Gene expression profiling of imatinib and PD166326-resistant CML cell lines identifies Fyn as a gene associated with resistance to BCR-ABL inhibitors

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Abstract

Imatinib is used to treat chronic myelogenous leukemia (CML), but resistance develops in all phases of this disease. The purpose of the present study was to identify the mode of resistance of newly derived imatinib-resistant (IM-R) and PD166326-resistant (PD-R) CML cells. IM-R and PD-R clones exhibited an increase in viability and a decrease in caspase activation in response to various doses of imatinib and PD166326, respectively, as compared with parental K562 cells. Resistance involved neither mutations in BCR-ABL nor increased BCR-ABL, MDR1 or Lyn expression, all known modes of resistance. To gain insight into the resistance mechanisms, we used pangenomic microarrays and identified 281 genes modulated in parental into the resistance mechanisms, we used pangenomic microarrays and identified 281 genes modulated in parental versus IM-R and PD-R cells. The gene signature was similar for IM-R and PD-R cells, accordingly with the cross-sensitivity observed for both inhibitors. These genes were functionally associated with pathways linked to development, cell adhesion, cell growth, and the JAK-STAT caspase-8 (24–33). Imatinib has emerged as the leading compound to treat CML. It targets the ATP-binding site of different tyrosine kinases including BCR-ABL, the platelet-derived growth factor receptor (5), and C-KIT (6). Imatinib selectively induces growth arrest and apoptosis of BCR-ABL–positive leukemia cells with minimal effect on normal hematopoietic progenitors (7–9). Of note, this agent has proven very effective in patients with chronic phase or blast crisis (8). Although treatment with imatinib achieves complete hematologic remission in the great majority of patients with CML, total cytogenetic and molecular responses are relatively rare events (11). Moreover, resistance to imatinib is a common future in patients with accelerated phase or blast crisis (12). To investigate the possible mechanisms of resistance to imatinib, several groups have reported the isolation and characterization of resistant human CML cell clones following prolonged culture in progressively increasing concentrations of the BCR-ABL inhibitor (13, 14). Careful studies of these clones and of cells from imatinib-resistant patients have led to the conclusion that resistance to imatinib can be accounted for by point mutations in the bcr-abl gene, resulting in single amino-acid changes in the tyrosine kinases AXL and Fyn as well as CD44 and HMGA2. Small interfering RNA experiments and pharmacologic approaches identified FYN as a candidate for resistance to imatinib. Our findings provide a comprehensive picture of the transcriptional events associated with imatinib and PD166326 resistance and identify Fyn as a new potential target for therapeutic intervention in CML. [Mol Cancer Ther 2009;8(7):1924–33]

Introduction

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disease characterized by an acquired cytogenetic abnormality resulting from the translocation t(9;22) (q34;q11). This translocation fuses the bcr and the c-abl genes (1). The resulting p210 BCR-ABL protein encodes an oncoprotein endowed with constitutive tyrosine kinase activity that phosphorylates numerous substrates, and activates multiple signaling pathways, including PI3K/Akt, Ras/MEK/ERK, JAK/STAT, and nuclear factor-κB. By doing so, p210BCR-ABL confers growth factor–independent proliferation, survival of myeloid progenitor cells, and inhibition of apoptosis (2–4). Imatinib has emerged as the leading compound to treat CML. It targets the ATP-binding site of different tyrosine kinases including BCR-ABL, the platelet-derived growth factor receptor (5), and C-KIT (6). Imatinib selectively induces growth arrest and apoptosis of BCR-ABL–positive leukemia cells with minimal effect on normal hematopoietic progenitors (7–9). Of note, this agent has proven very effective in patients with chronic phase or blast crisis (8). Although treatment with imatinib achieves complete hematologic remission in the great majority of patients with CML, total cytogenetic and molecular responses are relatively rare events (11). Moreover, resistance to imatinib is a common future in patients with accelerated phase or blast crisis (12). To investigate the possible mechanisms of resistance to imatinib, several groups have reported the isolation and characterization of resistant human CML cell clones following prolonged culture in progressively increasing concentrations of the BCR-ABL inhibitor (13, 14). Careful studies of these clones and of cells from imatinib-resistant patients have led to the conclusion that resistance to imatinib can be accounted for by point mutations in the bcr-abl gene, resulting in single amino-acid substitutions that generally occur in the ATP binding pocket, rare BCR-ABL, gene amplification leading to increased expression of p210 BCR-ABL (15–18), or increased expression of the MDR1 gene-encoded P-glycoprotein 1 (19). More recently, other types of resistance independent of BCR-ABL and linked to an overexpression of the Src tyrosine kinases
Lyn and Hck have been reported (20, 21). Therefore, multiple causes of resistance seem to exist and warrant being searched for. In the present study, we generated several imatinib- and PD166326 (dual BCR-ABL and Src kinase inhibitor)-resistant K562 clones that exhibit drastic resistance to both inhibitors. Resistance does not seem to be linked to either mutations or increased expression of BCR-ABL, or up-regulation of MDRI or Lyn kinase, all previously reported modes of resistance. Gene expression profiling reveals the modulation of a specific set of genes which includes the Src kinase Lyn. The possible function of these different genes is discussed in relation with resistance to BCR-ABL inhibitors.

Materials and Methods

Reagents and Antibodies
Imatinib and PD166326 were provided by Novartis Pharma and Pfizer, respectively. RPMI 1640 medium and FCS were purchased from Gibco BRL. Sodium fluoride, sodium orthovanadate, phenylmethylsulfonyl fluoride, aprotonin, and leupeptin were purchased from Sigma. Ac-DEVD-AMC, Ac-LEHD-AMC, Ac-DEVD-CHO, and Ac-LEHD-CHO were from Alexis Biochemicals. Anti-phospho-C-ABL (Tyr 245), anti-SPARC, and anti-MCAM antibodies were from Cell Signaling Technology, Haematologic Technologies Inc., and BD Biosciences, respectively. Anti-HSP60, HSP90, VDAC1, cABL, PIM1, AXL, LYN, Fyn, HMGA2, and anti-p27 antibodies were purchased from Santa Cruz Biotechnology. Anti-phospho-Akt (Ser 473), anti-CD44-FITC and anti-CD36-FITC monoclonal antibodies were purchased from Dako.

Cell Lines
The human CML cell line K562 was purchased from the American Type Culture Collection and was grown at 37°C under 5% (v/v) CO2 in RPMI 1640 medium supplemented with 5% (v/v) FCS, 50 units/mL penicillin, 50 μg/mL streptomycin, and 1 mmol/L sodium pyruvate.

Generation of Imatinib and PD166326 K562 Cell Lines
From the K562 cell line, we established several imatinib and PD166326-resistant clones by addition in the culture medium of increasing concentrations of imatinib or PD166326, respectively. Briefly, cells were gradually exposed to increasing concentrations of imatinib or PD166326 starting from 100 nmol/L imatinib and 1 nmol/L PD166326. Concentrations of inhibitors were doubled every 3 mo. Cells that continued to grow in the presence of 10 μmol/L imatinib and 30 nmol/L PD166326 were obtained and cloned by limited dilution. The IC50 value for the more resistant imatinib cell clone (III-F5) was >20 μmol/L versus 0.5 μmol/L in parental cells, and the IC50 value for the more resistant PD166326 cell clone (IV-H2) was >20 nmol/L versus 1 nmol/L in parental cells. The parental sensitive cell line was maintained in parallel cultures without imatinib and PD166326.

Caspase Activity Measurement
After stimulation, cells were lysed for 30 min at 4°C in lysis buffer (22), and lysates were cleared at 10,000 g for 15 min at 4°C. Each assay was done with 25 μg of protein. Cellular extracts were incubated in a 96-well plate with Ac-DEVD-AMC (7-amino-4-methylcoumarin) or Ac-LEHD-AMC (0.2 mmol/L) for various times at 37°C as described earlier (22).

Cell Viability (XTT)
Cells (15 × 103 cells/100 μL) were incubated in 96-well plates with different effectors for the times indicated. Fifty microliters of XTT reagent (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate) were added to each well.

Flow Cytometry
After stimulation, cells were washed with ice-cold PBS and incubated at 4°C for 30 min in 100 μL of PBS containing 0.1% (w/v) bovine serum albumin with anti-CD44 or anti-CD36-FITC monoclonal antibodies. After a wash with PBS, fluorescence was measured by using the FL1 channel of a fluorescence-activated cell sorting apparatus (FACScan; Becton-Dickinson).

Western Blot
Western blot analysis has been described elsewhere (22).

Assessment of the Cell Cycle by Flow Cytometry
Cells were exposed to either 1 μmol/L imatinib or nmol/L PD166326 for 24 h at 37°C. Thereafter, cells were washed, fixed in citrate buffer, and finally left a minimum of 18 h at ~20°C. Next, cells were incubated in a Glycine/NaCl buffer containing 0.05% (w/v) NP-40, 7.5 μg/mL RNase A, and 16 μg/mL of propidium iodide for 1 h at 4°C. Cell distribution across the different phases of the cell cycle was analyzed with a FACScan (Becton-Dickinson).

Knockdown of Fyn by siRNA
Parental or imatinib-resistant cells were transfected with control siRNA or siRNA-Fyn using the Lipofectamine RNAiMax protocol. Cells were seeded in medium without antibiotics at 1 × 105 cells per well in 6-well tissue culture plates with different effectors for the times indicated. Fifty microliters of siRNA reagent was added to each well.

Microarray Experiments
RNAs were extracted using the RNeasy kit Mini (Qiagen) and quantified by nanodrop spectrophotometry. RNA quality was evaluated using the Agilent Bioanalyzer 2100 and Lab-on-Chip Nano 6000 chip (ratio of the 28S/18S RNA ≥ 1.5). Pangenomic microarrays were printed using human Réseau National des Génopoles/Medical Research Council oligonucleotide collection as described earlier (23). The list of the 25,299 probes spotted on the microarray is available on http://www.microarray.fr (follow the link to “human national set”). RNA were labeled and hybridized as described previously (24). Two biological replicates were done for each comparison inverting Cyanine dyes to reduce the impact of dye bias, following a design in which the three cell lines have been compared in pairs. Experimental data and associated microarray designs have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO).
Candidate Genes for Resistance to BCR-ABL Inhibitors

Statistical Analysis

The data were normalized by the print tip lowess method (within-array normalization method) and by quantile (between-array normalization method) using the LimmaGUI package in the software package R Bioconductor (25). Means of ratios from all comparisons were calculated for each gene, and B test analysis was done using the Limma package available from Bioconductor. We voluntarily restricted our analysis to genes that exhibited $\geq 1.5$-fold modulation and a “B value” (Log Odds) >0. All normalized data sets were registered in the GEO database under the accession number GSE10382.

Biological Theme Analysis

Ontologies attached to each modulated gene were then used to classify them according to main biological themes. To this end, we used the Expression Analysis Systematic Explorer (EASE) program, available at http://david.niaid.nih.gov/david/ease.htm (26). The data were analyzed for themes using the gene ontology cellular compartment, molecular function, and biological process provided by NCBI (27). All significant ($P \leq 0.05$) categories resulting from each list produced the overrepresented biological themes shown.
in Supplementary Table S1. Ingenuity software (http://www.ingenuity.com/) and Mediante (28), an information system containing diverse information about our probes set and the data sets (http://www.microarray.fr), were also used to find gene links and build biological networks.

Results

Selection and Characterization of BCR-ABL Inhibitor-resistant K562 Cell Clones

We generated two sets of K562 cell clones resistant to imatinib or PD166326, two BCR-ABL inhibitors. All the tested clones showed a strong increase in cell viability and a decrease in caspase 3 activation in response to 1 μmol/L imatinib or 3 nmol/L PD166326 compared with parental K562 cells (Fig. 1A to F). Among these clones, an imatinib-resistant (IM-R; III-F5) and a PD166326-resistant (PD-R; IV-H2) were selected for further studies because they exhibited the highest degree of resistance and gain of viability in the presence of imatinib and PD166326, respectively. Both clones exhibited a >20-fold reduction in imatinib sensitivity compared with K562 cells together with >12-fold decrease in PD166326 sensitivity (29). We (30) and others (7, 31, 32) have shown that imatinib induced caspase activation in parental and K562-resistant clones (Fig. 1C and D). Resistance to inhibitors was illustrated by the abrogation of caspase 3 and 9 activities (Fig. 1E and F) and PARP cleavage in IM-R and PD-R clones (Fig. 1G).

BCR-ABL Inhibitor-mediated Cell Cycle Arrest Is Impaired in IM-R and PD-R Clones

Imatinib blocks K562 cells in the G0/G1 phase of cell cycle (33). Hence, we investigated the effect of imatinib and PD166326 on the regulation of cell cycle in parental, IM-R, and PD-R K562 cells. A decrease of the cell fraction with fully replicated DNA (G2/M) occurs within 24 hours following imatinib or PD166326 addition into K562 cell cultures (Fig. 2A). This correlated with an increased proportion of cells in the G0/G1 phase of cell cycle. As also shown in Fig. 2A, BCR-ABL inhibitor–mediated growth arrest preceded apoptosis because no significant increase in the number of cells in the Sub-G1 phase of the cell cycle was detected in the presence of imatinib or PD166326. Imatinib- and PD166326-mediated growth arrest correlated with accumulation of the p27 cyclin inhibitor in K562 cells (Fig. 2B). Neither a decrease in G2/M nor a p27 accumulation was detected in IM-R and PD-R cells upon imatinib or PD166326 treatment. These findings show that BCR-ABL inhibitor–induced cell cycle arrest is impaired in resistant clones.

Resistance of IM-R and PD-R Cell Clones Likely Occurs Independently of BCR-ABL

The molecular mechanisms of IM-R and PD-R clone resistance did not involve mutations in the BCR-ABL kinase domain (not shown), and BCR-ABL expression was identical in parental and IM-R and PD-R clones (Fig. 3A). Importantly, dephosphorylation of BCR-ABL by imatinib and PD166326 occurred similarly in parental and resistant K562 cells. Although the phospho-ABL status is identical in the three series of cells, the overall phosphotyrosine protein status was different. Noticeably, phosphorylation of proteins with molecular mass in the 50 to 60 kDa range was altered in resistant versus parental K562 cells treated with the BCR-ABL inhibitors (not shown). Importantly, phosphoproteins in the 50 to 60 kDa range likely corresponded to Src kinases, the implications of which in imatinib resistance have been previously reported (34). Indeed, Lyn is overexpressed and activated in the acute phase of CML and its up-regulation correlates with disease progression and resistance in patients treated with imatinib (20, 21, 35). Therefore, we analyzed Lyn expression in parental, IM-R, or PD-R K562 cell clones. We were unable to detect any increase in Lyn expression in IM-R or PD-R cells (Fig. 3B). Unexpectedly, we found a drastic reduction in the level of the 56-kDa Lyn isoform. MDR-1 overexpression is another reported mode of resistance in CML cell lines (19, 36). However, we failed to detect any modification of MDR-1 level in resistant clones compared with parental K562 cells.

Figure 2. Imatinib and PD166326 fail to induce growth arrest in IM-R and PD-R. K562 WT, PD-R, and IM-R cells (0.25 × 10^6/mL) were incubated for 24 h at 37°C with 1 μmol/L imatinib or 3 nmol/L PD166326. For analysis of cell cycle by flow cytometry (A), cells were harvested, washed, and then fixed in citrate buffer at −20°C for a minimum of 18 h. Cells were then incubated for 45 min with labeling buffer containing propidium iodide and immediately analyzed by flow cytometry. Histograms represent the percentage of cells in each phase of cell cycle (subG1, G0/G1, S, and G2/M). Results represent one representative experiment done three times. B, for Western blot analysis of p27, 100 μg of protein were subjected to SDS-PAGE (10% polyacrylamide) followed by immunoblot analysis. HSP60 was used as a loading control.
In conclusion, resistance to BCR-ABL inhibitors in IM-R and PD-R cells does not rely on increased expression of BCR-ABL, Lyn, or MDR-1, strongly suggesting additional modes of resistance, which remain to be identified.

Comparison of Gene Expression Profiles in Parental, IM-R, and PD-R K562 Cells

To gain insight into the potential mechanisms of resistance to BCR-ABL inhibitors, we next analyzed gene expression profiles of parental, IM-R, and PD-R K562 clones using pangenomic microarrays (23). These experiments were done on two independent cell cultures for each clone and gave rise to the diagram presented in Fig. 4A. We found that 281 genes, corresponding to 1% of the total set of genes present on the biochip, were differentially expressed between parental and IM-R or PD-R (Supplemental Table S1). Importantly, a majority of these genes was found similarly modulated in the two sets of clones (Fig. 4A). Indeed, of the 194 up-regulated genes, 110 were common to imatinib- and PD166326-resistant clones, whereas 54 genes were down-regulated in both resistant clones (Fig. 4B). The nature of the modulated genes is depicted in Supplemental Table S1. The EASE program (1, 26) associated with our information system “Mediante” (28) was used to identify important biological themes overrepresented among differentially expressed genes between wild-type and resistant clones. These themes were essentially linked to “development,” “regulation of cell growth,” “cell adhesion,” and “regulation of JAK-STAT cascade.” We were also interested in understanding how individual genes were integrated into specific regulatory and signaling networks. Biologically relevant networks were drawn from the lists of genes that were similarly up-regulated or down-regulated in the two resistant clones through the use of Ingenuity Pathways Analysis. The pathway shown in Supplemental Fig. S1 was identified as being the most significant among the up-regulated genes, containing 21 focus genes, and illustrated the increased proportion of genes associated with the P53/ATF and p38 MAPK pathways. The expression of several highly modulated genes was next evaluated by Western blotting or flow cytometry. As shown in Fig. 5A, we confirmed the overexpression of p59 Fyn, AXL, SPARC, MCAM, and HMGA2 in IM-R and PD-R resistant cell lines as well as the down-regulation of PIM1, in agreement with the microarray data. Finally, flow cytometric measurements showed up-regulation of CD44 and CD36 in both resistant clones (Fig. 5B). Overexpression of some of these proteins was confirmed in several independent IM-R and PD-R clones as illustrated in Fig. 5C and D for CD44 and HMGA2, confirming that overexpression is due to the resistant status of our cell clones and not to clonal differences.

Involvement of Fyn Overexpression in the Resistance to Imatinib Mesylate

Recently Ban et al. reported that Fyn was significantly increased in CML blast crisis as compared with chronic phase and that BCR-ABL overexpression results in up-regulation of Fyn protein and mRNA (37). We thus investigated the

![Figure 3](http://example.com/Fig3.png)
effect of Fyn overexpression on imatinib resistance of our IM-R and PD-R clones using both pharmacologic approaches and siRNA silencing. A complete knockdown of Fyn was evidenced 3 days following the addition of Fyn siRNA (SiFyn; Fig. 6A). Parental K562, IM-R, and PD-R were therefore incubated for 2 days with control (si-CT) or siFyn and cell metabolism was determined 2 days later. Fyn knockdown was sufficient to resensitize IM-R and PD-R cells to imatinib (Fig. 6). Importantly, treatment of IM-R and PD-R cells with the Src kinase inhibitor PP2 also significantly reduced cell metabolism in IM-R and PD-R K562 cells (Fig. 6C). Altogether, our findings show that overexpression of Fyn is an important determinant of imatinib resistance in BCR-ABL inhibitor-resistant K562 cell clones. We next used the phospho-Src(Y416) antibody to analyze the phosphorylation status of Lyn and Fyn in parental, IM-R, and PD-R clones treated with PD166326 (Fig. 6D). Three phosphorylated bands corresponding to P59Fyn and P56/53Lyn were detected in K562 cells. The 56-kDa band was not seen in IM-R and PD-R cells in agreement with the decreased expression of Lyn observed in Fig. 3B. Of note, P59Fyn was strongly phosphorylated in IM-R and PD-R cells treated with PD166326, indicating that Fyn is activated in these cells and is further increased in the presence of PD166326. Finally, sequencing of Fyn in the three cell lines ruled out the possibility that constitutive activation of P59Fyn in resistant cells could be due to activating mutations (not shown).

Discussion

Here we investigated the mode of resistance of IM-R and PD-R K562 cell clones to imatinib and PD166326 treatment. PD166326 is a nanomolar pyridopyrimidine inhibitor of ABL kinase effective against resistant BCR-ABL mutants. Conversely to imatinib, PD166326 also inhibits Src kinases and as such may exert additional effects on CML cell lines (38, 39). IM-R and PD-R cells exhibited a drastic decrease in imatinib and PD166326 sensitivity as shown by cell metabolism and caspase assays. In addition, they showed cross-resistance to both kinase inhibitors, highlighting similarities in their mode of inhibition of BCR-ABL. Importantly,
resistance was linked neither to overexpression or increased activity of P210 BCR-ABL, nor to mutations in the tyrosine kinase domain of BCR-ABL in IM-R and PD-R clones (not shown). These findings strongly suggest that resistance of both clones is independent of BCR-ABL. We therefore looked for other possible modes of resistance, and in a first approach we focused our attention on downstream signaling pathways. Of note, increased expression and activity of the Src kinases Hck and Lyn are responsible for some forms of imatinib resistance in patients treated with imatinib (20, 21). Moreover, Lyn overexpression is associated with the advanced phase of the disease (21). Obviously, overexpression of the tyrosine kinase Lyn is not involved in the lack of sensitivity of our IM-R and PD-R clones to BCR-ABL inhibitors because we failed to detect any increase in the expression of Lyn. Rather, Lyn expression was reduced in imatinib-resistant versus sensitive CML cell lines and especially the p56 Lyn isoform (Fig. 4C). The preferential extinction of p56 Lyn expression in resistant clones is currently not understood.

To gain insight into the possible mechanisms of resistance, we carried out pangenomic microarrays to identify genes differentially modulated in parental versus IM-R and PD-R cells. The gene signature was very similar for both IM-R and PD-R clones. Imatinib targets BCR-ABL, c-KIT, and the platelet-derived growth factor receptor, whereas PD166326 behaves as a dual BCR-ABL and Src kinase inhibitor (2, 3, 38, 39). The observation that most of the differentially expressed genes are comodulated in IM-R and PD-R clones is consistent with the action of both inhibitors at the level of BCR-ABL. Regarding PD166326, the difference in gene expression could be ascribed to its ability to inhibit Src kinases. Several gene profiling data have been

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**Figure 5.** Protein expression of some modulated genes. A, K562 WT, PD-R, and IM-R cells \(1 \times 10^6\) were harvested, washed, and finally lysed. Protein \((100 \mu g)\) was subjected to SDS-PAGE followed by immunoblot analysis. HSP60 were used as a loading control. B, cells \((1 \times 10^6)\) were harvested, washed, and labeled using anti-CD44-FITC or anti-CD36-FITC antibody. Cells were analyzed by flow cytometry. C, the same clones were analyzed for CD44 expression by Western blotting. HSP60 served as an invariant loading control.
Fyn expression was analyzed by Western blot 2, 3 and 4 d after si-RNA addition. HSP60 was used as a loading control. B, cells (10^5/mL) were incubated for a total of 4 d at 37°C with si-CT or si-Fyn. Imatinib 1 μmol/L was added for the last 2 d before determination of cell viability using the XTT assay as described in Materials and Methods. Results are the mean ± SE of three different experiments done in triplicate. C, cells (10^5/mL) were incubated for 2 d with 3 μmol/L PP2, a Src kinase inhibitor, and cell metabolism was measured by the XTT assay. Results are the mean ± SE of three different experiments made in triplicate. D, cells (10^5/mL) were incubated for 30 min at 37°C with various concentrations of PD166326. Fyn and Lyn phosphorylation was determined by immunoblot analysis with anti-phospho-Src (Y416) or anti-Fyn antibody.

Recently reported that are aimed at comparing sensitive with resistant CML cell lines or blood cells from CML patients (40, 41). Tipping et al. identified by Affymetrix microarray analysis of the imatinib-resistant KCL22 CML cell line, several genes associated with the suppression of apoptosis (42). Among the 29 up-regulated genes found, only PGST1 was detected in our list of differentially expressed genes. In another study, Mahadevan et al. reported the up-regulation of 20 genes in an imatinib-resistant gastrointestinal stromal tumor cell line (43). Overexpression of AXL was also detected in our screening. The genes for which modulation has been confirmed (Fig. 5) have all been previously involved in cell proliferation. This is consistent with the resistant status of the IM-R and PD-R clones. Moreover, most of these genes are known oncogenes or are modulated in human cancers (44–46).

Among all the potential resistance genes identified in the present study we focused our attention on Fyn. Indeed, if the contribution of the Src kinases Lyn and Hck in BCR-ABL–mediated leukemogenesis and resistance to BCR-ABL inhibitors is well established, little is known about the role of Fyn in this context. Recently Ban et al. (37) reported, using tissue arrays, that Fyn expression is increased in CML blast crisis as compared with chronic-phase patients. Moreover, BCR-ABL overexpression results in an up-regulation of Fyn protein and mRNA. These authors also show that knockdown of Fyn with small hairpin RNA dampens cell growth and leads to increased sensitivity to imatinib, suggesting that BCR-ABL mediates up-regulation of Fyn in CML blast crisis compared with chronic phase. In agreement with these results, we show that knockdown of Fyn with specific siRNA or inhibition of Src kinase activity by the Src inhibitor PP2, a Src kinase inhibitor, and cell metabolism was measured by the XTT assay. Results are the mean ± SE of three different experiments made in triplicate. D, cells (10^5/mL) were incubated for 30 min at 37°C with various concentrations of PD166326. Fyn and Lyn phosphorylation was determined by immunoblot analysis with anti-phospho-Src (Y416) or anti-Fyn antibody.

Figure 6. Fyn knockdown or inhibition resensitizes IM-R and PD-R K562 cells to imatinib. K562, IM-R, and PD-R cells (10^5/mL) were incubated for various times with a control si-RNA (si-CT) or a si-RNA specifically directed against Fyn (si-Fyn) at 37°C. A, Fyn expression was analyzed by Western blot 2, 3 and 4 d after si-RNA addition. HSP60 was used as a loading control. B, cells (10^5/mL) were incubated for a total of 4 d at 37°C with si-CT or si-Fyn. Imatinib 1 μmol/L was added for the last 2 d before determination of cell viability using the XTT assay as described in Materials and Methods. Results are the mean ± SE of three different experiments done in triplicate. C, cells (10^5/mL) were incubated for 2 d with 3 μmol/L PP2, a Src kinase inhibitor, and cell metabolism was measured by the XTT assay. Results are the mean ± SE of three different experiments made in triplicate. D, cells (10^5/mL) were incubated for 30 min at 37°C with various concentrations of PD166326. Fyn and Lyn phosphorylation was determined by immunoblot analysis with anti-phospho-Src (Y416) or anti-Fyn antibody.
Regarding Fyn overexpression, it is tempting to speculate that during the selection process, which is initiated with low concentrations of BCR-ABL inhibitors, emergent resistant cells slowly increase the expression of Fyn to counteract the effect of BCR-ABL inhibition. Importantly, this augmentation in Fyn expression is accompanied by hyperphosphorylation of Fyn, which is further enhanced in the presence of BCR-ABL inhibitors. This activation of Fyn might allow cells to resist increasing doses of imatinib or PD166326, leading to selection of clones with high level of Fyn expression. Altogether, our findings show that overexpression and increased activity of Fyn is an important determinant of imatinib resistance in BCR-ABL inhibitor–resistant K562 cell clones.

In conclusion, gene expression profiling of resistant cells identifies several new genes associated with resistance to BCR-ABL inhibitors. Our results underline the important role of Fyn in resistance to imatinib and PD166326, although further studies are needed to better define the importance of the proteins encoded by these genes in CML progression and resistance to tyrosine kinase inhibitors. But our findings bring new and important information concerning the mechanisms of resistance of CML cell lines, and reveal new proteins potentially involved in resistance to BCR-ABL inhibitors. This may have important implication for the establishment of future therapies in CML, and possibly, other hematopoietic malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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