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

Sébastien Grosso,1,2,5 Alexandre Puissant,1,2,5 Maeva Dufies,1,2,5 Pascal Colosetti,1,2,5 Arnaud Jacquel,6 Kevin Lebrigand,2,7 Pascal Barbry,2,7 Marcel Deckert,2,3 Jill Patrice Cassuto,4 Bernard Mari,2,7 and Patrick Aubeger1,2,5,4

1INSERM U895, Mediterranean Center for Molecular Medicine, Cell Death, Differentiation and Cancer Team 2, Faculté de Médecine, Nice Cedex 2, France; 2Université de Nice Sophia-Antipolis, and 3INSERM U576 and 4Service d’Hématologie Clinique, Hopital de l’Archet, Nice, France; 5Équipe Labellisée par la Ligue Nationale contre le Cancer, Paris, France; 6INSERM U876, Faculté de Médecine, Dijon, France; and 7CNRS UMR 6097, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France

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 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 cascade. Especially relevant were the increased expression of 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 p210BCR-ABL protein encodes an oncoprotein endowed with constitutive tyrosine kinase activity that phosphorylates numerous substrates, and activates multiple signaling pathways, including PI3Kinase/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 in chronic phase of CML (10) and to a lesser extent, in patients in accelerated phase and 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 feature 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,
aprotinin, and leupeptin were purchased from Sigma.
Ac-DEVD-AMC, Ac-LEHD-AMC, Ac-DEVD-CHO, and
Ac-LEHD-CHO were from Alexis Biochemicals. Anti-phos-
pho-C-ABL (Tyr 245), anti-SPARC, and anti-MCAM antibodies
were from Cell Signaling Technology, Haematologic Technolo-
gies 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-CD44-FITC and anti-CD36-FITC monoclonal antibodies
were from BD Biosience, and horseradish peroxi-
dase-conjugated antibodies were 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 strep-
tomycin, 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 ex-
posed to increasing concentrations of imatinib or
PD166326 starting from 100 nmol/L imatinib and 1 nmol/L
PD166326. Concentrations of inhibitors were doubled every
week. After 3 mo, cells that continued to grow in the presence
of 10 μmol/L imatinib and 30 nmol/L PD166326 were ob-
tained 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 × 10^3 cells/100 μL) were incubated in 96-well
plates with different effectors for the times indicated. Fifty
microliters of XTT reagent (sodium 3-[1-(phenylamino-
carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sul-
fonylic 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 ana-
lyzed 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 × 10^5 cells per well in 6-well tissue culture
plate and transfected with siRNA at a final concentration
of 50 nmol/L. Cells were harvested 0, 2, 3, or 4 d after
the beginning of the transfection for protein expression. Two
days after transfection, cells were treated or not with imati-
nib for 2 supplemental days before addition of XTT reagent.
The siRNA sequences used were CT: CUGGAGUUGUCC-
CAAUUCU, and Fyn: GAUGUGAGCGACGCUAU.

Microarray Experiments

RNAs were extracted using the RNeasy kit Mini (Qiagen)
and quantified by nanodrop spectrophotometry. RNA qual-
ity 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 hu-
man 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
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. Ex-
perimental data and associated microarray designs have
been deposited in the National Center for Biotechnology
Information (NCBI) Gene Expression Omnibus (GEO)
**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 ≥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 ≤ 0.05) categories resulting from each list produced the overrepresented biological themes shown.
in Supplementary Table S1. Ingenuity software (http://www.ingenuity.com/) and Mediane (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 K562 cells, through a mitochondria-dependent pathway. Therefore, we analyzed the effect of imatinib and PD166326 on caspase 3 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.
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. 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 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 PI3K/AKT 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](https://example.com/fig3.png) BCR-ABL expression and phosphorylation is not modified in IM-R and PD-R cells. A, K562 WT, PD-R, and IM-R cells (0.25 x 10⁶/mL) were incubated for 30 min at 37°C with 1 μmol/L imatinib or 3 nmol/L PD166326. One hundred micrograms of protein were subjected to SDS-PAGE (8% polyacrylamide) followed by immunoblot analysis. BCR-ABL phosphorylation and expression were visualized using anti-phospho-ABL and anti-ABL antibodies, respectively. HSP90 was used as a loading control. B, cells (10⁶/mL) were harvested, washed, and finally lysed. Proteins (100 μg) were subjected to SDS-PAGE followed by immunoblot analysis with a specific anti-Lyn antibody. HSP60 was used as a loading control. C, cells (10⁷/mL) were harvested, washed, and labeled with anti-PGP-PE antibody. They were then analyzed by flow cytometry. Doxorubicin-resistant K562 cells were used as positive control of PGP expression.
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

![Figure 5](https://example.com/figure5.png)

**Figure 5.** Protein expression of some modulated genes. A, K562 WT, PD-R, and IM-R cells (1 x 10^6) were harvested, washed, and finally lysed. Protein (100 µg) was subjected to SDS-PAGE followed by immunoblot analysis. HSP60 were used as a loading control. B, cells (1 x 10^6) were harvested, washed, and labeled using anti-CD44-FITC or anti-CD36-FITC antibody. Cells were analyzed by flow cytometry. C, cell clones were harvested, washed, and finally labeled using anti-CD44-FITC antibody and analyzed by flow cytometry. D, the same clones were analyzed for HMGA2 expression by Western blotting. HSP60 served as an invariant loading control.
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 1μmol/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 done 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 resensitized IM-R and PD-R K562 cells to imatinib. Taken together, our findings are in favor of an important role of Fyn in the resistance to BCR-ABL inhibitors. Ban et al. also reported that most of the CML cells from patients in blast crisis were positive for Fyn, whereas the majority of chronic and accelerated specimens were negative (37). Of note, BaF3 cells expressing imatinib point mutants of BCR-ABL retained the ability to up-regulate Fyn (37). Thus, it seems that overexpression of Fyn is linked to acutization. We established here that Fyn is up-regulated in imatinib- and PD166326-resistant K562 cell lines, linking Fyn overexpression to acquisition of resistance to BCR-ABL inhibitors. This is coherent with the fact that resistance to imatinib is a common feature in patients with accelerated phase or blast crisis (12) and with its potential role in transformation (37). The IM-R K562 cells described by Donato et al. retained active BCR-ABL kinase activity that was inhibitable by imatinib (21), suggesting that BCR-ABL was not coupled to proliferation or survival of K562-R cells. They concluded that acquired resistance may be associated with BCR-ABL independence and to up-regulation of Lyn. Interestingly, following an identical protocol, we generated IM-R and PD-R K562 cells that developed BCR-ABL–independent resistance but likely through the overexpression of the tyrosine kinase Fyn. The reasons for the selection of IM-R clones with elevated level of Lyn (21) or Fyn are unclear, but our findings strengthen the notion that increased Src kinase expression is important for acquired resistance to BCR-ABL inhibitors. In conclusion, alternative signaling pathways may be engaged in CML cells as a compensatory mechanism to BCR-ABL inhibition. In the continued presence of BCR-ABL inhibitors, CML cells may increase expression of different tyrosine kinases in order to resist the antileukemic effect of imatinib or PD166326. This may favor independent tyrosine kinase pathways such as those described in the present study (increase in Fyn and AXL expression, for instance) that can reduce the BCR-ABL dependence and allow the cell to survive in adverse conditions, i.e. in the presence of the drugs.
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.

Acknowledgments
We are indebted to Pr Emmanuel Van-Obberghen (INSERM U907, Nice, France) for reviewing the manuscript.

References


Molecular Cancer Therapeutics

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

Sébastien Grosso, Alexandre Puissant, Maeva Dufies, et al.


Updated version  Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-09-0168

Cited articles  This article cites 46 articles, 30 of which you can access for free at:
http://mct.aacrjournals.org/content/8/7/1924.full.html#ref-list-1

Citing articles  This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/8/7/1924.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.