Evidence that Resistance to Nilotinib May Be Due to BCR-ABL, Pgp, or Src Kinase Overexpression

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Abstract

Targeting the tyrosine kinase activity of Bcr-Abl is an attractive therapeutic strategy in chronic myeloid leukemia (CML) and in Bcr-Abl–positive acute lymphoblastic leukemia. Whereas imatinib, a selective inhibitor of Bcr-Abl tyrosine kinase, is now used in frontline therapy for CML, second-generation inhibitors of Bcr-Abl tyrosine kinase such as nilotinib or dasatinib have been developed for the treatment of imatinib-resistant or imatinib-intolerant disease. In the current study, we generated nilotinib-resistant cell lines and investigated their mechanism of resistance. Overexpression of BCR-ABL and multidrug resistance gene (MDR-1) were found among the investigated mechanisms. We showed that nilotinib is a substrate of the multidrug resistance gene product, P-glycoprotein, using verapamil or PSC833 to block binding. Up-regulated expression of p53/56 Lyn kinase, both at the mRNA and protein level, was found in one of the resistant cell lines and Lyn silencing by small interfering RNA restored sensitivity to nilotinib. Moreover, failure of nilotinib treatment was accompanied by an increase of Lyn mRNA expression in patients with resistant CML. Two Src kinase inhibitors (PP1 and PP2) partially removed resistance but did not significantly inhibit Bcr-Abl tyrosine kinase activity. In contrast, dasatinib, a dual Bcr-Abl and Src kinase inhibitor, inhibited the phosphorylation of both BCR-ABL and Lyn, and induced apoptosis of the Bcr-Abel cell line overexpressing p53/56 Lyn. Such mechanisms of resistance are close to those observed in imatinib-resistant cell lines and emphasize the critical role of Lyn in nilotinib resistance. [Cancer Res 2008;68(23):9809–16]

Introduction

Chronic myeloid leukemia (CML) is characterized by the presence of the Philadelphia (Ph) chromosome (22q−) that results from a t(9;22)(q34;q11) reciprocal translocation (1, 2). Twenty-five percent of acute lymphoblastic leukemias (ALL) in adults are also characterized by this translocation. The Ph chromosome contains a BCR-ABL hybrid gene, the molecular hallmark of CML and Ph-positive ALL (3). BCR-ABL encodes an oncogenic fusion protein of 190, 210, or 230 kDa, depending on the breakpoint on the BCR gene. The unifying feature of all these Bcr-Abl fusion proteins is their deregulated protein tyrosine kinase activity that is responsible for leukemogenesis in vitro and in vivo (4, 5). Targeting the tyrosine kinase activity of Bcr-Abl is an attractive therapeutic strategy in CML or in BCR-ABL–positive ALL that has recently found success with the development of new drugs such as tyrosine kinase inhibitors. Imatinib mesylate (previously known as STI571) is a Bcr-Abl tyrosine kinase inhibitor that competes with ATP for binding to the Ab1 kinase domain and stabilizes the protein in its closed, inactive conformation, thereby inhibiting its activity (6, 7). The impressive effects of imatinib in CML have been clinically demonstrated and confirmed in several large trials (8), and now imatinib is the common treatment of CML in chronic phase. However, imatinib resistance, which was predicted by previous in vitro studies, is now a well-recognized problem, particularly in the advanced phase of the disease. One of the main mechanisms of resistance in patients with CML is mutation in the Bcr-Ab1 tyrosine kinase domain. Nilotinib and dasatinib, the second-generation tyrosine kinase inhibitors, have been developed to override this phenomenon (9, 10).

In an attempt to study the potential mechanism of resistance to nilotinib, we generated nilotinib-resistant cells from different Ph-positive cell lines (K562, AR230, and LAMA84). Characterization of nilotinib-resistance mechanisms identified Bcr-Abl and/or multidrug resistance P-glycoprotein (Pgp) overexpression. In addition, up-regulated expression of the Src family kinase, p53/56 Lyn, was detected in nilotinib-resistant K562 cells. Silencing of p53/56 Lyn by small interfering RNA (siRNA) in nilotinib-resistant K562 cells restored their sensitivity to nilotinib. Finally, we investigated Lyn expression by quantitative reverse-transcriptase PCR in nilotinib-resistant patients. Our data suggest that BCR-ABL–positive cells can evade the inhibitory effect of nilotinib by several mechanisms, similar to those observed with imatinib.

Materials and Methods

Reagents. RPMI 1640, FCS, PBS, trypan blue, and antibiotics were purchased from Invitrogen. Imatinib and nilotinib were kindly provided by Novartis Pharma. MTS was purchased from Promega. The inhibitors PP1 and PP2 were purchased from Calbiochem (VWR). Verapamil and PSC833 were purchased from Sigma (St. Louis, MO) and from Novartis Pharma. The following antibodies were used: 4G10 anti-phosphotyrosine and anti-phosphorylated STAT5 (1:1H2; Euromedex), anti-Abl 8E9 (PharMingen, Becton Dickinson), A-2066 anti-actin (Sigma Aldrich), anti-Hck (N-30), anti-Lyn (H-6 and 44), anti-Becl2 (L-19), anti-STAT5 (G-2) and Bcl-2 (8C8) (Santa Cruz Biotechnology), and antihuman Pgp (clone UIC2; Immunotech).
Cell lines. Three BCR-ABL–positive human cell lines were used in this study: K562, LAMA84, and AR230. Nilotinib-resistant cell lines were derived as previously described for imatinib and designated LAMA84-rn, AR230-rn, and K562-rn (11). Cells were maintained in RPMI 1640 supplemented with 10% FCS, 2 mmol/L of L-glutamine, 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin (referred to in this article as "RF-10") at 37°C in a humidified atmosphere containing 5% CO2. Aliquots were taken at 24 h intervals for assessment of cell viability by trypan blue exclusion (Sigma Aldrich).

Generation of nilotinib-resistant cell lines in liquid culture. Cell lines maintained in culture as described above were gradually exposed to increasing concentrations of nilotinib, at a rate of 0.1-nmol/L increments for every 10 days of culture. After approximately 3 months, sublines of cells growing in 20 nmol/L of nilotinib were maintained continuously in culture with this dose of the inhibitor. The parental, sensitive cell lines were maintained in parallel cultures without nilotinib to be used as controls.

Cell proliferation assay. Cell proliferation was measured by the number of viable cells using MTS tetrazolium (Cell Titer96 Aqueous, Promega). Washed cells in RPMI 1640 were plated (10⁴ cells) in quadruplicate into microtiter-plate wells plus various doses of inhibitors as indicated (imatinib, nilotinib, verapamil, PSC833, PP1, or PP2). The measure of viability was performed on a 4-day kinetic, and each day, 20μL of MTS was added to the wells. After 2 h of incubation at 37°C, the plates were read in a microplate autoreader (Dynex Technologies) at a 490 nm wavelength. The mean results of the four-well set are standardized in comparison with the initial absorbance at day 0. All the experiments were repeated at least three times.

Lyn silencing. To inhibit Lyn protein expression, 2×10⁶ cells were washed twice in cold PBS and transfection was performed according to the manufacturer's instructions using the protocol T03 (Amaza AG). Cells were seeded at 2×10⁵/mL in RF-10 for 24 h, after which cells were separated into two batches and incubated in the absence or in the presence of 20 nmol/L of nilotinib. Aliquots of the culture were harvested daily for protein expression analysis and measurement of cell viability by triplicate trypan blue exclusion counts. Experiments were performed no longer than 4 days and were repeated twice. Cell viability is presented as the mean of the triplicate for one representative experiment.

Western blot analysis. Protein lysates were prepared according to Kabarowski and colleagues (12). Protein concentration was measured by the bicinchoninic acid protein assay (Pierce) and the lysates were stored at −80°C. Approximately 25 μg of proteins were resolved on 8% SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes (Bio-Rad) by semidry electrophoretic transfer, probed with individual antibodies and visualized by the enhanced chemiluminescence system (Perkin-Elmer). For immunoprecipitation of Lyn, 400 μg of protein lysates were preclared with 30 μL of 50% slurry protein A–sepharose by incubation for 1 h at 4°C. After centrifugation, the supernatant was incubated with 2 μg of Lyn

Figure 1. Proliferation of nilotinib-resistant cell lines. Proliferation of the nilotinib-sensitive (s) and resistant cell lines (rn) AR230-rn, K562-rn, and LAMA84-rn were tested in MTS assays in the presence of increasing concentrations of nilotinib (A). Points, mean of the absorbance of the four-well set standardized in comparison with the starting absorbance at day 0, which is directly proportional to the number of viable cells. Bcr-Abl expression was detected by Western blots. Immunoblots using anti-Abl and anti-actin antibodies (B) were performed on cell lysates from parental (s) and nilotinib-resistant (rn) cells. Arrows, target proteins detected. C, quantification of Bcr-Abl expression was performed by densitometry. Bcr-Abl level expression was normalized with actin as loading control. Columns, mean fold increase expression of Bcr-Abl by calculating the ratio of the resistant cells to their sensitive counterparts.
Relativquantification was performed by using GUS resolved as described above. The A–sepharose was solubilized with SDS loading buffer and samples were after three washes with lysis buffer, the pellet of protein A–sepharose. After three washes with lysis buffer, the pellet of protein A–sepharose was solubilized with SDS loading buffer and samples were resolved as described above. The in vitro kinase assay was performed as described (13).

Patients and BCR-ABL or Lyn quantification. Seven CML patients in chronic phase (n = 2) or in accelerated phase (n = 5; two males, five females) with Ph chromosome and BCR-ABL–positive CML following the failure of imatinib or dasatinib were investigated. The duration of therapy was 3 to 36 months, and all patients were analyzed at the beginning or before nilotinib and under treatment at the moment of failure. Resistance was defined by progression or absence of hematologic and cytogenetic response. To assess molecular responses, total RNA was extracted from peripheral blood cells and BCR-ABL transcript levels were quantified and normalized to endogenous Abh using quantitative real-time reverse-transcriptase PCR, according to recommendations recently proposed for harmonization of results and as we have already reported (11). Quantitative real-time reverse-transcriptase PCR to amplify LYN transcripts was carried out on the same cDNA using a forward primer 5’-AAGTTGGTGAAAAGGCTTGG-3’ and a reverse primer 5’-GCCACCTTGGTACTTGTGA-3’ from LYN gene in the Universal ProbeLibrary (UPL#60, MGB probe) according to the manufacturer’s instructions (Roche Diagnostics). With a serial 10-fold dilution series for cDNA from a patient’s sample, the assay was found to be linear over at least five orders of magnitude (slope: −3.414; intercept, 35.81). Expression of relative quantification was performed using GUS as endogenous control as previously described (14). Analysis was done by comparative Ct method giving the amount of target normalized to the endogenous reference and relative to the same pool of peripheral blood lymphocytes as the calibrator. For K562 cell lines, LYN gene expression was measured using the method described above. Statistical analysis. A Mann-Whitney test was used to calculate differences between means; differences were considered significant at P < 0.05.

Results

Generation of nilotinib-resistant cell lines. Ph-positive cell lines (AR230, LAMA84, and K562) were grown in the presence of increasing concentrations of nilotinib over a period of 3 months to generate resistant cell lines. Resistance was defined as the capacity to survive in the continuous presence of the highest dose of nilotinib. Indeed, the final concentration of 20 nmol/L of nilotinib was chosen in relation to the dose administered for CML patient treatment. The proliferation of the parental cell lines AR230, LAMA84, and K562 were compared, respectively, with the nilotinib-resistant lines AR230-rn, LAMA84-rn, and K562-rn, which were able to grow in liquid culture until 20 nmol/L of nilotinib (Fig. 1A). For instance, the IC50s for AR230-rn and K562-rn were 15 and 30 nmol/L, respectively, for nilotinib compared with 1 and 5 nmol/L, respectively, for their sensitive counterparts. We also tested the effect of imatinib on nilotinib-resistant cell lines, showing that the sensitivity to imatinib was also modified in the nilotinib-resistant cell lines (results not shown). However, LAMA84-rn exhibits a pattern of proliferation different from the other cell lines probably due to its mechanism of resistance whereas its sensitive parental cell line LAMA84 is the most sensitive (Fig. 1A).

Some resistant cell lines overexpress Bcr-Abl. The level of Bcr-Abl protein in the different nilotinib-sensitive and -resistant cell lines was studied by immunoblotting. Albeit both AR230 and LAMA84 cell lines did not express endogenous Abl, the nilotinib-resistant cell lines showed a significant increase of Bcr-Abl compared with the sensitive parental line as we have previously described for imatinib, although it was less pronounced. With regard to the human cell lines, the overall results show that
resistance to nilotinib may be mediated at least by up-regulated expression of Bcr-Abl (Fig. 1B and C). The K562 nilotinib-resistant cell line, K562-rn, expressed a similar level of Bcr-Abl compared with its parental counterpart (no significant difference in the level of Bcr-Abl was detected on four separate experiments) and this was confirmed by flow cytometry detection of Abl (data not shown). The 2-fold and 3-fold increased expression of Bcr-Abl detected, respectively, in AR230-rn and LAMA84-rn were statistically significant on four separate experiments and was accompanied by an increase in the phosphorylation status of STAT5 (Supplementary Fig. S2A). In addition, no mutation in the BCR-ABL kinase domain was detected.

**Nilotinib is a substrate of Pgp.** Overexpression of Pgp (encoded by the multidrug resistance gene; MDR-1) can functionally modify the uptake of several drugs including imatinib as we have already reported (15). We have tested the well-characterized K562/DOX cell line that displays resistance to several drugs by overexpressing Pgp. The resistance of K562/DOX to nilotinib was reversed by simultaneous incubation with either verapamil (Ip50 = 0.5 mmol/L) or PSC833 (Ip50 = 0.1 mmol/L) confirming that nilotinib is a substrate of the Pgp (Fig. 2A). We therefore studied the pattern of Pgp expression by flow cytometry in the different nilotinib-resistant cell lines and found that LAMA84-rn expressed the MDR-1 gene product, Pgp, indicating that at least two mechanisms of resistance operate in this cell line, both a significant overexpression of Bcr-Abl and Pgp (Fig. 2B). Indeed, verapamil incubation of LAMA-rn cells only partially restores the sensitivity to nilotinib as this resistant cell line also overexpressed Bcr-Abl. The two other nilotinib-resistant cell lines, AR230-rn and K562-rn, were not positive for Pgp expression as tested by flow cytometry (results not shown).

The K562 nilotinib-resistant cell line exhibits Lyn kinase overexpression. In order to investigate whether resistance to nilotinib is associated with tyrosine kinase expression, Western blot analysis was performed using antiphosphotyrosine antibody. Nilotinib-resistant cell lines, such as AR230-rn and LAMA84-rn, harbored a phosphotyrosine pattern similar to their sensitive counterpart. In contrast, in K562-rn cells, the pattern of protein tyrosine phosphorylation shows different phosphorylated bands at 27, 40, 53, 56, 75, and 100 kDa (Fig. 3A). Investigation of kinase identity shows an overexpression of the tyrosine kinase Lyn (Fig. 3B) with a 10-fold increase (n = 6, P < 0.05), whereas no significant difference was detected for Hck. According to the report of Dai and colleagues, Lyn overexpression...
was accompanied by an increase of Bcl-2 expression (16). This overexpression was also detected at the mRNA level by quantitative reverse-transcriptase PCR showing that the normalized Lyn expression was 0.48 and 4.84 in K562 and K562-rn, respectively. We detected the level of Lyn expression and activity after nilotinib withdrawal for 5 days. Both Lyn expression and activity were similar in K562-rn grown with or without nilotinib, although the \textit{in vitro} kinase activity of Lyn was higher in K562-rn in comparison with the nilotinib-sensitive K562 (Fig. 4C and D). In an attempt to prove that Lyn kinase is involved in the resistance to nilotinib in K562-rn cells, two inhibitors, PP1 and PP2, were tested. K562 cells were sensitive to nilotinib as previously shown but the proliferation was not significantly affected by the presence of both Src kinase inhibitors PP1 or PP2 (Fig. 4A). In contrast, PP1 and PP2 (2 μmol/L) dramatically modified the sensitivity of K562-rn cells (Fig. 4B). The IC\textsubscript{50} of nilotinib for K562 was 2 to 5 nmol/L and not modified after either PP1 or PP2 addition. For K562-rn and K562-rn plus PP1 or PP2 the IC\textsubscript{50} were 40 nmol/L and decreased to 20 nmol/L, respectively.

The K562 nilotinib-resistant cell line overexpressing Lyn kinase is sensitive to dasatinib. We also investigated the effect of dasatinib, a multitargeted kinase inhibitor with activity against Bcr-Abl and the Src family kinases, on cell viability by proliferation assay. Both K562 and K562-rn proliferation were inhibited by dasatinib, suggesting that this inhibitor overrides the advantage of Lyn overexpression (Fig. 5A). At the same time, we compared the effects of 2 μmol/L of imatinib, 20 nmol/L of nilotinib, 10 μmol/L of PP1, 10 μmol/L of PP2, and 200 nmol/L of dasatinib in both K562 and K562-rn cells on whole cell tyrosine phosphorylation using Western blotting. Following incubation for 3 hours, dasatinib strongly decreased the whole pattern of tyrosine phosphorylation in both cell lines, especially the bands at 210, 140, 55, and 39 kDa (Fig. 5B). Imatinib and nilotinib in the same conditions were able to decrease Bcr-Abl and Abl phosphorylation in K562 cells but weakly so in K562-rn, suggesting the participation of other kinases in Bcr-Abl phosphorylation. These inhibitors did not change the phosphorylation of Lyn. The incubation of K562-rn cells with Src kinase inhibitors PP1 or PP2 weakly decreased the whole phosphorylation pattern, although Lyn phosphorylation was not decreased, suggesting that Lyn overexpression masks the inhibitory effect of PP1 or PP2 (Fig. 5). However, a decrease of Lyn and STAT5 tyrosine phosphorylation was seen with higher concentrations of PP1 or PP2 (40 μmol/L; data not shown; Supplementary Fig. S2B and C). As suspected, the two inhibitors, PP1 and PP2, did not significantly modify the proliferation of LAMA-rn or AR230-rn cell lines.

Silencing Lyn restored the sensitivity to nilotinib. Cell lines (K562 and K562-rn) were transfected by control or targeted siRNA and grown for 24 hours in RF-10. After this period, each sample was divided into two parts and grown in the absence or in the presence of 20 nmol/L of nilotinib. In parallel, a proliferation assay was set up on both transfected cell lines either with control siRNA or anti-Lyn siRNA. From day 2, Lyn expression was measured by Western blot each day until day 4. The siRNA anti-Lyn induced a large decrease of Lyn expression in the K562 cell line, whereas the control siRNA did not modify the expression. The proliferation of both the control and anti-Lyn siRNA-transfected K562 cells was inhibited by nilotinib (Fig. 6B). In contrast, the sensitivity to nilotinib was not modified by the control siRNA in K562-rn cells, whereas the anti-Lyn siRNA dramatically decreases the expression of Lyn (60%) which restored the sensitivity to nilotinib. These results confirm the critical role of Lyn in the nilotinib-resistant K562-rn cell line.

\textit{Lyn} and \textit{Bcr-Abl} transcript expression in nilotinib-resistant patients. Using Quantitative reverse-transcriptase PCR, we analyzed \textit{Lyn} kinase mRNA expression in seven nilotinib-resistant
patients before and after resistance to nilotinib (Supplementary Table S1). For two patients (nos. 4 and 7), a significant increase of Lyn expression was observed after resistance acquisition. For two patients (nos. 1 and 3), a slight increase was found. In three other patients (nos. 2, 5, and 6), no significant variation was observed. Of note, two out of three patients displayed mutations in the Bcr-Abl kinase domain. Bcr-Abl mRNA was increased under nilotinib treatment, illustrating the progression of the disease of these patients.

Discussion

In the work reported here, we used Bcr-Abl–positive cell lines to generate sublines with differential sensitivity to nilotinib and investigated the mechanisms of resistance. Using a strategy similar to the one used previously to characterize imatinib-resistant cells, we compared the different clones and studied the mechanisms involved. Regarding MDR-1 gene expression, this mechanism has already been reported in imatinib-resistant cell lines (15, 17, 18). Similar behaviors in the development of resistance to other chemotherapeutic agents have been described (19, 20). The most extensively studied mediator of this phenomenon is the Pgp protein encoded by the MDR-1 gene, which affects the uptake of a soluble compound by “pumping out” the drugs through the plasma membrane. Measurement of Pgp expression in the three nilotinib-resistant cell lines showed that one of them, LAMA84-rn, did indeed overexpress Pgp, in comparison to the LAMA84 nilotinib-sensitive line. The inhibition of Pgp with verapamil or PSC833, potent blockers of the pump, led to an improved uptake of nilotinib in LAMA84-rn and resulted in a weak reduction of its IC$_{50}$ (21). It is important to note that inhibition of Pgp resulted in the enhancement of the sensitivity of LAMA84-rn to higher doses of nilotinib, but was not sufficient to fully overcome their resistance to the concentration in which they normally survive. This is most likely due to the fact that this cell line carries a second, independent mechanism of resistance, i.e., Bcr-Abl overexpression, which, on its own, is able to sustain the basic resistant phenotype as has previously been described (15, 22). We recently reported that polymorphisms of the MDR-1 gene are involved in the response to imatinib in patients with CML, and it will be worthwhile to study its involvement in nilotinib response, although a similar role is clearly suspected (23).

Both AR230-rn and LAMA84-rn cell lines showed up-regulated expression of Bcr-Abl (2-fold and 3-fold, respectively) and such Bcr-Abl overexpression could be bypassed by the additional use of Src inhibitors such as CGP76030 (22). Up-regulated expression of Bcr-Abl was accompanied by an increase of the phosphorylated form of STAT5 in nilotinib-resistant cells. In addition to the higher level of Bcr-Abl detected in LAM84-rn than in the AR230-rn cell line, the expression of the MDR-1 gene in LAM84-rn emphasized the results

Figure 5. Dasatinib is able to overcome resistance to nilotinib Lyn overexpressing cells. Proliferation of nilotinib-sensitive K562 and the nilotinib-resistant cell line K562-rn was tested using the MTS assay in the presence of increasing concentration of dasatinib (A). Results are expressed as the mean absorbance of the four-well set standardized in comparison with the starting absorbance at day 0, which is directly proportional to the number of viable cells. The results are representative of three independent experiments. The whole tyrosine phosphorylation pattern was detected on nilotinib-sensitive K562 and nilotinib-resistant K562-rn cells incubated for 3 h at 37°C with vehicle alone, 2 µmol/L of imatinib, 20 nmol/L of nilotinib, 10 µmol/L of PP1, 10 µmol/L of PP2, and 200 nmol/L of dasatinib. After one wash, cell pellets were solubilized and proteins separated by SDS-PAGE. After immunoblotting with antiphosphotyrosine, membranes were stripped and reprobed for the kinase Lyn and actin as loading control (B). Arrows, target proteins detected.
of the proliferation assay. Indeed, our results suggest that as more Bcr-Abl is expressed, more inhibitor is required. In addition to the already known mechanisms of resistance, the most interesting one was the up-regulated expression of the Lyn tyrosine kinase. An overexpression of two Src kinases, Lyn and Hck, was reported by Donato and colleagues in the same cell line resistant to imatinib and in CML blast-crisis patients (24). However, the peculiar K562 cell line resistant to imatinib in their study underepressed Bcr-Abl, as shown both by the decrease of Bcr-Abl expression and signaling. In this study, the nilotinib-resistant K562 cell line expresses a similar level of Bcr-Abl, suggesting that a down-regulation of Bcr-Abl is not a direct consequence of Lyn overexpression. The overexpression of Lyn detected in the K562-rn cell line was quantified as a 10-fold increase. It will be interesting to elucidate the mechanism of overexpression and the triggered modification of signaling pathways. The role of Lyn in the resistance to nilotinib was emphasized by the effect of PP1 or PP2, two compounds well characterized for Src kinase inhibition, although the former also inhibits Bcr-Abl (25). Moreover, treatment of the K562-rn cell line with SU6656, a Src kinase inhibitor, decreased both the whole cell tyrosine phosphorylation and viability (data not shown). A partial restoration of nilotinib sensitivity was detected in proliferation assays of K562-rn incubated with PP1 or PP2. Although Src kinase inhibitors PP1 or PP2 weakly decreased the whole phosphorylation pattern, Lyn phosphorylation was not decreased, suggesting that Lyn overexpression masks the inhibitory effect of PP1 or PP2 (Fig. 5). However, a decrease of Lyn and STAT5 tyrosine phosphorylation was seen with higher concentrations of PP1 or PP2 (40 μmol/L; data not shown; Supplementary Fig. S2B and C). Although dasatinib inhibits STAT5 in both K562 and K562-rn cells, high concentrations of nilotinib had no effect (results shown in Supplementary Fig. S2D). Another study, reported by Dai and colleagues, described two Bcr-Abl-independent imatinib-resistant cell lines (K562 and LAMA) in which Lyn overexpression was associated with Bcl-2 expression, an antiapoptotic protein involved in imatinib resistance (16). In the nilotinib-resistant K562-rn cells, the 10-fold increase in Lyn expression was associated with an increase of Bcl-2 expression, whereas no significant modification of Bcl-xL was detected. It is well known that Lyn kinase is involved in cytokine signaling pathways in many hematopoietic cell types and such overexpression may be able to bypass the inhibitory effect of nilotinib on Bcr-Abl (26, 27). A possible role of Lyn may be to increase the oncogenic potential of Bcr-Abl by the phosphorylation of specific domains, as it was reported for the SH3-SH2 domains or the Y177 site for the recruitment of adaptors (28, 29). It is noticeable that the whole tyrosine phosphorylation of Bcr-Abl is similar in both cell lines although K562-rn overexpressed the kinase Lyn. However, the detection of specific phosphorylation(s) may help to discern whether some are more phosphorylated in K562-rn cells. Targeting Lyn in K562-rn did not change the total BCR-ABL phosphorylation. However, dasatinib, the dual Src and Bcr-Abl inhibitor strongly reduces the whole tyrosine phosphorylation

Figure 6. Silencing Lyn restores nilotinib sensitivity. K562 and K562-rn cells were transfected with 5 μg of siRNA corresponding to a negative control siRNA (Neg) or Lyn siRNA (Lyn). Cells were seeded at 2 × 10⁵/mL in RF-10 for 24 h after which they were separated into two batches and incubated in the absence (−) or in the presence (+) of 20 nmol/L of nilotinib. Aliquots of the culture were harvested daily for protein expression analysis and measurement of cell viability by triplicate trypan blue exclusion counts. Proteins were solubilized and separated by SDS-PAGE. After electrotransfer, membranes were probed for the kinase Lyn and actin as a loading control (A). Arrows, proteins detected. Proliferation of nilotinib-sensitive K562 and nilotinib-resistant K562-rn cells transfected with a negative control siRNA (Control) or Lyn siRNA (Lyn) was tested by the MTS assay in the presence of increasing concentrations of nilotinib (B). Points, mean of the absorbance of the four-well set standardized in comparison with the starting absorbance at day 0, which is directly proportional to the number of viable cells, and presented as the mean of the quadruplicate for one representative experiment.
pattern of K562-rn cells and triggers apoptosis. This result strongly suggests that dasatinib targets are involved in the resistance to nilotinib in K562-rn cells. In light of the recent report concerning the chemical proteomic profiles of Bcr-Abl inhibitors, dasatinib is able to interact with at least 32 kinases (30). Among these, the tyrosine kinases Btk and Tec were inhibited by nanomolar concentrations of dasatinib (31). However, a similar level of Btk was detected in both cell lines. The critical role of Lyn in nilotinib resistance is shown by silencing Lyn expression in K562-rn cells that is accompanied by a complete restoration of nilotinib sensitivity. However, it will be worthwhile to investigate the potential involvement of a Btk pathway downstream of Lyn, as it is already known in B cell signaling.

Overall, the results presented in this study show that most, similar to those reported for imatinib such as Bcr-Abl overexpression, MDR-1 expression, or Src kinase up-regulated expression. However, we cannot exclude the participation of another tyrosine kinase in the mechanism of nilotinib resistance in the peculiar K562-rn cell line, in which a critical role of Lyn was shown. Moreover, this is the first study showing an overexpression of Lyn kinase and its implication in nilotinib resistance. From a clinical point of view, the three tyrosine kinase inhibitors exhibit different patterns of side effects. In addition, imatinib-resistant CML patients who develop resistance against nilotinib may still show a response to dasatinib.

Disclosure of Potential Conflicts of Interest

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