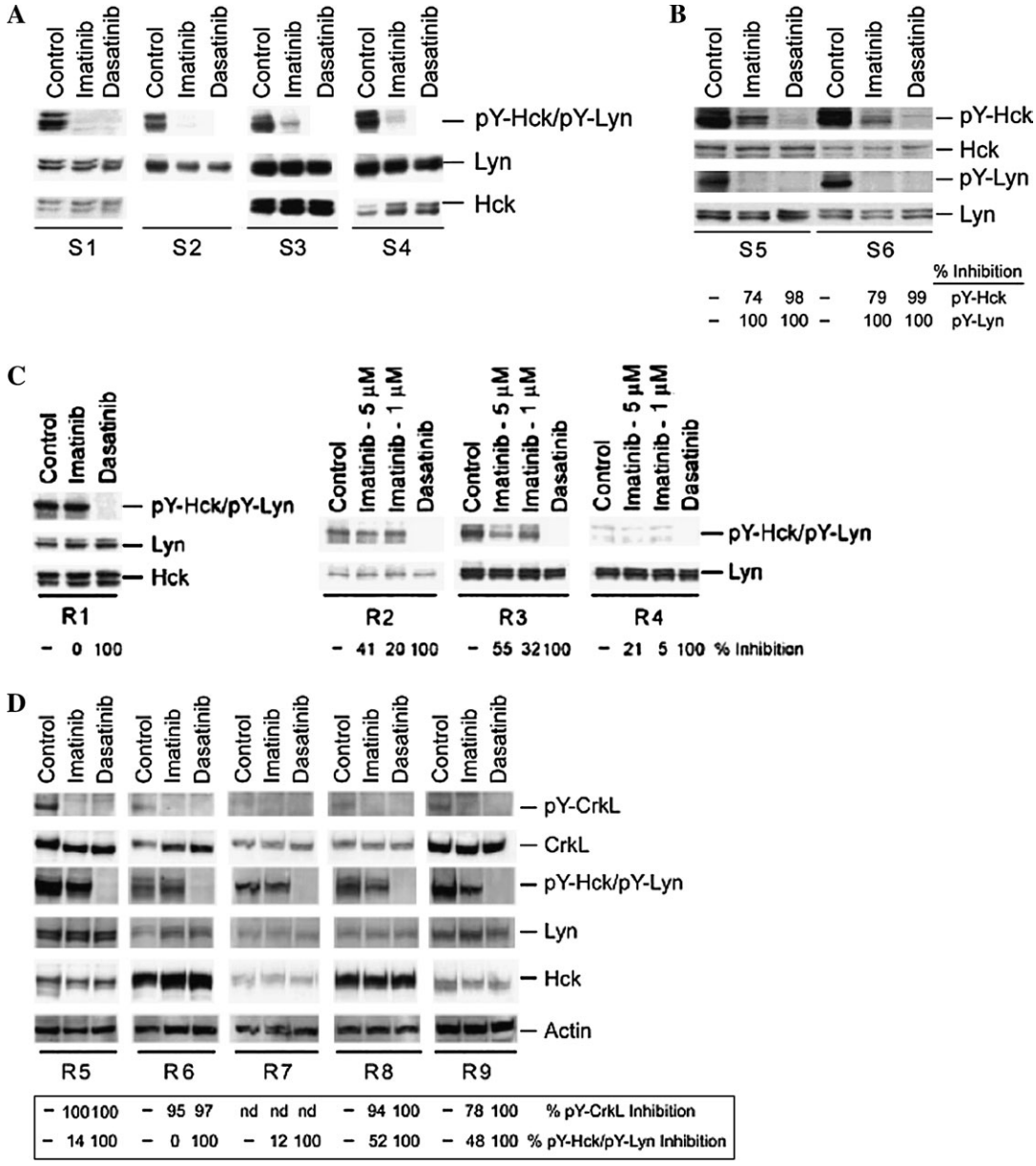

Figure 4. Regulation of LYN activation in imatinib-sensitive and imatinib-resistant chronic myelogenous leukemia (CML) patients. **A**) The role of BCR-ABL kinase and phosphorylation of LYN and HCK in CML cells from four imatinib-sensitive (intolerant) patients (S1–S4). Mononuclear CML cells isolated from these four patients were treated with 5 μM imatinib or 0.5 μM dasatinib for 2 hours. Phosphorylation of LYN and HCK kinases was then assessed by immunoblotting first with phosphorylation site-specific antibodies pY411-HCK and pY396-LYN, respectively, and then with antibodies against total LYN and HCK protein. **B**) Densitometric analysis of the effect of imatinib or dasatinib treatment on LYN and HCK phosphorylation in CML cells from two imatinib-sensitive CML patients (S5 and S6). Mononuclear cells were treated with 5 μM imatinib or 0.5 μM dasatinib for 1 hour. Lysates were prepared, and 250 μg of protein from each lysate was immunoprecipitated with anti-HCK or anti-LYN as indicated. The immunoprecipitates were subjected to immunoblot analysis by first probing for tyrosine phosphorylation of LYN or HCK by immunoblotting with antibodies against phosphotyrosine (pY) and then by reprobing for total LYN or HCK with anti-LYN or anti-HCK, respectively. **C**) Kinase inhibitors and LYN and HCK phosphorylation in CML cells from four imatinib-resistant patients (R1–R4). Mononuclear cells from imatinib-resistant patients who expressed a mutation-negative form of BCR-ABL (Table 1) were treated with 1 or 5 μM imatinib or with 0.5 μM dasatinib for 2 hours. Phosphorylation of LYN and HCK was examined by immunoblotting first with phosphorylation site-specific antibodies pY411-HCK or pY396-LYN, respectively, and then with antibodies against total LYN or HCK protein. **D**) LYN and HCK activation and BCR-ABL kinase activity in five imatinib-resistant CML patients (R5–R9). Mononuclear cells from patients R5–R9 were treated with imatinib or dasatinib, and phosphorylation of CRKL, LYN, and HCK was measured by immunoblotting with anti-pY-CRKL, anti-pY396-LYN, and anti-pY411-HCK antibodies. In addition, total CRKL, total LYN, and total HCK were measured by immunoblotting with anti-CRKL, anti-LYN, and anti-HCK antibodies. Actin was used as the protein loading control for interpatient comparison. In panels **B**–**D**, band density was measured by densitometry and the percentage inhibition, compared with control cells, was calculated for each kinase inhibitor and is presented at the bottom of the panel.

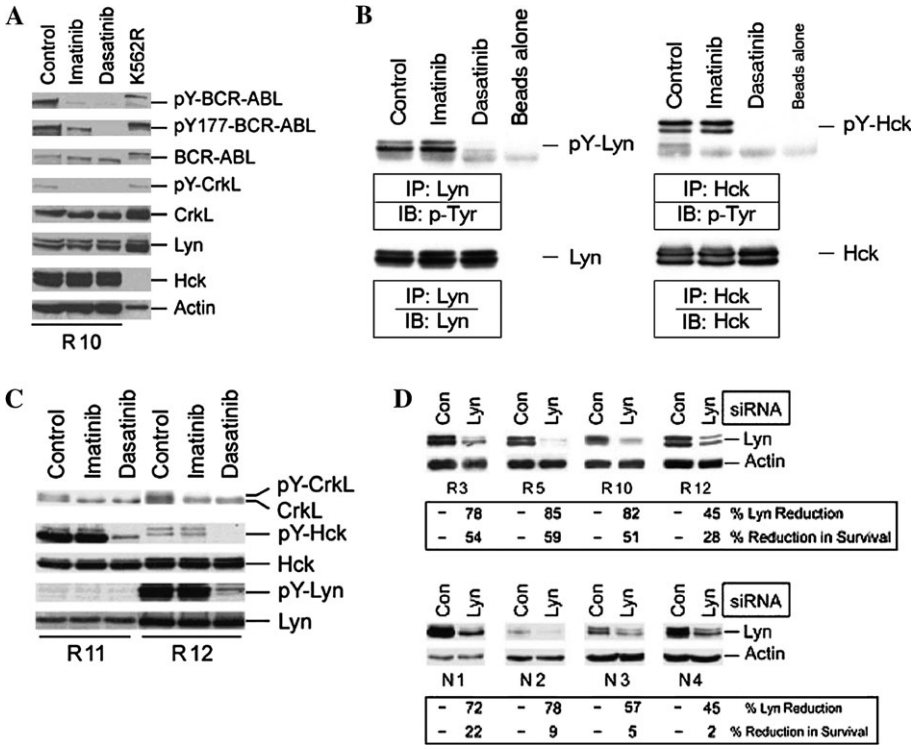

Figure 5. BCR-ABL–independent LYN kinase activation in myeloid and lymphoid chronic myelogenous leukemia (CML) cells. **A**) Kinase inhibition in an imatinib-resistant patient with CML in blast crisis (T-cell lymphoblastoid) (R10), whose disease progressed during imatinib treatment. Mononuclear cells isolated from this patient were incubated with 5 μM imatinib or 1 μM dasatinib for 1 hour. Lysates were prepared from these cells, and phosphorylation of BCR-ABL, CRKL, LYN, and HCK was measured by immunoblotting with antibodies for phosphorylation of BCR-ABL (pY-BCR-ABL), site-specific phosphorylation at tyrosine-177 in BCR-ABL kinase (pY177-BCR-ABL), phosphorylated CRKL (pCRKL), as well as total CRKL, LYN, HCK, and actin. K562R cell lysate (lane K562R) was used as a positive control for phosphorylation of BCR-ABL, LYN, and CRKL. **B**) Imatinib and dasatinib treatment and phosphorylation of LYN or HCK in CML cells from patient R10. Cells were treated with imatinib or dasatinib, and lysates were prepared. Protein (0.5 mg) from each lysate was immunoprecipitated (IP) with anti-LYN or anti-HCK bound to immunobeads. The location of any antibody bands was identified by use of controls containing only immunobeads (lanes Beads alone). Immunoprecipitates were assessed by immunoblotting (IB) first for phosphorylated tyrosines in LYN and HCK with antibodies against phosphorylated tyrosine (bands pY-LYN and pY-HCK, respectively) and then for total LYN and HCK protein with antibodies against LYN or HCK protein. **C**) Phosphorylation of LYN and HCK in cells from imatinib-resistant CML patients R11 and R12. Mononuclear cells from these patients were treated with 5 μM imatinib or 0.5 μM dasatinib for 1 hour. Lysates were prepared and 30 μg of protein was separated by electrophoresis and examined for CRKL expression and phosphorylation (electrophoretic mobility) by immunoblotting with anti-CRKL. Another 400 μg of lysate protein was immunoprecipitated with antibodies against total HCK or total LYN and then immunoblotted for phosphorylated HCK or LYN (pY-HCK and pY-LYN, respectively) and then total LYN and total HCK with anti-LYN and anti-HCK, respectively. **D**) The effect of silencing LYN expression on survival of mononuclear cells from imatinib-resistant CML patients (R3, R5, R10, and R12) and newly diagnosed CML patients (N1–N4). Cells from each patient were subjected to electroporation with 100 nM LYN short interfering RNA (siRNA) or with a control siRNA (Con). After 48 hours, cell lysates were prepared from some cells from each patient and LYN and actin levels were measured by immunoblotting. LYN bands were quantitated by densitometry and normalized to actin levels to estimate the percentage reduction in LYN expression in treated vs control cells. After 96 hours, cell survival was estimated in the remaining cells by counting total and trypan blue–stained cells. Data are the average of triplicate samples, with no more than 5% variability between samples.

Because SRC family kinase knockout mice that lack *LYN*, *HCK*, and *FGR* have been used to demonstrate a critical role for one or all three kinases in lineage-specific stem-cell BCR-ABL transformation (22), we examined the phosphorylation of LYN and HCK in mononuclear cells from imatinib-resistant patient R10, who was in lymphoid blast crisis and who expressed the V299L mutant p190 form of BCR-ABL kinase. Survival of these cells 48 hours after treatment was reduced to 42% of that of untreated cells by 5 μM imatinib and to 50% by 100 nM dasatinib (data not shown). Treatment with either imatinib or dasatinib inhibited phosphorylation of BCR-ABL kinase and CRKL (Figure 5, A). Phosphorylation at tyrosine-177, the activation-specific site in BCR-ABL kinase, was partially inhibited by imatinib treatment but was inhibited completely by dasatinib treatment, as previously reported (26,29). LYN and HCK phosphorylation were assessed individually by immunoprecipitation from control and kinase inhibitor–treated cells followed by immunoblotting for phosphorylated tyrosine residues (Figure 5, B). Imatinib treatment did not alter phosphorylation of LYN or HCK, but dasatinib treatment fully suppressed phosphorylation of CRKL, LYN, and HCK (Figure 5, A and B).

We next investigated whether phosphorylation of LYN and HCK differs in CML cells from lymphoid lineages (R10) and myeloid lineages; myeloid cells from two imatinib-resistant CML patients (patients R11 and R12) with mutation-negative BCR-ABL genes were studied. Although both LYN and HCK proteins were detected in cell lysates from both patients, only phosphorylated HCK could be detected in the lysate from patient R11, and phosphorylated LYN was more prominent than phosphorylated HCK in the lysate from patient R12 (Figure 5, C). Both imatinib and dasatinib suppressed phosphorylation of the BCR-ABL substrate CRKL in cells from patients R11 and R12, but imatinib had minimal effect on LYN or HCK activation (Figure 5, C). As described above (Figure 5, A and B), dasatinib fully suppressed phosphorylation of both proteins, indicating that the phosphorylation of LYN and HCK activation was independent of BCR-ABL kinase in imatinib-resistant myeloid and lymphoid disease.

Persistent activation of LYN and/or HCK kinase may result from a structural change induced by point mutation, and so we sequenced the regions encoding the kinase and carboxyl-terminal (negative regulatory) domains of *LYN* and *HCK* genes from all cells isolated from imatinib-resistant patients (R1–R12). No mutations in these domains in either gene were detected in cells from any patient. Tyrosine phosphorylation was then directly assessed with phosphorylation site–specific antibodies against

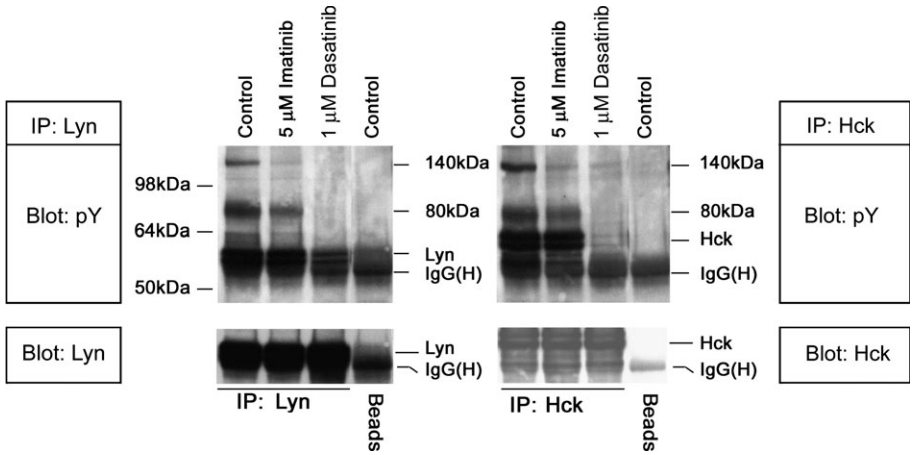

Figure 6. Phosphotyrosyl protein complexes containing phosphorylated LYN and HCK from a patient with chronic myelogenous leukemia (CML). Mononuclear cells were isolated from imatinib-resistant patient R10 with CML in blast crisis (T-cell lymphoblastoid). Cells were treated as described in Figure 5, A. Lysates (0.5 mg of protein) from untreated and treated cells were immunoprecipitated (IP) with antibodies against LYN or HCK and immunobeads. Controls containing only immunobeads (lanes Beads) were used to assess nonspecific binding and identify locations of immunoglobulin heavy chain proteins [IgG(H)]. First, proteins containing phosphotyrosine that were complexed with either LYN or HCK were detected by immunoblotting (IB) with antiphosphotyrosine antibodies (pY), blots were stripped, and then total LYN and HCK were detected with anti-LYN antibodies or anti-HCK antibodies, respectively. Positions of molecular weight standards are indicated on the left; molecular weights of the bands are indicated on the right.

LYN and HCK and mass spectroscopy analysis of tryptic peptides derived from LYN immunoprecipitates. From these analyses, we found evidence for unique site modifications in lysates of cells isolated from patients R10 and R12. Phosphorylation of LYN at residue tyrosine-396 in the activation site was detected in untreated specimens from all resistant patients (R1–R12), but phosphorylation at residue tyrosine-507 in the negative regulatory site was not detected in any patient. In two specimens, LYN tyrosine phosphorylation at an additional site—tyrosine-459 in patient R10 and tyrosine-193 in patient R12—was detected. The importance of phosphorylation at these sites is unknown. However, a homologous site in the SH2 domain of c-SRC (tyrosine-215) has been associated with the persistent activation of c-SRC kinase and with poor prognosis in breast cancer patients (44).

Decreased expression of LYN kinase mediated by siRNA induced CML cells taken from imatinib-sensitive and -resistant patients to apparently undergo apoptosis (42). We have previously noted (31) similar activity in cells overexpressing LYN kinase that were treated with LYN antisense oligonucleotide and have now confirmed these results by use of LYN siRNA silencing (Figure 1, C). We investigated whether LYN kinase affects survival of CML cells from both imatinib-sensitive and -resistant patients by use of mononuclear cells isolated from four imatinib-resistant CML patients (R3, R5, R10, and R12) and from four newly diagnosed CML patients (N1–N4). Cells from each patient were electroporated with a control or LYN siRNA, and then LYN kinase expression and cell survival were measured. LYN expression levels in all cell lysates were reduced 45%–85% by LYN siRNA treatment. For imatinib-resistant patients, LYN expression was reduced by 73% (95% CI = 43% to 100%), and for newly diagnosed CML patients, LYN expression was reduced by 63% (95% CI = 39% to 87%). Survival of cells from imatinib-resistant patients was reduced 48% (95% CI = 26% to 70%); however, survival of cells from newly diagnosed CML patients was reduced only 10% (95% CI = −5% to 24%) (Figure 5, D). These results were obtained from a single experiment (due to sample availability) and are averaged across all four patients. These results support an association between LYN expression and/or activation in the survival of cells from imatinib-resistant patients but not from newly diagnosed patients.

Because the mechanisms of action and key substrates associated with LYN-mediated CML cell survival have not been described, we examined the phosphoproteins complexed with LYN or HCK kinases in primary mononuclear cells from imatinib-resistant CML patient R10. Cells from this patient were treated with imatinib or dasatinib, and lysates were prepared. Complexes containing LYN and/or HCK kinases were isolated by use of immunobeads and anti-LYN or anti-HCK antibodies; phosphotyrosine-containing proteins, as well as LYN and HCK kinases, were identified by immunoblot analysis of the immunoprecipitates. Phosphorylation of both HCK and LYN was not affected by imatinib treatment but was suppressed by dasatinib treatment (Figure 6). Phosphoproteins of 80 and140 kDa were detected in both LYN and HCK complexes, indicating that common SRC family kinase phosphoprotein clusters may be present in primary CML cells. The association of the 140-kDa protein with LYN and HCK complexes or its tyrosine phosphorylation appeared to be more sensitive to imatinib treatment than the association or phosphorylation of the 80-kDa protein. In contrast, dasatinib treatment reduced the phosphorylation of both LYN and HCK kinases as well as the association or phosphorylation of both 80- and 140-kDa phosphoproteins. We have identified the 140-kDa protein as the E3-ligase, c-Cbl (through tryptic-phosphopeptide analysis by mass spectrometry of the excised phosphoprotein band) but have not been able to identify the 80-kDa phosphoprotein.

Discussion

In this report, we describe an association between LYN activation and imatinib resistance in some CML patients who had mutation-negative BCR-ABL genes. In the 12 imatinib-resistant CML patients studied, we detected highly activated LYN kinase and HCK kinase that was not suppressed by imatinib treatment. In the six imatinib-sensitive (intolerant) patients studied, imatinib fully suppressed phosphorylation of LYN and HCK kinases. Thus, the

failure of some CML patients to respond to imatinib appears to be associated with a BCR-ABL–independent mechanism of LYN and HCK activation. Activated LYN appears to play a direct role in the survival of imatinib-resistant CML cells but not in the survival of CML cells from newly diagnosed patients, for whom we do not have sufficient follow-up to determine imatinib response. These studies expand the spectrum of cellular changes that occur during imatinib therapy beyond outgrowth of cells with BCR-ABL point mutations and support the use of other tyrosine kinase inhibitors to treat a broad spectrum of imatinib-resistant CML patients. CML patients who do not respond to imatinib therapy (ie, hematologic resistance) frequently express a nonmutated form of BCR-ABL (12–16). Although we previously demonstrated (31) LYN overexpression and activation in cells isolated from CML patients with progressing disease and acquired resistance to imatinib, the role and extent of LYN associated with clinical response to imatinib was not fully defined.

Overexpression of LYN kinase in CML cells results in loss of imatinib sensitivity and characteristics (Figure 1, B) that resemble specific mutations in the BCR-ABL kinase domain (41). A three- to fourfold reduction in imatinib sensitivity was previously described for several common BCR-ABL mutations (including M244I, F317L, M351I, and E450K), which can be mimicked by LYN overexpression (Figure 1, B). However, a change in sensitivity to kinase inhibitors was not detected in LYN-overexpressing imatinib-resistant cells treated with dasatinib, indicating that agents targeting LYN and BCR-ABL kinases appear to overcome LYN-mediated imatinib resistance. This observation does not support previous hypotheses (32) that LYN overexpression induces global changes in apoptotic responsiveness and cell survival.

It has been proposed (45) that BCR-ABL expression in hematopoietic stem cells induces "oncogene addiction," which ultimately underlies the clinical activity of agents such as imatinib. When cells are able to survive BCR-ABL inhibition, this addiction may be transferred to other targets (46). In imatinib-resistant cells, LYN kinase may play a role in this phenomenon because its targeted silencing induces apoptosis, as observed in imatinib-resistant K562R cells (Figure 1) and imatinib-resistant cells from patients R3, R5, R10, and R12 (Figure 5, D), all of which have high levels of LYN expression. Others have shown (42) that LYN silencing decreases survival of CML cells from patients who are resistant to imatinib treatment or chemotherapy. However, we could not link LYN silencing to decreased survival of cells from newly diagnosed CML patients N1–N4 (Figure 5, D), indicating a distinct role for LYN in imatinib-resistant patients compared with imatinib-naive patients. The level of LYN expression may be an important threshold determinant of "addictive" LYN activity because, although full or partial LYN silencing was achieved by LYN siRNA in K562 cells and cells from imatinib-naive patients, respectively, there was limited apoptotic or proliferative change in these cells when compared with control cells (Figures 1 and 5, D). Analyses of LYN expression and LYN silencing in CML cells with distinct lineage characteristics from other well-characterized patients may resolve some of these issues because threshold LYN expression could have been altered by previous chemotherapies, exposure to imatinib, coexpression of other kinases, and other stress mechanisms (31,32,47,48). The use of compounds with a broader spectrum of action may prevent transferred addiction to other pathways, such as those involving LYN kinase and other SRC-family kinases.

Before enrollment in a clinical trial of dasatinib, we obtained the disease status of 68 CML patients and determined their BCR-ABL mutation status to assess whether LYN or HCK kinase was involved in clinical imatinib resistance. For the study of LYN regulation, we compared imatinib-sensitive patients with imatinib-resistant patients who expressed a nonmutated form of BCR-ABL, a mutated form that was not predominant (ie, 30% or less of the sequenced clones express the mutation) (patients R4 and R9), or a mutated form that was predicted from in vitro studies (43) to not affect imatinib activity (patients R4 and R10). In all imatinib-sensitive cell lines and patients, LYN and/or HCK activation was suppressed by imatinib (Figure 4, A–C). However, in mononuclear cells from imatinib-resistant patients who expressed nonmutated BCR-ABL, phosphorylation of BCR-ABL was inhibited by imatinib, whereas LYN and HCK phosphorylation was fully (or partially) independent of BCR-ABL. The inability of imatinib to inhibit LYN or HCK phosphorylation in these cells may prevent disengagement of all survival or "addictive" signals (Figures 1 and 6) (32,42). The higher clinical activity of dasatinib than of imatinib or inhibitors with higher BCR-ABL affinity (49) in patients with specific types of imatinib-resistant CML (41,50,51) indicates that SRC-family kinase inhibition may be an important therapeutic determinant.

Our analysis of patient specimens had several limitations. First, sample availability and access to patient material were limited and repetitive analyses were not usually possible. Therefore, sample analysis and response to kinase inhibitors were largely based on single experiments averaged across a patient population with similar clinical characteristics. Second, the concentration of tyrosine kinase inhibitors used in these ex vivo experiments to affect BCR-ABL and other kinases was in line with pharmacologically achievable levels of imatinib and dasatinib. However, the cellular concentration of each inhibitor may vary widely between patients and may only partially reflect the concentrations used in our experiments. Therefore, the effects described for kinase inhibitor activities in our patient specimen analysis may only partially reflect the clinical activity of imatinib or dasatinib.

Our analysis indicates that BCR-ABL kinase is not the primary upstream mediator of LYN or HCK activation in some CML patients. We did not detect *LYN* or *HCK* gene mutations or deletions but did detect unique LYN tyrosine phosphorylation sites—tyrosine-193 and -459—in cells from imatinib-resistant CML patients with myeloid or lymphoid disease, respectively. The clinical significance of this observation is unknown, but at least one homologous tyrosine phosphorylation site in c-SRC at tyrosine-215 has been associated with elevated kinase activity, increased tumorigenicity, and poor patient prognosis (44). It should be noted that these two LYN phosphotyrosine sites were recently detected by phosphoproteomic analysis of leukemic cells and normal and other tumor tissues (http://www.phosphosite.org), but no additional experimental details have been reported.

Two tyrosine phosphoproteins were detected in LYN and HCK immunoprecipitates from CML cells. One phosphoprotein was identified as the E3-ligase, c-Cbl, which has previously been shown to complex with BCR-ABL (52) and, through site-specific

tyrosine phosphorylation, to mediate the formation of signaling complexes (53). Other SRC-family kinases, including LYN, have been shown to regulate c-Cbl phosphorylation and stability (49,54,55), indicating some association among LYN, BCR-ABL, and c-Cbl (p140) (Figure 6). Evidence for a primary role for BCR-ABL in c-Cbl tyrosine phosphorylation was obtained with a CML primary specimen because c-Cbl phosphorylation was inhibited by both imatinib and dasatinib (Figure 6) (56). Tyrosine phosphorylation of both LYN and HCK kinases and their association with an 80-kDa protein was sensitive only to dasatinib; the importance of the latter observation has not yet been determined.

In this study, we observed BCR-ABL–independent activation of LYN in imatinib-resistant patients who had mutation-negative BCR-ABL. We also observed that LYN appears to play a direct role in CML cell survival. These results support the presence of more complex mechanisms of targeted drug resistance in CML patients than was expected from mutational studies of BCR-ABL. The association and mechanism of LYN activation in imatinib resistance warrants further study in additional patients.

References

1. Goldman JM, Melo JV. Chronic myeloid leukemia—advances in biology and new approaches to treatment. *N Engl J Med.* 2003;349(15):1451–1464.
2. Kantarjian H, Sawyers C, Hochhaus A, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. *N Engl J Med.* 2002;346(9):645–652.
3. Sawyers CL, Hochhaus A, Feldman E, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood.* 2002;99(10): 3530–3539.
4. Kantarjian HM, Cortes J, O'Brien S, et al. Imatinib mesylate (STI571) therapy for Philadelphia chromosome-positive chronic myelogenous leukemia in blast phase. *Blood.* 2002;99(10):3547–3553.
5. Gorre ME, Mohammed M, Ellwood K, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. *Science.* 2001;293(5531):876–880.
6. Branford S, Rudzki Z, Walsh S, et al. Detection of BCR-ABL mutations in patients with CML treated with imatinib is virtually always accompanied by clinical resistance, and mutations in the ATP phosphate-binding loop (P-loop) are associated with a poor prognosis. *Blood.* 2003;102(1): 276–283.
7. Shah NP, Nicoll JM, Nagar B, et al. Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell.* 2002;2(2):117–125.
8. von Bubnoff N, Schneller F, Peschel C, Duyster J. BCR-ABL gene mutations in relation to clinical resistance of Philadelphia-chromosome-positive leukaemia to STI571: a prospective study. *Lancet.* 2002;359(9305):487–491.
9. White DL, Saunders VA, Dang P, et al. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. *Blood.* 2006;108(2):697–704.
10. Gambacorti-Passerini C, Barni R, le Coutre P, et al. Role of alpha1 acid glycoprotein in the in vivo resistance of human BCR-ABL(+) leukemic cells to the abl inhibitor STI571. *J Natl Cancer Inst.* 2000;92(20): 1641–1650.
11. Jorgensen HG, Elliott MA, Allan EK, Carr CE, Holyoake TL, Smith KD. Alpha1-acid glycoprotein expressed in the plasma of chronic myeloid leukemia patients does not mediate significant in vitro resistance to STI571. *Blood.* 2002;99(2):713–715.
12. Hochhaus A, Kreil S, Corbin AS, et al. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. *Leukemia.* 2002; 16(11):2190–2196.
13. Lahaye T, Riehm B, Berger U, et al. Response and resistance in 300 patients with BCR-ABL-positive leukemias treated with imatinib in a single center: a 4.5-year follow-up. *Cancer.* 2005;103(8): 1659–1669.
14. Lange T, Park B, Willis SG, Deininger MW. BCR-ABL kinase domain mutations in chronic myeloid leukemia: not quite enough to cause resistance to imatinib therapy? *Cell Cycle.* 2005;4(12):1761–1766.
15. Khorashad JS, Anand M, Marin D, et al. The presence of a BCR-ABL mutant allele in CML does not always explain clinical resistance to imatinib. *Leukemia.* 2006;20(4):658–663.
16. Wei Y, Hardling M, Olsson B, et al. Not all imatinib resistance in CML are BCR-ABL kinase domain mutations. *Ann Hematol.* 2006;85(12): 841–847.
17. Radich JP, Dai H, Mao M, et al. Gene expression changes associated with progression and response in chronic myeloid leukemia. *Proc Natl Acad Sci USA.* 2006;103(8):2794–2799.
18. Neviani P, Santhanam R, Trotta R, et al. The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell.* 2005;8(5):355–368.
19. Miething C, Grundler R, Mugler C, et al. Retroviral insertional mutagenesis identifies RUNX genes involved in chronic myeloid leukemia disease persistence under imatinib treatment. *Proc Natl Acad Sci USA.* 2007;104(11): 4594–4599.
20. Danhauser-Riedl S, Warmuth M, Druker BJ, Emmerich B, Hallek M. Activation of Src kinases p53/56Lyn and p59hck by p210bcr/abl in myeloid cells. *Cancer Res.* 1996;56(15):3589–3596.
21. Roginskaya V, Zuo S, Caudell E, Nambudiri G, Kraker AJ, Corey SJ. Therapeutic targeting of Src-kinase Lyn in myeloid leukemic cell growth. *Leukemia.* 1999;13(6):855–861.
22. Hu Y, Liu Y, Pelletier S, et al. Requirement of Src kinases Lyn, Hck and Fgr for BCR-ABL1-induced B-lymphoblastic leukemia but not chronic myeloid leukemia. *Nat Genet.* 2004;36(5):453–461.
23. Wilson MB, Schreiner SJ, Choi HJ, Kamens J, Smithgall TE. Selective pyrrolo-pyrimidine inhibitors reveal a necessary role for Src family kinases in Bcr-Abl signal transduction and oncogenesis. *Oncogene.* 2002;21(53): 8075–8088.
24. Klejman A, Schreiner SJ, Nieborowska-Skorska M, et al. The Src family kinase Hck couples BCR/ABL to STAT5 activation in myeloid leukemia cells. *EMBO J.* 2002;21(21):5766–5774.
25. Lionberger JM, Wilson MB, Smithgall TE. Transformation of myeloid leukemia cells to cytokine independence by Bcr-Abl is suppressed by kinase-defective Hck. *J Biol Chem.* 2000;275(24):18581–18585.
26. Warmuth M, Bergmann M, Priess A, Hauslmann K, Emmerich B, Hallek MJ. The Src family kinase Hck interacts with Bcr-Abl by a kinase-independent mechanism and phosphorylates the Grb2-binding site of Bcr. *J Biol Chem.* 1997;272(52):33260–33270.
27. Stanglmaier M, Warmuth M, Kleinlein I, Reis S, Hallek M. The interaction of the Bcr-Abl tyrosine kinase with the Src kinase Hck is mediated by multiple binding domains. *Leukemia.* 2003;17(2):283–289.
28. Miething C, Mugler C, Grundler R, et al. Phosphorylation of tyrosine 393 in the kinase domain of Bcr-Abl influences the sensitivity towards imatinib in vivo. *Leukemia.* 2003;17(9):1695–1699.
29. Meyn MA, Wilson MB, Abdi FA, et al. Src family kinases phosphorylate the Bcr-Abl SH3-SH2 region and modulate Bcr-Abl transforming activity. *J Biol Chem.* 2006;281(41):30907–30916.
30. Donato NJ, Wu JY, Stapley J, et al. Imatinib mesylate resistance through BCR-ABL independence in chronic myelogenous leukemia. *Cancer Res.* 2004;64(2):672–677.
31. Donato NJ, Wu JY, Stapley J, et al. BCR-ABL independence and Lyn kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. *Blood.* 2003;101(2):690–698.
32. Dai Y, Rahmani M, Corey SJ, Dent P, Grant S. A Bcr/Abl-independent Lyn-dependent form of imatinib mesylate (STI-571) resistance is associated with altered expression of Bcl-2. *J Biol Chem.* 2004;279(33): 34227–34239.
33. Ito T, Tanaka H, Kimura A. Establishment and characterization of a novel imatinib-sensitive chronic myeloid leukemia cell line MYL, and an

imatinib-resistant subline MYL-R showing overexpression of Lyn. *Eur J Haematol.* 2007;78(5):417–431.
34. Hu Y, Swerdlow S, Duffy TM, Weinmann R, Lee FY, Li S. Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph+ leukemia in mice. *Proc Natl Acad Sci USA.* 2006;103(45):16870–16875.
35. Kitamura T, Koshino Y, Shibata F, et al. Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp Hematol.* 2003;31(11):1007–1014.
36. Besse A, Lamothe B, Campos AD, et al. TAK1-dependent signaling requires functional interaction with TAB2/TAB3. *J Biol Chem.* 2007;282(6): 3918–3928.
37. Schindler T, Bornmann W, Pellicena P, Miller WT, Clarkson B, Kuriyan J. Structural mechanism for STI-571 inhibition of abelson tyrosine kinase. *Science.* 2000;289(5486):1938–1942.
38. Buchdunger E, Zimmermann J, Mett H, et al. Inhibition of the Abl protein-tyrosine kinase in vitro and in vivo by a 2-phenylaminopyrimidine derivative. *Cancer Res.* 1996;56(1):100–104.
39. Shah NP, Tran C, Lee FY, Chen P, Norris D, Sawyers CL. Overriding imatinib resistance with a novel ABL kinase inhibitor. *Science.* 2004; 305(5682):399–401.
40. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med.* 2006;354(24): 2531–2541.
41. Azam M, Latek RR, Daley GQ. Mechanisms of autoinhibition and STI-571/imatinib resistance revealed by mutagenesis of BCR-ABL. *Cell.* 2003; 112(6):831–843.
42. Ptasznik A, Nakata Y, Kalota A, Emerson SG, Gewirtz AM. Short interfering RNA (siRNA) targeting the Lyn kinase induces apoptosis in primary, and drug-resistant, BCR-ABL1(+) leukemia cells. *Nat Med.* 2004; 10(11):1187–1189.
43. Burgess MR, Skaggs BJ, Shah NP, Lee FY, Sawyers CL. Comparative analysis of two clinically active BCR-ABL kinase inhibitors reveals the role of conformation-specific binding in resistance. *Proc Natl Acad Sci USA.* 2005;102(9):3395–3400.
44. Vadlamudi RK, Sahin AA, Adam L, Wang RA, Kumar R. Heregulin and HER2 signaling selectively activates c-Src phosphorylation at tyrosine 215. *FEBS Lett.* 2003;543(1-3):76–80.
45. Weinstein IB, Joe AK. Mechanisms of disease: oncogene addiction—a rationale for molecular targeting in cancer therapy. *Nat Clin Pract Oncol.* 2006;3(8):448–457.
46. Sharma SV, Gajowniczek P, Way IP, et al. A common signaling cascade may underlie "addiction" to the Src, BCR-ABL, and EGF receptor oncogenes. *Cancer Cell.* 2006;10(5):425–435.
47. Hibbs ML, Harder KW. The duplicitous nature of the Lyn tyrosine kinase in growth factor signaling. *Growth Factors.* 2006;24(2):137–149.
48. Kaabeche K, Lemonnier J, Le Mee S, Caverzasio J, Marie PJ. Cbl-mediated degradation of Lyn and Fyn induced by constitutive fibroblast growth factor receptor-2 activation supports osteoblast differentiation. *J Biol Chem.* 2004;279(35):36259–36267.
49. Quintas-Cardama A, Kantarjian H, Jones D, et al. Dasatinib (BMS-354825) is active in Philadelphia chromosome-positive chronic myelogenous leukemia after imatinib and nilotinib (AMN107) therapy failure. *Blood.* 2007;109(2):497–499.
50. Hochhaus A, Kantarjian HM, Baccarani M, et al. Dasatinib induces notable hematologic and cytogenetic responses in chronic-phase chronic myeloid leukemia after failure of imatinib therapy. *Blood.* 2007;109(6): 2303–2309.
51. Cortes J, Rousselot P, Kim DW, et al. Dasatinib induces complete hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in blast crisis. *Blood.* 2007;109(8): 3207–3213.
52. Gaston I, Johnson KJ, Oda T, et al. Coexistence of phosphotyrosine-dependent and -independent interactions between Cbl and Bcr-Abl. *Exp Hematol.* 2004;32(1):113–121.
53. Bhat A, Kolibaba K, Oda T, Ohno-Jones S, Heaney C, Druker BJ. Interactions of CBL with BCR-ABL and CRKL in BCR-ABL-transformed myeloid cells. *J Biol Chem.* 1997;272(26):16170–16175.
54. Tanaka S, Neff L, Baron R, Levy JB. Tyrosine phosphorylation and translocation of the c-cbl protein after activation of tyrosine kinase signaling pathways. *J Biol Chem.* 1995;270(24):14347–14351.
55. Grishin A, Sinha S, Roginskaya V, et al. Involvement of Shc and Cbl-PI 3-kinase in Lyn-dependent proliferative signaling pathways for G-CSF. *Oncogene.* 2000;19(1):97–105.
56. Patel H, Marley SB, Gordon MY. Detection in primary chronic myeloid leukaemia cells of p210BCR-ABL1 in complexes with adaptor proteins CBL, CRKL, and GRB2. *Genes Chromosomes Cancer.* 2006;45(12):1121–1129.
57. O'Hare T, Walters DK, Stoffregen EP, et al. In vitro activity of Bcr-Abl inhibitors AMN107 and BMS-354825 against clinically relevant imatinib-resistant Abl kinase domain mutants. *Cancer Res.* 2005;65(11):4500–4505.

Funding

National Institutes of Health (PO1 CA49639); Leukemia and Lymphoma Society (CF01-313, 6118).

Notes

The authors wish to thank the patients for agreeing to participate in this study and the clinical staff and faculty in the Department of Leukemia, The M. D. Anderson Cancer Center, for assistance with specimen collection. The authors had full responsibility for designing the study, the collection of data, analysis and interpretation of the data, the decision to submit the manuscript for publication, and writing of the manuscript. M. Talpaz is on the speakers list for Novartis and Bristol Myers Squibb and participated in clinical studies funded by these two companies.

Manuscript received May 30, 2007; revised April 24, 2008; accepted May 8, 2008.