Mutation in the ATP-binding Pocket of the ABL Kinase Domain in an STI571-resistant BCR/ABL-positive Cell Line

Clara Ricci, Barbara Scappini, Vladimir Divoky, Simona Gatto, Francesco Onida, Srdan Verstovsek, Hagop M. Kantarjian, and Miloslav Beran

Abstract

The major mechanism of action of STI571 is a competitive interference with the ATP-binding site of the Bcr/Abl tyrosine kinase. In the BCR/ABL-positive cell line KBM5, we studied cellular events associated with the in vitro acquisition of resistance to STI571. The emergence of the STI571-resistant phenotype was accompanied by only a marginal increase in the number of copies of the BCR/ABL gene and its level of expression. The activity of the Bcr/Abl kinase (level of autophosphorylation) in resistant cells was, however, incompletely inhibited by STI571, and the acquisition of the high degree of resistance was associated with a single-point mutation leading to a substitution of a threonine-to-isoleucine at position 315 of Abl. In the resistant KBM5-STI571R1.0 cells, 20% of the BCR/ABL transcripts and 10% of BCR/ABL gene copies on the DNA level were mutated. The mutation was present in all 10 STI571-resistant clones derived from low density clonogenic assay, confirming its presence in all colony-forming cells but only in a fraction of the BCR/ABL gene copies in each cell. The contribution of this mutation to STI571-resistant phenotype remains unknown. Preliminary data showing partial reversibility of resistance in these cells suggest that resistance may be multifactorial. No other mutations were identified in the kinase domain of the BCR/ABL gene.

Introduction

The lack of response to STI571 or the recurrence of disease after a transient response in patients with variously advanced stages of BCR/ABL-positive CML indicates an STI571-resistant phenotype that develops either by the selection of preexisting resistant clones or the de novo induction of resistance. In vitro studies in STI571-resistant BCR/ABL-positive cell lines demonstrated an association between resistance to STI571 and quantitative changes in the Bcr/Abl protein expression (1–3). To date, no qualitative changes such as BCR/ABL gene rearrangements or mutations in the kinase domain have been identified in cell lines with STI571-induced resistance in vitro (1–3). Recently, however, point mutations within the DNA sequences coding for the ATP-binding pocket of the BCR/ABL gene were identified in cells from some patients with CML who had STI571-refractory disease or who had a relapse during the treatment (4–10). A point mutation resulting in a threonine-to-isoleucine change at amino acid position 315 (T315I) has been described in detail (4). This mutation, when engineered into wild-type p210 Bcr/Abl and transiently transfected into 293T cells or to Ba/F3 cells, interfered with the inhibition of Bcr/Abl kinase activity in cells exposed to STI571 (4, 7). However, the extent to which this mutation contributes to STI571 resistance in vivo remains unknown. An in vitro model that allows study of the role of this mutation in human cells has not been available, and neither this nor any other mutation has been reported in any BCR/ABL-positive cell lines.

In this study, we describe the occurrence of the T315I mutation in a proportion of copies of the BCR/ABL gene within cells in a cell line rendered resistant to STI571 by in vitro exposure to the drug. The STI571-resistant cells represent a model for additional investigation of the role of this mutation in the development of STI571-resistant CML phenotype. These cells may be also useful in development of methods to overcome STI571 resistance in experimental systems.

Materials and Methods

Cell Lines and STI571 Treatment. KBM5 cells were derived from a patient with myeloid blastic phase of CML; the cells contain multiple copies of the Philadelphia chromosome (11) while lacking the normal ABL gene (12). To select for the resistant phenotype, the KBM5 cells were exposed to increasing concentrations of STI571 (Novartis, Basel, Switzerland), starting with 0.05 μM and increasing by 0.1 μM when the cells resumed a near normal growth kinetics. Parental cells were maintained concomitantly without the drug. After 4 months, the treated cells, designated KBM5-STI571R1.10, were able to grow in the presence of 1.0 μM STI571 and were maintained at this concentration.

The reported studies were performed after the cells had grown in the presence of this drug concentration for 6–8 months. During this period, no changes in the sensitivity of parental cells to STI571 were observed.

Isolation of Clones and Clonally Derived Cell Lines by Cloning in Methyllumellose. To obtain single cell-derived clonal sublines, leukemic cells were cultured at low cell density using semisolid medium in CFU colony assay. The resistant KBM5-STI571R1.10 cells were plated at low density (0.5 × 10^3 cell/ml) in Iscove’s methylcellulose medium (Methocult H4230; Stem Cell Technologies, Vancouver, Canada) containing 1 μM concentration of STI571. After 8 days of culture, well-separated individual colonies were removed under sterile conditions and either analyzed directly or transferred to liquid cultures and expanded. Five single cell-derived clones, expanded in the presence, and five expanded in the absence of 1.0 μM STI571 were used for additional studies of the cellular changes associated with the resistant phenotype, including the presence of BCR/ABL mutations.

Stability of Resistance. The KBM5-STI571R1.10-resistant cell line and two clonally derived cell lines were grown for 25 passages either in the presence of STI571 (1 μM) or in the absence of the drug. The degree of resistance was then estimated by the MTT assay.

Cell Proliferation Assay and the Measurement of STI571 Resistance. The cell proliferation was measured using the MTT (Sigma Chemical, St. Louis, MO) colorimetric reduction method. The exponentially growing cells were washed, plated in triplicate in 96-well plates at a cell density of 4 × 10^4 cells/ml, and STI571 was added at various concentrations. After 72 h of exposure, the level at which proliferation was inhibited was measured as a percentage of control growth (no drug in the sample). The survival curves were constructed, and the drug concentration resulting in IC_{50} was determined.
resistance index (RI) was calculated by dividing IC_{50} for resistant cells by IC_{50} for the parental, STI571-sensitive cells.

Western Blot Analysis. Cells were harvested in the presence or absence of graded concentrations of STI571. After 2 h, cells were washed twice with cold PBS containing protease inhibitors [protease inhibitor mixture (Complete, Mini Roche Molecular Biochemical, Indianapolis, IN), 10 μg/ml leupeptin, 10 μg/ml aprotinin], and phosphatase inhibitors (AEBSSF 1 mm, sodium fluoride 10 mM, orthovanadate 1 mM), and 1 × 10^7 cells were lysed in 1 ml of lysis buffer [0.125 m Tris-HCL (pH 6.8), 1% SDS, 0.01% bromphenol blue, 5% glycerol, 2% 2-mercaptoethanol, protease and phosphatase inhibitors]. Cell lysates corresponding to 5 × 10^6 were boiled for 10 min, resolved on 7.5% SDS-PAGE gels, and transferred onto a polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). This procedure proved optimal for the detection of Bcr/Abl and minimized its degradation which, although absent in the control K562 cell line, was consistently observed in KBM5 parental and resistant cells. Anti-c-Abl (Pharmingen, San Diego, CA) and antiphosphotyrosine (4G10 monoclonal; Upstate Biotechnology, Lake Placid, NY) were used for Western blot analyses. After overnight incubation at 4°C with primary antibody, the membranes were washed and incubated with secondary antibody (antimouse, Bio-Rad) horseradish peroxidase-conjugated antibodies at room temperature for 1 h. The membranes were washed, and bound antibodies were detected with enhanced luminol and oxidizing reagent by chemiluminescence as specified by manufacturer (Amersham, Arlington Heights, IL). After stripping the membranes in the stripping buffer [0.5 mm Tris-HCL (pH 6.7), 2% SDS, 100 mM 2-mercaptoethanol] for 30 min at 56°C, they were reprobed with anti-β-actin antibodies (Sigma Chemical) to assess the comparability of the protein loading. Intensity of Bcr/Abl bands was evaluated using BioMax 1D software and compared with intensity of the corresponding β-actin bands on the same membranes.

Results

Generation and Characterization of STI571-resistant Cell Line. After 4 months of exposure of KBM5 cells to increasing concentrations of STI571, the cells were able to grow in the presence of 1 μM concentration of the drug (KB5M-STI571R10). There were no apparent differences in the growth kinetics of the parental cells growing in the absence and the resistant cells growing in the presence of 1 μM STI571.

The IC_{50} of parental and resistant cell lines was 0.55 and 15.5 μM, respectively, as determined by the MTT assay. The calculated RI was 28.2. The IC_{50} of the 10 single CFU-derived, clonal sublines, assayed by the same methodology, varied between 7.0 and 30.3 μM and the corresponding RIs varied between 12.7 and 30.3.

The growth of KBM5-STI571R10-resistant cell line and of the two resistant clonal derivatives was comparable in the presence and absence of 1 μM STI571. Removal of the drug from the growth media for 25 passages resulted in a partial reversal of the resistance in all of the lines studied (Fig. 1).

The fluorescence in situ hybridization analysis of KBM5 and KBM5-STI571R10 cells documented only a marginal increase in the number of BCR/ABL fusion signals. The proportion of parental cells containing 2, 3, and 4–8 fusion signals was 5, 20, and 75%; the corresponding RIs varied between 12.7 and 30.3.

Expression and Phosphorylation of the p210 Bcr/Abl Protein. The acquisition of resistance to STI571 was characterized by a slight overexpression of Bcr/Abl as assessed by immunoblotting (Fig. 2). Using densitometry and correction against β-actin expression, the expression of Bcr/Abl in KBM5-STI571R10 was found to be increased 3.8-fold (Fig. 2).

Inhibition of p210 Bcr/Abl Phosphorylation by STI571. Exponentially growing sensitive and resistant cells were washed and incubated for 2 h in 1.5, and 10 μM concentrations of STI571. The Western blot analysis with antiphosphotyrosine antibody demonstrated a dose-

![Fig. 1. The degree and stability of STI571 resistance. KBM5-STI571R10 cells (●, ○) and two clonal derivatives, KBM5-STI571R10C1 (■, □) and KBM5-STI571R10C6 (●, ○) were grown in the presence (closed symbols) or absence (open symbols) of 1.0 μM STI571 for 25 passages. The level of resistance was assessed by MTT assay. The sensitivity of the parental KBM5 cells (●) is shown for comparison. The mutational status of clonal cell lines C1 and C6 at the time of the removal from the STI571-containing media is shown in Fig. 3 on cDNA level (D) and on gDNA level (E).](image-url)
STI571 resistance comparable with that of the KBM5-STIR1.0 cell line. The analysis of cDNA with direct sequencing of the 177-bp PCR product; the mutation abolishes the recognition site DdeI. The same mutation was suggested by restriction analysis of plasmids, containing cDNA-derived PCR product (for details see Materials and Methods). It involves both quantitation of the BCR/ABL gene copies in all 10 clones (Fig. 3E). To determine proportion of wild-type and mutated gDNA copies, the 169-bp PCR products obtained from two CFU-derived KBM5-STI571R1.0 clones growing in the continuous presence of STI571; the acquisition of the resistant phenotype was characterized by only a marginal increase in the number of BCR/ABL gene copies and the level of expression of Bcr/Abl protein. The resistant cells display, however, a Bcr/Abl tyrosine kinase that is resistant to STI571. Using gDNA as a template, a 344-bp PCR product consisting of the whole exon 3 of the ABL was obtained from 10 clonal derivatives of KBM5-STI571R1.0 cell line. Direct sequencing in both forward and reverse directions showed presence of both wild-type and mutated sequences in the STI571-resistant cell line and all 10 resistant clones. Ddel digestion of 169-bp gDNA PCR product obtained from KBM5-STI571R1.0-derived clones verified the presence of both wild-type and mutant BCR/ABL gene copies in all 10 clones (Fig. 3E).

**Discussion**

The cellular and molecular mechanisms involved in the development of STI571-resistance in vitro and in vivo involves both quantitative and qualitative changes of the Bcr/Abl tyrosine kinase. We report development of STI571 resistance in p210Bcr/Abl-positive myeloid cell line KBM5-STI571R1.0 growing in the continuous presence of STI571; the acquisition of the resistant phenotype was characterized by only a marginal increase in the number of BCR/ABL gene copies and the level of expression of Bcr/Abl protein. The resistant cells display, however, a Bcr/Abl tyrosine kinase that is resistant to STI571.
STI571 inhibition. The uniqueness of the resistant cells lies in the presence of a specific mutation within the ATP-binding domain of Bcr/Abl. Whereas such mutation has recently been found by Gore et al. (4) in patients with a STI571-resistant blastic phase of CML, this is the first report of mutation in the ATP-binding pocket of the Abl kinase domain, which has been found in BCR/ABL-positive cell line rendered resistant to STI571 exposure in vitro. When studied in patients, the mutation was detected in 17–80% of RT-PCR-derived BCR/ABL plasmid clones (4). The presence of both mutated and wild-type BCR/ABL transcripts was postulated to reflect mutant and wild-type BCR/ABL patients’ cells (4, 7). Possible different proportions of mutant and wild-type BCR/ABL DNA copies within individual cells have not been evaluated.

Our study revealed a mutation only in KBM5-STI571R1.0 cells and not in parental KBM5, suggesting that the mutation arose during the drug exposure. These data favor a de novo acquisition of the mutation and the preferential survival of mutant cells under selective drug pressure albeit not completely ruling out the possibility of a preexistent BCR/ABL mutation in a parental cell. Of interest is the question of how only a minor fraction of mutant Bcr/Abl molecules can account for the observed level of STI571 resistance. Is this minor fraction of mutant Bcr/Abl copies involved in more critical pathways required for survival/growth of these cells, or is it acting in a dominant negative fashion? A relatively modest increase of p210 expression in cells containing high number of BCR/ABL copies of which only a minority is mutated suggests a possibility of a dominant negative effect. A slightly lower proportion of mutant copies on gDNA than cDNA level suggests such an effect on the transcriptional levels. A partial suppression of the kinase phosphorylation in KBM5-STIR1.0 cells by STI571 suggests that the wild-type molecules might still be blocked by the drug and that only the mutant fraction of Bcr/Abl molecules is sufficient to trigger the kinase-dependent activation of proliferation/survival. Additional studies are required to answer these questions. There is still a remote possibility that there are other undetected mutations within the pseudo wild-type transcripts.

We tested the possibility that the point mutation may have resulted from a PCR error. Besides including a DNA polymerase with proof-reading activity to our PCR, we tested the reproducibility of our findings in repeated experiments involving a single PCR round and different primer combinations. In all instances, we confirmed the presence of the C-T point mutation of the KBM5-STIR1.0 cells and absence of any nucleotide change involving the ATP-binding pocket coding sequence in the parental cells. No other mutations, recently reported in patients clinically resistant to STI571 (5–10), were identified in KBM5 parental or any STI571-resistant KBM5 sublines. Interestingly, growth of KBM5-STIR1.0 cells and two clonally derived sublines in the absence of STI571 for 25 passages resulted in a partial reversal of resistance (Fig. 1). Additional studies will be required to explain such partial reversal of the STI571 resistance and to assess biological significance of this mutation, occurring in a fraction of amplified BCR/ABL genes in resistant cells, and the role of this mutation in the STI571-resistant phenotype.

In summary, systematic analysis of the DNA sequence in the region corresponding to the ATP-binding pocket of the kinase domain of the BCR/ABL gene identified a single nucleotide substitution C-T at ABL position 944 that results in the T315I mutation reported in a proportion of STI571-resistant CML patients (4). The analysis of gDNA and cDNA yielded results documenting that each clonogenic cell contains both wild-type- and mutated BCR/ABL copies and transcripts.

Acknowledgments

We thank Dr. Hui Lin and Dr. Ralph Arlinghaus (M. D. Anderson Cancer Center, TX) for initial advice and for providing us with the positive control for Dde1 digests of mutant BCR/ABL and some of the primers. We also thank Dr. Arlinghaus for advice and critical review of this manuscript. We thank Li Dong for technical assistance with the initial parts of the study.

References

Mutation in the ATP-binding Pocket of the ABL Kinase Domain in an STI571-resistant BCR/ABL-positive Cell Line

Clara Ricci, Barbara Scappini, Vladimir Divoky, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/21/5995

Cited articles
This article cites 9 articles, 7 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/21/5995.full.html#ref-list-1

Citing articles
This article has been cited by 17 HighWire-hosted articles. Access the articles at:
/content/62/21/5995.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.