Clinical Resistance to STI-571 Cancer Therapy Caused by BCR-ABL Gene Mutation or Amplification

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Clinical studies with the Abl tyrosine kinase inhibitor STI-571 in chronic myeloid leukemia demonstrate that many patients with advanced stage disease respond initially but then relapse. Through biochemical and molecular analysis of clinical material, we find that drug resistance is associated with the reactivation of BCR-ABL signal transduction in all cases examined. In six of nine patients, resistance was associated with a single amino acid substitution in a threonine residue of the Abl kinase domain known to form a critical hydrogen bond with the drug. This substitution of threonine with isoleucine was sufficient to confer STI-571 resistance in a reconstitution experiment. In three patients, resistance was associated with progressive BCR-ABL gene amplification. These studies provide evidence that genetically complex cancers retain dependence on an initial oncogenic event and suggest a strategy for identifying inhibitors of STI-571 resistance.

Cancers are characterized by multiple oncogenic events that collectively contribute to the phenotype of advanced stage disease. With the advent of new drugs that target specific molecular abnormalities, it is important to know whether the initial oncogenic event continues to play a functional role at later stages of tumor progression and at relapse with the development of chemotherapy resistance. This question has been addressed in transgenic mice through regulated expression of the initial oncogene. In three models testing different oncogenes in different tissues, the primary oncogene was required to maintain the tumor phenotype, despite the presence of numerous additional oncogene and tumor suppressor mutations (1–3). Recent clinical trials of the Abelson tyrosine kinase (Abl) inhibitor STI-571 in chronic myeloid leukemia (CML) allow this question to be addressed directly in human cancer (4, 5).

CML is a pluripotent hematopoietic stem cell disorder characterized by the Philadelphia (Ph) chromosome translocation (6, 7). The resulting BCR-ABL fusion gene encodes a cytoplasmic protein with constitutive tyrosine kinase activity (8). Numerous experimental models have established that BCR-ABL is an oncogene and is sufficient to produce CML-like disease in mice (9, 10). CML progresses through distinct clinical stages. The earliest stage, termed the chronic phase, is characterized by the expansion of terminally differentiated neutrophils. Over several years the disease progresses to an acute phase termed blast crisis, characterized by maturation arrest with excessive numbers of undifferentiated myeloid or lymphoid progenitor cells. The BCR-ABL oncogene is expressed at all stages, but blast crisis is characterized by multiple additional genetic and molecular changes. STI-571 is a 2-phenylaminopyrimidine that targets the adenosine triphosphate (ATP) binding site of the kinase domain of ABL (11). In phase I clinical trials, STI-571 induced remissions in patients in chronic phase as well as blast crisis (4, 5). Whereas responses in chronic phase have been durable, remissions observed in blast crisis patients have usually lasted only 2 to 6 months, despite continued drug treatment (4).

To characterize the mechanism of relapse in STI-571–treated patients, we first assessed the status of BCR-ABL signaling in primary leukemia cells (12). Our goal was to distinguish between BCR-ABL–dependent and BCR-ABL–independent mechanisms of relapse. If BCR-ABL remains critical for the proliferation of the leukemia clone, then the BCR-ABL signaling pathway should be reactivated. Alternatively, if the expansion of the leukemia clone is independent of BCR-ABL, then signaling through the BCR-ABL pathway should remain impaired by STI-571. The most direct measure of signaling through the BCR-ABL pathway is through the enzymatic activity of BCR-ABL protein itself (8, 13, 14). Although readily measured in cell lines, this assay is difficult to perform in a reproducible, quantitative fashion with clinical material because BCR-ABL is subject to rapid degradation and dephosphorylation upon cell lysis (15). In a search for alternative measures of BCR-ABL kinase activity, we found that the phosphotyrosine content of Crkl, an adaptor protein that is specifically and constitutively phosphorylated by BCR-ABL in CML cells (16–18), could be measured reproducibly and quantitatively in clinical specimens. Crkl binds BCR-ABL directly and plays a functional role in BCR-ABL transformation by linking the kinase signal to downstream effector pathways (19). When phosphorylated, Crkl migrates with altered mobility in SDS–polyacrylamide gel electrophoresis (PAGE) gels and can be quantified by means of densitometry. As expected, Crkl phosphorylation in primary CML patient cells was inhibited in a dose-dependent manner when exposed to STI-571 and correlated with dephtosphorylation of BCR-ABL (Fig. 1A) (20).

To establish the dynamic range of this assay in patient material, we measured Crkl phosphorylation in cells from BCR-ABL–negative individuals (n = 4), untreated CML patients (n = 4), and patients who responded to STI-571 therapy but whose bone marrow cells remained 100% Ph chromosome–positive (n = 8). The mean level of Crkl phosphorylation in cells from CML patients before STI-571 treatment was 73 ± 13.3% (Fig. 1B). At the time of response the mean was 22 ± 9.9% (Fig. 1B), similar to the mean level of Crkl phosphorylation in cells from BCR-ABL–negative individuals (22 ± 6.0%) (21). We next measured levels of Crkl phosphorylation in primary leukemia cells from 11 patients who responded to STI-571 but subsequently relapsed on treatment. In these cases, which included one patient with lymphoid blast crisis, three with Ph+ acute lymphoid leukemia, and seven with myeloid blast crisis, the mean level of Crkl phosphorylation at relapse was 59 ± 12.5% (Fig. 1C). Antibodies to phosphotyrosine (anti-phosphotyrosine) immunoblot analysis of a subset of these samples confirmed that BCR-ABL was phosphorylated on tyrosine at relapse (Fig. 1C). Longitudinal analysis of blood or bone marrow samples obtained from a subset of these patients before and throughout the course of STI-571 treatment confirmed that Crkl phosphorylation fell during the response to treatment but increased at the time of relapse (Fig. 1D). Therefore, disease progression in patients who initially respond to STI-571 is associated with the failure to maintain effective inhibition of BCR-ABL kinase activity.

A recent preclinical study of STI-571 resistance in mice engrafted with a human blast crisis CML cell line demonstrated that Crkl is a key negative regulator of proliferation (22). This observation raises the possibility that STI-571 resistance in patients is due to a host-mediated response against the drug. Alternatively, resis-
Some CML cell lines that develop resistance to STI-571 after months of in vitro growth in subtherapeutic doses of the drug show amplification of the BCR-ABL gene (24–26). We performed dual-color fluorescence in situ hybridization (FISH) experiments to determine if BCR-ABL gene amplification could be similarly implicated in STI-571 resistance in human clinical samples (27). Multiple copies of the BCR-ABL gene were detected in interphase nuclei in three (two myeloid blast crisis, one lymphoid blast crisis) of the 11 patients who relapsed after initially responding to STI-571 (Fig. 3). Further cytogenetic and FISH characterization of metaphase spreads from these patients showed a unique inverted duplicate Ph chromosome with interstitial amplification of the BCR-ABL fusion gene (Fig. 3C). In one patient, the inverted duplicate Ph chromosome could be detected before the initiation of STI-571. In all three cases, additional copies of the aberrant Ph chromosome were observed as STI-571 treatment continued, as well as ring chromosomes harboring multiple copies of the BCR-ABL. Patient MB14 was reevaluated by FISH 1 month after receiving alternative treatment for her leukemia. BCR-ABL amplification was no longer detectable 4 weeks after discontinuation of STI-571, raising the possibility that persistent STI-571 administration might select for increased copies of the BCR-ABL gene in some patients. Quantitative polymerase chain reaction (QPCR) analysis of genomic DNA (gDNA) obtained from these three patients confirmed increased ABL gene copy number at relapse when compared to a patient without BCR-ABL gene amplification (Fig. 3D) (28).

We also considered the possibility that mutations in BCR-ABL might confer resistance to STI-571. A 579-base pair (bp) region corresponding to the ATP binding pocket and the activation loop of the kinase domain of BCR-ABL was sequenced in the nine patients for whom RNA was available at the time of relapse (Fig. 4A) (29). A single, identical C → T nucleotide (nt) change was detected at ABL nt 944 in six of nine cases examined (Fig. 4A). In all six patients a mixture of wild-type and mutant cDNA clones was found, with the frequency of mutant clones ranging from 17 to 70%. The mutation was found in three of three patients with lymphoid disease [two Ph+ acute lymphoid leukemia (ALL), one lymphoid blast crisis] and
in three of six patients with myeloid blast crisis. The presence of the mutation was confirmed by analysis of gDNA (Fig. 4A) (30). Analysis of RNA or gDNA from pretreatment samples failed to provide evidence of the mutation before STI-571 therapy; however, we cannot rule out the possibility that rare cells bearing the mutation exist before treatment.

This single nucleotide C → T change results in a threonine to isoleucine substitution at position 315 (Thr315 → Ile; T315I) of c-Abl. The recently solved crystal structure of the catalytic domain of Ab1 complexed with a variant of STI-571 identified both the amino acid residues within the ATP binding site and the activation loop of c-Abl that are required for STI-571 binding and, thus, the inhibition of Ab1 kinase activity (31). Thr315 is among those that form critical hydrogen bonds with STI-571. The potential consequence of the T315I substitution on the STI-571 binding pocket was modeled based on the crystal structure of the wild-type Ab1 kinase domain in complex with STI-571 (Fig. 4B). The absence of the oxygen atom normally provided by the side chain of Thr315 would preclude formation of a hydrogen bond with the secondary amino group of STI-571. In addition, isoleucine contains an extra hydrocarbon group in the side chain, which would result in a steric clash with STI-571 and presumably inhibit binding. Notably, the model predicts that the
T315I mutation should not interfere with ATP binding (32).

To confirm that this amino acid substitution interferes with STI-571 activity, we engineered the T315I mutation into wild-type p210 BCR-ABL (33). Cells were transfected with wild-type or T315I p210 BCR-ABL and cultured in the presence of increasing concentrations of STI-571 (34). As shown by Abl immunoblot analysis, the expression of wild-type and T315I mutant BCR-ABL proteins was similar and was not changed by STI-571 (Fig. 4C, bottom panels). On the basis of anti-phosphotyrosine immunoblot analysis, the kinase activities of wild-type BCR-ABL and the T315I mutant appear comparable in the absence of STI-571. Whereas wild-type BCR-ABL kinase activity was inhibited by STI-571, the T315I mutant retained high levels of phosphotyrosine at all concentrations of the inhibitor tested (Fig. 4C, top panels).

Our analysis of 11 patients with advanced stage ML who underwent disease progression after an initial response to STI-571 shows that reactivation of BCR-ABL signaling occurred in all patients, despite continued STI-571 treatment. Therefore, the primary explanation for disease progression in these patients appears to be BCR-ABL–dependent proliferation rather than secondary oncogenic signals that permit BCR-ABL–independent growth. It is possible that studies of a larger number of patients may identify exceptions to this theme, as has been reported in transgenic mice expressing conditional oncogenes where an occasional tumor can escape dependence on the initiating oncogene (1–3). In 8 of the 11 patients we studied, the mechanism of resistance was a consequence of mutation (six patients) or amplification (three patients) of the target oncogene BCR-ABL (one patient had both events). These results provide evidence in a genetically complex human cancer that a single molecular target remains relevant in late stage, relapsed disease.

The identity of the Abl kinase domain mutation found in these patients bears remarkable

![Fig. 4. Point mutation in the ATP binding pocket of the Abl kinase domain confers STI-571 resistance in relapsed patients.](image-url)
Reciprocal Regulation Between TOC1 and LHY/CCA1 Within the Arabidopsis Circadian Clock

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The interactive regulation between clock genes is central for oscillator function. Here, we show interactions between the Arabidopsis clock genes LATE ELONGATED HYPOCOTYL (LHY), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1), and TIMING OF CAB EXPRESSION 1 (TOC1). The MYB transcription factors LHY and CCA1 negatively regulate TOC1 expression. We show that both proteins bind to a region in the TOC1 promoter that is critical for its clock function. Conversely, TOC1 appears to participate in the positive regulation of LHY and CCA1 expression. Our results indicate that these interactions form a loop critical for clock function in Arabidopsis.

Circadian clocks represent a widespread, endogenous mechanism that allows organisms to time different processes appropriately throughout the day-night cycle. Among the activities controlled by the circadian clock are the regulation of transcription in cyanobacteria, the rhythmic movement of leaves in plants, and more complex activities such as...

References and Notes

12. Peripheral blood and/or bone marrow samples were obtained with appropriate informed consent from chronic myeloid leukemia patients at the University of California Los Angeles who were enrolled in multicenter clinical trials of STI-571 (sponsored by Novartis California Los Angeles who were enrolled in multicenter clinical trials of STI-571 (sponsored by Novartis). Two milligrams of peripheral blood with the Qiagen Blood Mini Kit (Qiagen). A 361-bp DNA fragment was amplified by PCR with a 5′-GAGTCTGGTACCTCAG-3′ and a 3′-TTGTTAAGGCGGTCCCGG-3′ primer, which are specific for sequences in exon 5 and exon 8 of GAPDH, respectively. Fold increase in ABL copy number was determined by calculating the difference between threshold cycle numbers of ABL and GAPDH for each sample (ΔCt). With control LB3 as reference sample, ΔCt from each sample was subtracted from ΔCt of control to determine Δ(ΔCt).
15. M. E. Gorre, C. L. Sawyer, unpublished data.
20. Cells were lysed in 1% Triton X-100 buffer with protease and phosphatase inhibitors (36). Equal amounts of protein, as determined by the BioRad DC protein assay (Bio-Rad, Hercules, CA), were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with phospho-tyrosines antibody 4G10, Upstate Biotechnologies, Lake Placid, NY), Abl antibody, T315I (Chemicon, CA), or anti-pTyr antibody (Sigma, St. Louis, MO), or Crk antisera (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized by ECL (Amersharm Biopack Biotech, Piscataway, NJ). The densitometric analyses were obtained to ensure linear range of signal intensity. Optimal exposures were quantified by densitometry with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).
21. M. E. Gorre, C. L. Sawyer, data not shown.