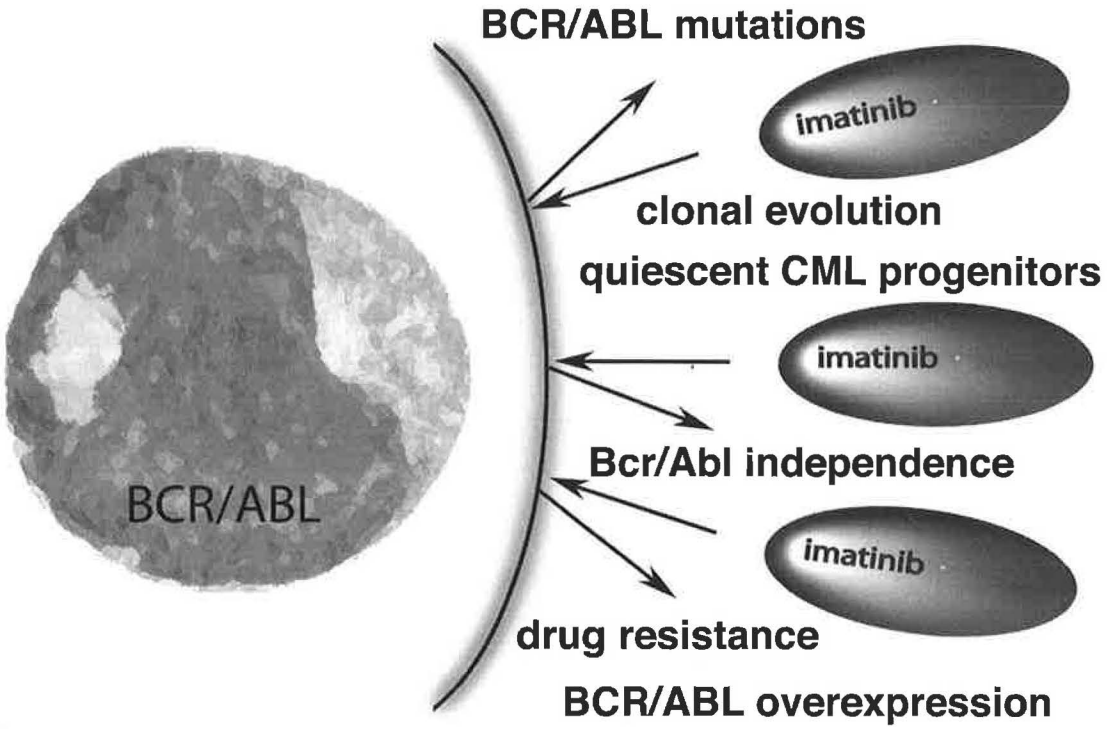
"Challenges for Chronic Myelogenous Leukemia Therapy in the Post-imatinib Era: A Kinase Strikes Back"



Internal Medicine Grand Rounds

Robert L. Ilaria, Jr., M.D.
July 1, 2004

This is to acknowledge that Robert Ilaria, Jr. has not disclosed any financial interests or other relationships with commercial concerns related directly or indirectly to this program. Dr. Ilaria will not discuss off-label uses in his presentation.

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Internal Medicine Grand Rounds, July 1, 2004

Research interests: Our laboratory studies the molecular biology of the oncogenes *BCR/ABL*, *EWS/FLI-1* and *PAX3/FKHR*. In our Bcr/Abl work, we focus on mouse models of Philadelphia chromosome positive hematological malignancy to test novel therapeutic approaches and to gain insight into the molecular pathogenesis of Bcr/Abl-induced leukemia. *EWS/FLI-1* and *PAX3/FKHR* are oncogenes that play central roles in the pediatric cancers Ewing's sarcoma and rhabdomyosarcoma. Both act as mutant transcription factors, but how these oncogenes transform cells is not well defined. We are interested in developing novel mouse models for these cancers to identify potential therapeutic targets and to develop a roadmap for how EWS/FLI-1 and PAX3/FKHR manipulate normal cellular processes to achieve tumorigenesis.

Chronic myelogenous leukemia: epidemiology and clinical manifestations

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder characterized by myeloid expansion with preserved granulocytic differentiation. CML comprises approximately 15% of leukemias in adults, with approximately 5000-7000 new cases diagnosed in the U.S. yearly, for an incidence of approximately 1 case per 75,000 persons. In large clinical trial series, the median age for CML has ranged between 45-55 years of age, although recent SEER data has reported that the highest incidence rate is in patients over the age of 65 [1]. The ratio of male to female cases is 1.3 to 1. There is no known hereditary component for the development of CML, even in monozygotic twins. Significant exposure to ionizing radiation has been associated with an increased incidence of CML, but the risk is significantly less than the development of other secondary cancers [2, 3]. No other environmental factors have been causally linked to CML. Historically, splenomegaly (due to extramedullary granulocytic infiltration) has been a cardinal sign of CML, but with the more widespread use of routine blood tests splenomegaly is detected in only about 40% of patients at diagnosis. Consistent with a trend towards earlier diagnosis, almost half of patients are asymptomatic at the time of presentation [4]. Of symptomatic patients, left upper quadrant discomfort, early satiety, anorexia, fatigue, and "B symptoms" such as weight loss, pruritus and sweats are the most commonly reported. Hepatomegaly is seen in fewer than 10% of patients. Symptoms of hyperviscosity may be observed in some patients presenting with more extreme degrees of granulocytosis.

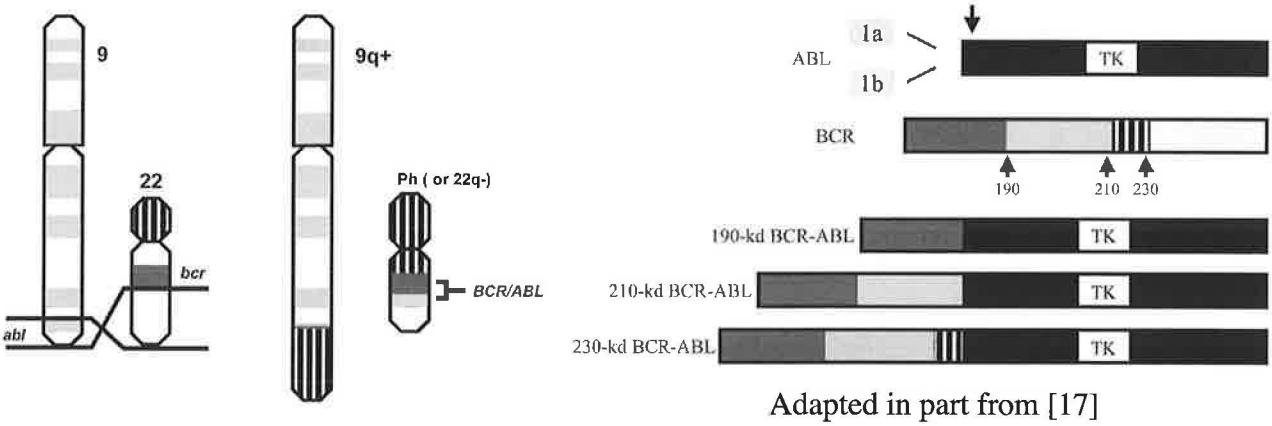
CML: laboratory features

On laboratory evaluation, an elevated WBC with a granulocytic predominance and left shift is seen in most cases. Variable degrees of eosinophilia and basophilia may also be observed on peripheral blood smear. Thrombocytosis is seen in approximately 40% of patients at diagnosis, and is greater than 700,000/μl in 15-20% of patients [4]. Anemia is generally modest, and is observed in approximately half of patients at presentation, but can be more severe in up to 20% of cases. In contrast to other causes of neutrophilia, the leukocyte alkaline phosphatase (LAP) score is normal or low in CML, but with the increasing availability of molecular diagnostic tests this test is less often used. The bone marrow (BM) evaluation in CML shows a marked increase in marrow cellularity and myeloid/erythroid ratio. Reticulin stain may reveal increased BM fibrosis, which has been associated with a worse prognosis [5].

CML: Molecular biology

CML is probably the best understood human malignancy. In 1960, investigators in Philadelphia noted the presence of a smaller than normal chromosome 22 (the "Philadelphia chromosome") in patients with CML [6]. In 1973, the Philadelphia chromosome (Ph) was fully characterized by cytogenetics and found to be the product of a reciprocal translocation (t(9;22) (q34.1;q11.21)) between chromosomes 9 and 22 [7]. Subsequent studies demonstrated that the Ph chromosome could be detected in granulocyte, monocyte, erythroid, megakaryocytic, and B-lymphoid lineage cells [8-10], but not in BM fibroblast or other mesenchymal cells [11]. These findings, together with the rare occurrence of T-lymphoid CML blast crisis [12, 13], illustrate the BM stem cell origins of CML.

Molecular analysis of the Ph chromosome led to the discovery that the proto-oncogene *ABL* comprised the chromosome 9-derived part of the genetic fusion, and that the *BCR* gene comprised the chromosome 22-derived portion (figure 1, left; [14, 15]). Three different Bcr/Abl fusions proteins can be transcribed from *BCR/ABL*, depending on the genomic breakpoint in the *BCR* gene. In CML, the *BCR* genomic breakpoint occurs within the 5.8 kb breakpoint cluster region (M-*bcr*) of *BCR*, resulting in a BCR/ABL transcript containing either 13 or 14 exons (e13 or e14) of *BCR*. The *ABL* breakpoint occurs anywhere within a ~300 kb region upstream of *ABL* exon 2, either before alternative exon 1b, between exon 1b and 1a, or between exon 1a and exon 2 [16]. Despite the heterogeneity of the *ABL* genomic breakpoint, all BCR/ABL mRNAs have a single ABL junction at exon 2 (a2) because of post-transcriptional processing. The resulting e13a2 or e14a2 BCR/ABL transcripts are translated into a 210 kd Bcr/Abl fusion protein, referred to as $P210^{BCR/ABL}$ or P210, which is expressed in virtually all cases of CML. It should be noted that originally *BCR* exons 13 and 14 were called b2 and b3, respectively, so some still refer to the *BCR/ABL* breakpoints as b2a2 or b3a2, which can be the source of some confusion.



Adapted in part from [17]

Figure 1. Reciprocal translocation between chromosomes 9 and 22 forms the Philadelphia (Ph) chromosome containing the *BCR/ABL* fusion (left). The three major forms of the Bcr/Abl fusion protein contain the same amount of *ABL*-derived sequences but different amounts of *BCR* (right). The ABL and BCR transcript junctions are indicated by arrows. BCR exon 1; exons 2-13 or 2-14; or 2-19 are denoted by the dark gray, light gray, and hatched rectangle, respectively. TK denotes the Abl tyrosine kinase domain.

Approximately 60% of Ph chromosome positive acute lymphoblastic leukemia (ALL) and very rare cases of CML express a shorter 190 kd Bcr/Abl chimeric protein. $P190^{BCR/ABL}$ is the product of a genomic breakpoint that occurs within m-*bcr*, a region in the 54 kb intron between the two alternative

BCR exons e2' and e2 [18]. RNA processing leads to a single BCR/ABL transcript (e1a2) that contains only exon 1 of *BCR* (figure 1, right). In some cases, a different *BCR/ABL* fusion leads to a longer BCR/ABL transcript containing sequences from the almost the entire BCR gene fused to Abl exon 2. The resulting $P230^{BCR/ABL}$ fusion protein has been associated with a neutrophilic form of CML, characterized by more mature granulocytic differentiation and a more indolent clinical course [19].

CML diagnosis

Advances in the molecular biology of CML has led to the general consensus that the presence of *BCR/ABL* by bone marrow and/or peripheral blood analysis is a prerequisite for the diagnosis of CML. Approximately 90% of patients with clinical features of CML will have evidence of *BCR/ABL* (Ph chromosome) by routine cytogenetics or *BCR/ABL* fluorescence-in situ hybridization (FISH). An additional 5-10% will have atypical BCR/ABL transcripts not detectable by routine cytogenetics but detectable by FISH and/or polymerase chain reaction (PCR)-based techniques. Patients with Ph chromosome negative but *BCR/ABL* positive myeloproliferative disorders experience the same general clinical course and response to therapy as the more conventional Ph chromosome positive CML patients [20]. Patients with myeloproliferative disorders resembling CML but lacking molecular evidence of BCR/ABL transcripts should be considered to have a disease distinct from CML [21], despite the fact that some refer to such patients as Ph chromosome negative or atypical CML.

CML biology

In contrast to other forms of leukemia, CML is comprised of distinct clinical stages. Chronic or stable phase CML is characterized by granulocytic expansion with relatively preserved differentiation, and the absence of significant peripheral blood basophilia, eosinophilia or blasts (figure 2, left). The median duration of chronic phase ranges between 3-6 years. Unfortunately, without definitive treatment, CML inevitably evolves into an acute or blastic phase (figure 2, right).

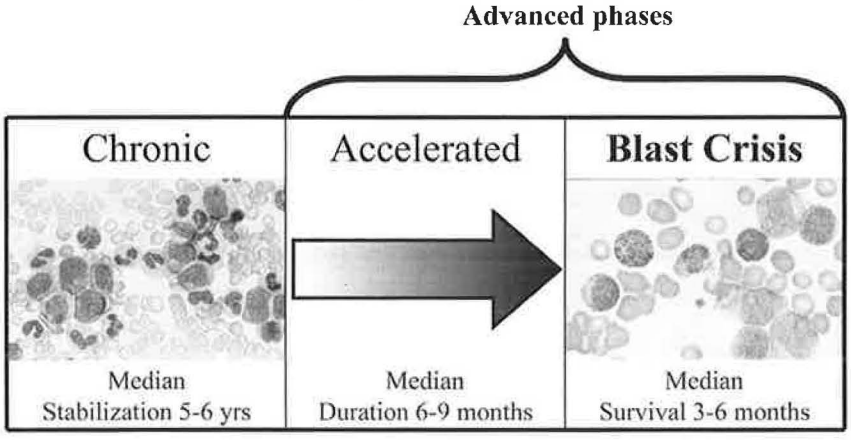


Figure 2. The clinical and biological phases of CML.

Two-thirds of patients develop myeloid blast crisis resembling acute myelogenous leukemia, while the remainder develop an ALL-like lymphoid blast crisis. It is likely that some cases of Ph chromosome positive ALL are really CML that had remained clinically silent until the development of lymphoid blast crisis. Prior to blastic phase, most-but not all-chronic phase patients develop an accelerated phase that is characterized by increasing degrees of basophilia (>20%); eosinophilia (eosinophils +basophils >20%); thrombocytosis (plts> one million/μl); increased presence of immature myeloid elements (blasts >15% or blasts +promyelocytes >30%); progressive splenomegaly; thrombocytopenia (plts< 100,000/μl); acquisition of additional chromosomal abnormalities by cytogenetics; or loss of

previously established disease control [22]. With recent trends in earlier diagnosis, over 80% of patients are diagnosed while in the chronic phase.

CML therapy

After the initial cloning of the *BCR/ABL* translocation breakpoint in CML, several important discoveries led investigators to the hypothesis that Bcr/Abl was a rational molecular target for CML therapy.

1. Abl is a nuclear-localized non-receptor protein tyrosine kinase [23, 24].
2. The presence of Bcr upstream of Abl constitutively activates Abl tyrosine kinase activity [25, 26].
3. The tyrosine kinase activity of Abl is required for Bcr/Abl hematopoietic cell transformation [27].
4. Bcr/Abl-transduced primary murine BM cells induce a CML-like disease in mice [28, 29].
5. Abl-deficient (knockout) mice are viable suggesting that cells could tolerate the pharmacological inhibition of ABL *in vivo*.

In the mid to late 1990's investigators screened compounds known to inhibit the platelet-derived growth factor (PDGF) receptor tyrosine kinase, a member of the type III tyrosine kinase family. The product of this search and subsequent chemical refinements led to CGP57148, later known as STI571, a highly water soluble 2-phenylaminopyrimidine tyrosine kinase inhibitor with a molecular weight of approximately 590 daltons (figure 3).

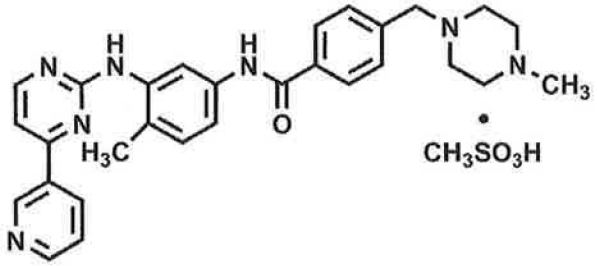


Figure 3. Chemical structure of the tyrosine kinase inhibitor imatinib mesylate (Gleevec), a 2-phenylaminopyrimidine.

Imatinib proved to be a potent inhibitor of Abl and Bcr/Abl kinase activity with an IC_{50} of approximately 0.025 μM [30]. Imatinib also inhibited the receptor tyrosine kinases PDGF and c-Kit at similar concentrations [31], and the tyrosine kinase ARG [32], but had little activity against several dozen receptor kinases and other signaling molecules. Initially it was felt that imatinib competed with ATP for binding to the Abl kinase domain, but subsequent co-crystallization structural analyses have indicated a more complex mechanism. According to the present model, the kinase domain of Abl must undergo certain conformational changes to accommodate imatinib in the binding cleft, and these structural changes lock Abl in the inactive state thereby preventing ATP binding and kinase activation (figure 4).

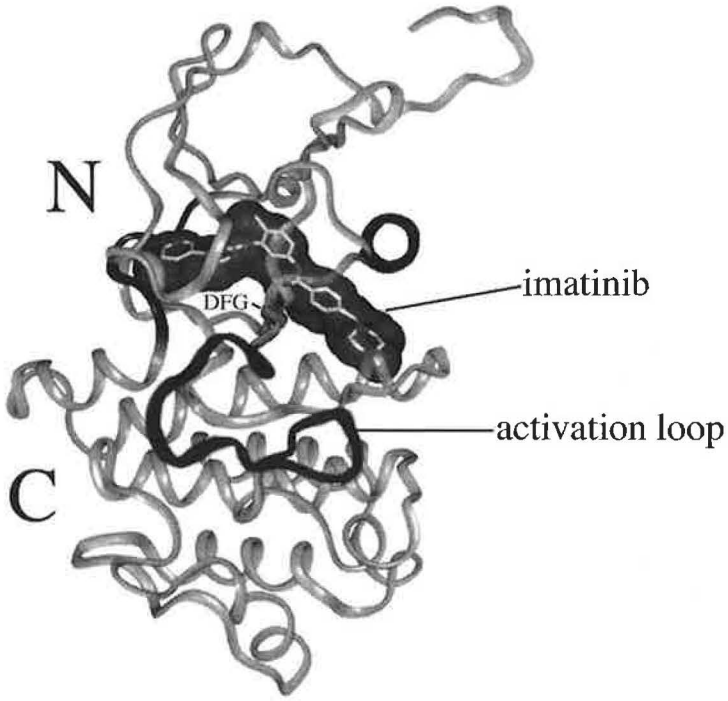


Figure 4. Ribbon model depicting imatinib interacting with Bcr/Abl. Note that imatinib binds in the cleft between the N and C lobes that contains the ATP-binding and catalytic regions. Imatinib binds Bcr/Abl when the activation loop is folded inward (non-tyrosine phosphorylated/inactive state).

Adapted from [33].

After encouraging findings in pre-clinical Bcr/Abl cell culture and xenograft studies (reviewed in [34], in the late 1990's STI571 (imatinib; Gleevec®, Novartis pharmaceuticals) entered phase I/II trials in CML patients who had failed or were intolerant of interferon-α, the standard therapy at the time [35]. Even in these early dose-finding trials imatinib exhibited therapeutic efficacy unprecedented in such unfavorable risk CML patients, including many patients with CML in blastic phase [36]. These studies led to a recent phase III randomized study comparing imatinib dosed orally at 400 mg a day to interferon-α plus ARA-C in newly diagnosed CML patients [37]. The initial analysis this study still in progress revealed that imatinib patients had an complete hematological response rate of 95% and an estimated major and complete cytogenetic response rate of 85% and 74%, respectively (for definitions of CML response, see table 1).

Table 1. Hematologic and cytogenetic criteria for response in CML

Response Category	Magnitude	Criteria
Hematological response	Complete	Normal peripheral WBC and differential
		Platelets < 450,000/μl
		Resolution of all associated CML signs and symptoms
	Partial	Incomplete normalization of peripheral blood counts
		Persistent splenomegaly or other associated disease symptoms
Cytogenetic response	Complete	No Ph+ cells by cytogenetics/FISH
	Partial	<35% Ph+ cells
Major cytogenetic response= CGR +PCR	Minor	≥35% Ph+ cells
	No	100% Ph+ cells

Patients randomized to interferon-α had a 56% complete hematologic response rate and an estimated major and complete cytogenetic response rate of only 22% and 9%, respectively (figure 5, left). The time to major cytogenetic response was more rapid in imatinib-treated patients and imatinib was superior to interferon-α/ARA-C in estimated time to progression (figure 5, right). There were no differences in overall survival between the two arms, but follow-up was short and the analysis was complicated by the large number of patients crossing from the interferon-α arm to imatinib (57%).

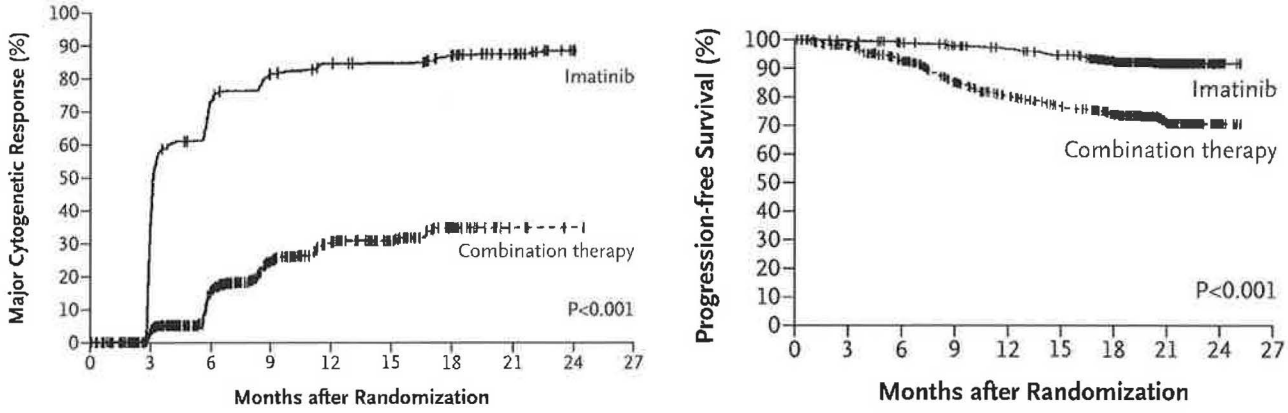


Figure 5. Kaplan Maier curve depicting the time to major cytogenetic response for CML patients treated on a large randomized phase III trial of imatinib versus interferon-α plus ARA-C.

Adapted from [37]

In contrast to the fairly unpleasant side-effects of interferon-α, imatinib was very well tolerated. The major side-effects included nausea, bloating, periorbital edema, muscle cramps, and skin rash [37]. These findings led to imatinib becoming the standard of care for the treatment of CML. Because of the initial efficacy and relatively favorable side effect profile of imatinib, initial studies did not establish the maximum tolerated dose. This, and the observation that imatinib rarely achieved BCR/ABL negativity by ultra-sensitive PCR detection techniques, led to a study of high dose imatinib therapy in newly diagnosed CML patients. At a dose of 800 mg a day, 90% of patients achieved a complete cytogenetic response and 28% achieved a reduction in BCR/ABL transcripts to undetectable levels ([37]; sensitivity $1:10^5$). The complete cytogenetic and molecular response rates were both statistically superior to a historical cohort receiving the standard 400 mg dose. Grade 3/4 hematological toxicity was significantly higher in the high dose group, but 82% of patients were able to maintain a dose of at least 600 mg a day. Long-term, randomized studies will be required to determine if the superior molecular responses observed with high dose imatinib ultimately translate into improved overall outcome.

Despite the inspiring success of imatinib thus far in CML, data from numerous studies have indicated that challenges still lie ahead. First, complete molecular responses by nested PCR at detection sensitivities of 10^6 or higher, commonly used to detect minimal residual disease in transplantation, have not been common with imatinib. Although this raises concerns for the inevitability of relapse, some argue that the eradication of all Bcr/Abl positive cells may not be required if they could be indefinitely suppressed by imatinib. It is not clear at present whether the

detection of BCR/ABL transcripts in a small percentage of otherwise normal individuals supports this hypothesis or not [38]. Second, results from the initial phase I/II and ongoing phase III studies have demonstrated that imatinib is less successful in advanced CML. In accelerated and blastic phase CML, imatinib has complete cytogenetic response rate of 34% and 8%, respectively [39, 40], indicating that additional approaches will be necessary to achieve durable disease control in advanced CML patients. Third, imatinib resistance has been reported in some chronic phase patients. Lastly, allogeneic bone marrow transplantation is the only established curative approach for CML, particularly when performed within the first year of diagnosis. Thus, CML patients with HLA-matched donors are faced with complex treatment choices until the long-term outcome of imatinib-treated patients has been determined.

Imatinib-resistant CML

In a recent German series of approximately 350 patients, 71 CML patients were classified as having imatinib-resistant CML. They included patients who relapsed while on imatinib therapy and those who never achieved a hematological response to imatinib (primary resistant CML; Table 2). Primary imatinib resistance did not occur in chronic phase CML patients, but was responsible for up to one half of the cases of imatinib-resistant CML observed in patients with more advanced phases of the disease [41, 42].

Table 2. Imatinib resistance by CML stage

CML phase		Relapse	1° resistance	Total	Percent
chronic		18	0	18	26%
accelerated		11	5	16	23%
blast	myeloid	17	17	34	48%
	lymphoid	2	0	2	3%
	Total	48	22	70	

Consistent with earlier phase II trials, over two-thirds of the imatinib-resistant CML cases occurred in patients with either accelerated or blast crisis CML. To determine possible mechanisms for CML relapse or primary resistance to imatinib, peripheral blood and bone marrow samples from these patients were analyzed by RT-PCR, FISH, and cytogenetics. About 13% of imatinib-resistant patients had evidence of increased BCR/ABL mRNA expression associated with at least one additional copy of the *BCR/ABL* gene. Point mutations in the *BCR/ABL* kinase domain predicted to interfere with imatinib binding were found in approximately 40% of imatinib-resistant CML patients. Complete cytogenetic data was available for only half of the patients, but about half of the evaluable imatinib-resistant patients had evidence of clonal evolution (acquisition of additional cytogenetic abnormalities besides t(9;22) in Ph positive cells). The most common cytogenetic abnormalities were duplication of the Ph chromosome, trisomy 8, and 17p alterations (loss of one p53 allele), all of which had been reported in advanced stages of CML prior to the availability of imatinib. Overall, a molecular or cytogenetic abnormality was detected in about 60% of imatinib-resistant CML patients that could explain the failure of imatinib [41, 42]. Interestingly, none of the primary imatinib-refractory CML patients had evidence of *ABL* kinase domain mutations. Although clonal evolution and increased Bcr/Abl expression were found in about 30% of imatinib-refractory patients, the mechanisms responsible for the remainder of the imatinib failures remained unresolved.

Imatinib-resistant *BCR/ABL* mutations

The prevalence of *BCR/ABL* kinase domain mutations in imatinib-resistant CML patients varies considerably among published series, perhaps due to differences in assay sensitivity. However, in all series *BCR/ABL* mutations have been more prevalent than *BCR/ABL* gene duplication [41, 43-46]. Over a dozen different *BCR/ABL* kinase domain mutations have been reported to date, with the vast majority clustered in the ATP-binding pocket (P-loop; P), activation (A) loop, and catalytic (C) regions of the Abl kinase domain (figure 6).

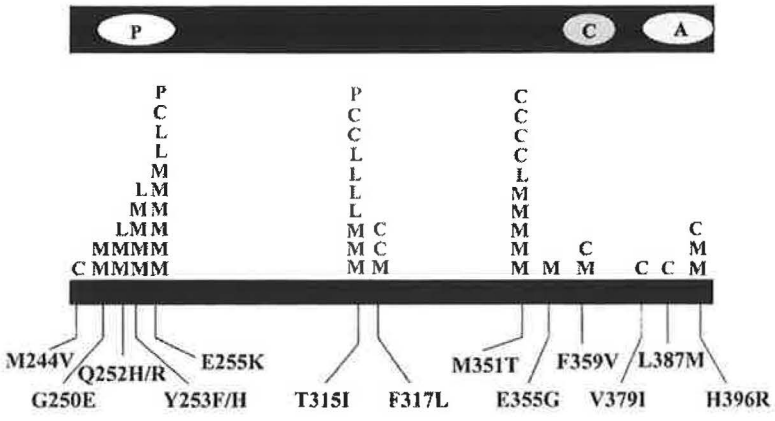


Figure 6. *BCR/ABL* mutations found in the leukemic cells of imatinib-resistant CML patients are clustered within a relatively short region of the kinase domain. The P-loop (P), catalytic (C) and activation (A) domains are depicted at top. At bottom, individual patients are depicted by letter indicating the stage of CML at time of relapse on imatinib: myeloid (M) or lymphoid (L) blast crisis, or chronic phase (C); P indicates patients in whom a mutation was present prior to imatinib therapy.

Adapted from [46].

In a recent series of 32 CML patients who had relapsed after an initial response to imatinib, mutations at amino acid 255 (glutamine changed to lysine), amino acid 315 (leucine changed to isoleucine), and amino acid 351 (methionine to threonine) comprised over 60% of the total mutations detected [46]. E255K (or E255V) and T315I have been among the most common mutations noted in other series of imatinib-resistant patients [43, 45, 47-49]. Modeling based of Abl and imatinib co-crystalization studies [33, 50] have predicted that the P-loop mutations E255K/V and Y253F/H ([48];figure 7), and others in the adjacent region, impair imatinib binding because they limit the flexibility of the kinase domain to accommodate imatinib [50, 51]. The T315I mutation is predicted to directly impair imatinib binding by disrupting a hydrogen bond that occurs between imatinib and the Abl kinase domain [50]. The precise mechanisms by which mutations such as M351I and H396P impair imatinib binding are less obvious. The activation loop mutation H396P is located near Y393, a critical tyrosine that is phosphorylated in the activated form of Bcr/Abl. It has been hypothesized that the H396P mutation causes the activation loop to adopt a more open conformation, mimicking the conformation adopted upon Y393 phosphorylation, and creating a steric clash for imatinib binding [33]. M351I is located in the Abl kinase domain C lobe near the activation loop, and it may limit the flexibility of this region to accommodate imatinib.

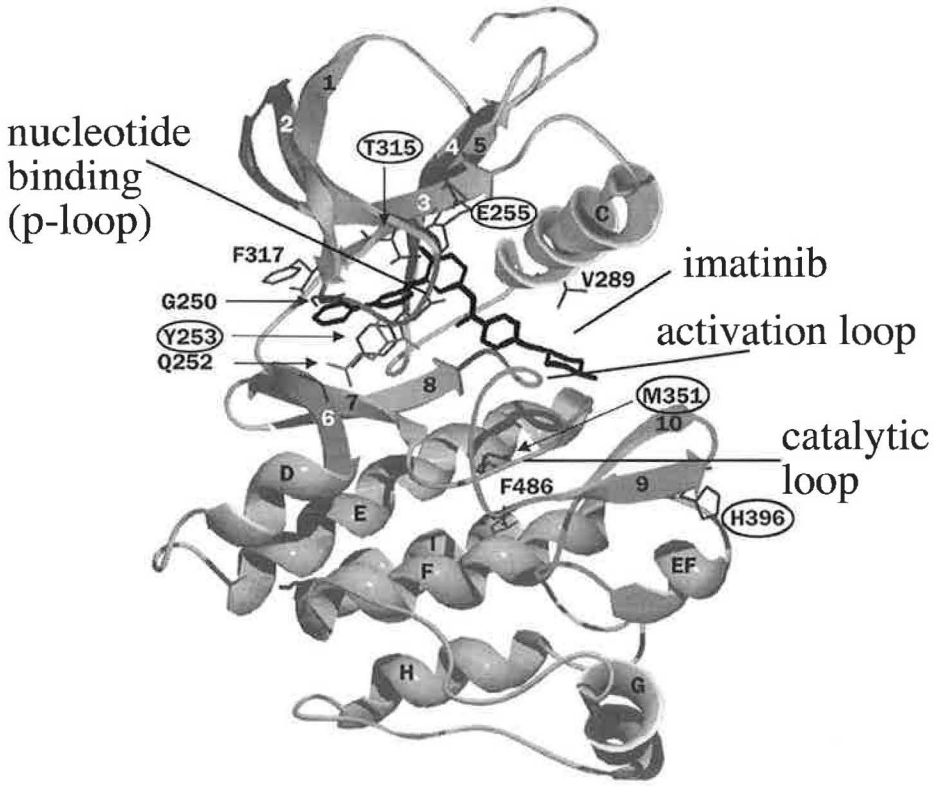


Figure 7. A ribbon model of the Abl kinase domain bound to imatinib (shown in black). The location of some Abl kinase domain mutations found in imatinib-resistant CML patients is shown relative to the β strands (numbers) and α helices (letters) comprising the P-, activation, and catalytic loops. The mutations discussed in the text are circled.

Adapted from [48].

Most imatinib resistance-associated Bcr/Abl mutants have the same kinase activity as wild-type Bcr/Abl, likely because the Bcr/Abl binding sites for imatinib and ATP overlap but are not identical [33, 50]. Despite the geographic proximity of most *BCR/ABL* kinase domain mutations, mutants vary in their degree of resistance to imatinib *in vitro*. For example, the mutations E255K/V and T315I are highly resistant to imatinib, with an IC_{50} of greater than 5 μM, while the mutants Y253H or Y253F have more modest degrees of imatinib resistance, with IC_{50}s of 3.7 μM and 1.8 μM, respectively (table 3).

Table 3. Characteristics of some of the most prevalent *BCR/ABL* kinase domain mutations

Bcr/Abl mutant	IC_{50}	location	postulated mechanism
Y253F	1.8 μM	P-loop	restricts conformation nec. to bind imatinib
Y253H	3.7 μM	P-loop	restricts ability of kinase domain to bind imatinib
E255K/V	> 5 μM	P-loop	
T315I	> 5 μM	kinase domain	alters a direct imatinib:kinase domain interaction
M351T	ND*	kinase domain	restricts activation loop conformation change necessary to bind imatinib
H396P	4.3 μM	activation loop	
H396R	5.4 μM	activation loop	

*kinase activity only 20% of WT; data compiled from [42, 52].

There is emerging evidence that clinical outcome may be influenced by the type of *BCR/ABL* mutation present at relapse. For example, in a recent study the median survival of CML patients with P-loop mutations was only 4.5 months after detection, while patients with non-P-loop mutations had only a 21% mortality rate at a median follow-up of 11 months [53]. This suggests that less potent Bcr/Abl mutants may be suppressible with higher doses of imatinib alone.

The molecular pathogenesis of *BCR/ABL* mutations in CML

The precise incidence of *BCR/ABL* mutations in newly diagnosed CML has yet to be fully elucidated. In a study of 144 imatinib-treated CML patients, patient samples were analyzed by direct RT-PCR-generated DNA sequencing for evidence of *ABL* kinase domain mutations. Thirty-three percent of accelerated phase patients were found to have a *BCR/ABL* kinase domain mutation, compared to 22% and 0% of late- and early-chronic phase patients, respectively [53]. *BCR/ABL* mutations were also more frequent in patients who had started imatinib more than 4 years after diagnosis compared to patients starting imatinib earlier. Others using more sensitive techniques have detected the presence of *BCR/ABL* mutations prior to the initiation of imatinib [44, 54-57]. In another study, investigators analyzed samples from patients with CML relapsing on imatinib by subcloning the RT-PCR products and sequencing multiple clones. The frequency of *BCR/ABL* mutation using this more sensitive approach was 91% [46]. A similar analysis of 13 patients who were having in a complete hematologic response to imatinib but failed to achieve a major cytogenetic response revealed that four patients harbored mutations. Three of these four patients ultimately progressed on imatinib within 18 months. These findings indicate that (1) *BCR/ABL* mutations can be present even at diagnosis, but highly sensitive techniques are required for their detection; (2) *BCR/ABL* mutations are more prevalent in advanced and relapsing imatinib-treated CML patients, suggesting a role for disease- and/or therapy-induced clonal selection.

Because Bcr/Abl has been implicated in mediating genomic instability [58-62], investigators have examined whether *BCR/ABL* mutations might indicate a more global increase in genomic mutations. In five patients with known *BCR/ABL* mutations, sequence analysis of 700 bp upstream of the Abl kinase domain and the c-Kit kinase domain (another tyrosine kinase with similar imatinib sensitivity [31]) revealed no additional mutations, suggesting that biologic selection for imatinib-resistant cells played a more important role than a global increase in mutagenesis [46].

Mechanisms of imatinib resistance other than *BCR/ABL* mutations and Bcr/Abl overexpression

BCR/ABL mutations and overexpression of wild type Bcr/Abl clearly play important roles in conferring resistance to imatinib. However, these two mechanisms do not explain all cases of CML relapsing or resistant to imatinib. Other potential mechanisms can be divided into two broad categories, based on their dependence on Bcr/Abl. Of the Bcr/Abl-dependent mechanism, probably the one of most concern is the observation that CML progenitors persist in many imatinib-treated patients [63, 64]. In a recent study, 11 of 15 CML patients in complete cytogenetic remission on imatinib were found to have 6.5-13.2% *BCR/ABL* + CD34+ cells by FISH [65]. The clinical significance of this finding remains to be determined since only two of the cohort has progressed to clinical relapse, and both patients were in accelerated phase at the start of imatinib.

The detection of BCR/ABL transcripts post-allogeneic BMT has been an important predictor of relapse [66]. However, in some CML patients with durable complete cytogenetic and molecular remissions on interferon-α, the presence of BCR/ABL + progenitors did not appear to guarantee relapse [67]. Thus, longer follow-up will be required to determine if persistent *BCR/ABL* + progenitors can be indefinitely suppressed by imatinib, or whether such cells will be potential reservoirs for the development of mutant Bcr/Abl clones or secondary molecular derangements. Nonetheless, the observation that CML patients on imatinib for two years or longer can still harbor significant numbers of BCR/ABL+ progenitors underscores the importance of closely monitoring imatinib-treated CML patients. Future challenges will be to determine how CML progenitors survive imatinib treatment. It is possible that Bcr/Abl is more important for myeloid expansion than CML progenitor survival, allowing for the persistence of CML progenitors in imatinib-treated patients [68]. Because imatinib is also a potent inhibitor of c-Kit [31], imatinib-mediated inhibition of c-Kit/stem cell factor signaling may contribute to the quiescence of CD34+ BCR/ABL+ cells. Lastly, CML progenitors, like other hematopoietic stem cells [69], may have altered expression of drug efflux pumps that interfere with the ability of imatinib to maintain therapeutic intracellular concentrations.

Bcr/Abl-independent mechanisms for imatinib resistance also represent an important, but still relatively undefined explanation for the failure of imatinib in some CML patients. It has been known for many years that additional chromosomal abnormalities may accompany CML progression, particularly alterations in chromosomes 7, 8, 17, 19, or 21 [70-73]. Alterations in p53 or Rb function are found in as many as one-third of cases of CML in blastic phase, particularly myeloid blast crisis [74-79], while deletion of the cell cycle inhibitor p16 has been associated more with lymphoid blastic transformation [80]. Thus, incomplete eradication of CML progenitors, or the initiation of imatinib in patients with advanced stages of CML could leave patients at risk for the development of Bcr/Abl-independent leukemic clones. Interestingly, cytogenetic abnormalities have recently been reported in Ph chromosome negative cells from imatinib-treated CML patients [81]. The clinical implications of this phenomenon, and the role of imatinib in this process, remain to be determined. Atypical cytogenetic findings have also been reported in some CML patients treated with interferon-α [82], suggesting that chronic alterations in BM cytokine milieu might provide a selective advantage for cells with certain chromosomal abnormalities.

The anti-leukemic effect of imatinib is likely mediated through the inhibition of multiple Bcr/Abl-dependent signal transduction pathways. Recent studies suggest that imatinib resistance may occur in some patients because of the constitutive activation of Bcr/Abl-independent signaling pathways such as Lyn and NF-κB that are not inhibited by imatinib [83, 84]. The role of the drug-binding plasma protein α1-acid glycoprotein in CML imatinib resistance has been controversial. Although α1-acid glycoprotein does bind imatinib with high affinity, it has been difficult to prove that it interferes with the therapeutic activity of imatinib in vivo [85-87].

Strategic approaches for the treatment of CML in the post-imatinib era

Since the adoption of imatinib as first line therapy for CML, treatment algorithms have become increasing complex. In the recent past, the formulation of CML treatment plans centered on the availability and feasibility of allogeneic BMT because it has been the only established curative therapy. Now, because all newly diagnosed CML patients will receive imatinib, attention has focused on developing an algorithm for separating patients into two groups: (1) patients who can be maintained on imatinib with a relatively low risk for disease progression (2) patients who are unlikely to be cured or have durable control of their disease with imatinib. Because allogeneic BMT is most effective when performed within the first year of diagnosis, an ideal monitoring schema would be sufficiently rigorous to detect imatinib failure early enough to allow patients with HLA-compatible donors to undergo BMT without compromising their outcome. Further, for those patients lacking transplantation options, sensitive assays of imatinib failure would allow sufficient time for patients to consider other therapeutic options or enroll on research protocols. Previous studies in CML patients treated with interferon-α showed that BCR/ABL transcript number correlated well with cytogenetic response and clinical outcome [88-91]. In imatinib-treated patients, quantitative RT-PCR analysis of BCR/ABL transcript level after two or three months of therapy has been demonstrated to correlate with the degree of cytogenetic response achieved at six months [92, 93]. Others have found that a 50% reduction in BCR/ABL transcript level after four weeks of imatinib predicted a major cytogenetic response at six months [94]. Investigators analyzing data from the IRIS imatinib trial used the combination of complete cytogenetic remission and major molecular remission (defined as a $\geq$ 3 log reduction in baseline BCR-ABL/ABL transcript ratio) to identify a particularly favorable subgroup of newly diagnosed CML patients who had not progressed in the first two years of therapy (figure 8).

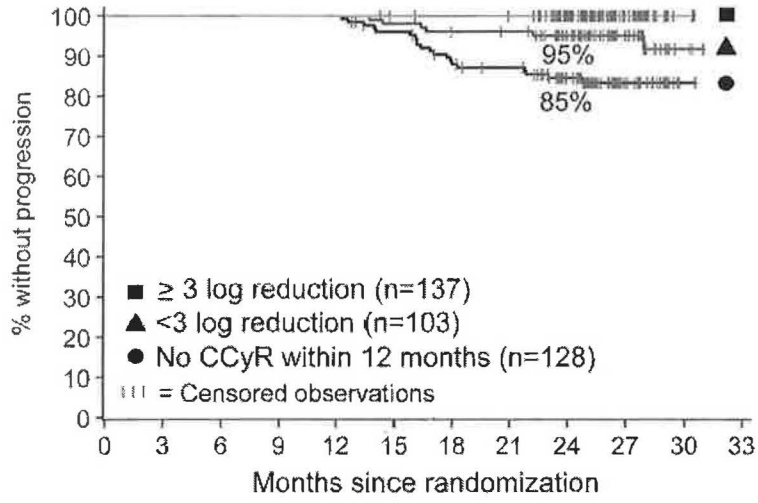


Figure 8. The influence of complete cytogenetic response (CCyR) and reduction in BCR/ABL transcripts on CML progression on imatinib.

Adapted from [95, 96].

Although these data cannot predict the ultimate success of imatinib in this favorable subgroup, it does reflect initial attempts to group CML patients into prognostic categories based on sophisticated molecular techniques. Until long-term follow up is available for imatinib-treated patients, the decision between imatinib and stem cell transplantation will remain largely a leap of faith influenced by evolving clinical and scientific data. As always, for patients at the far ends of the clinical spectrum the decision will be more straightforward, and for most patients in the middle decisions will be accompanied by considerable uncertainty (figure 9).

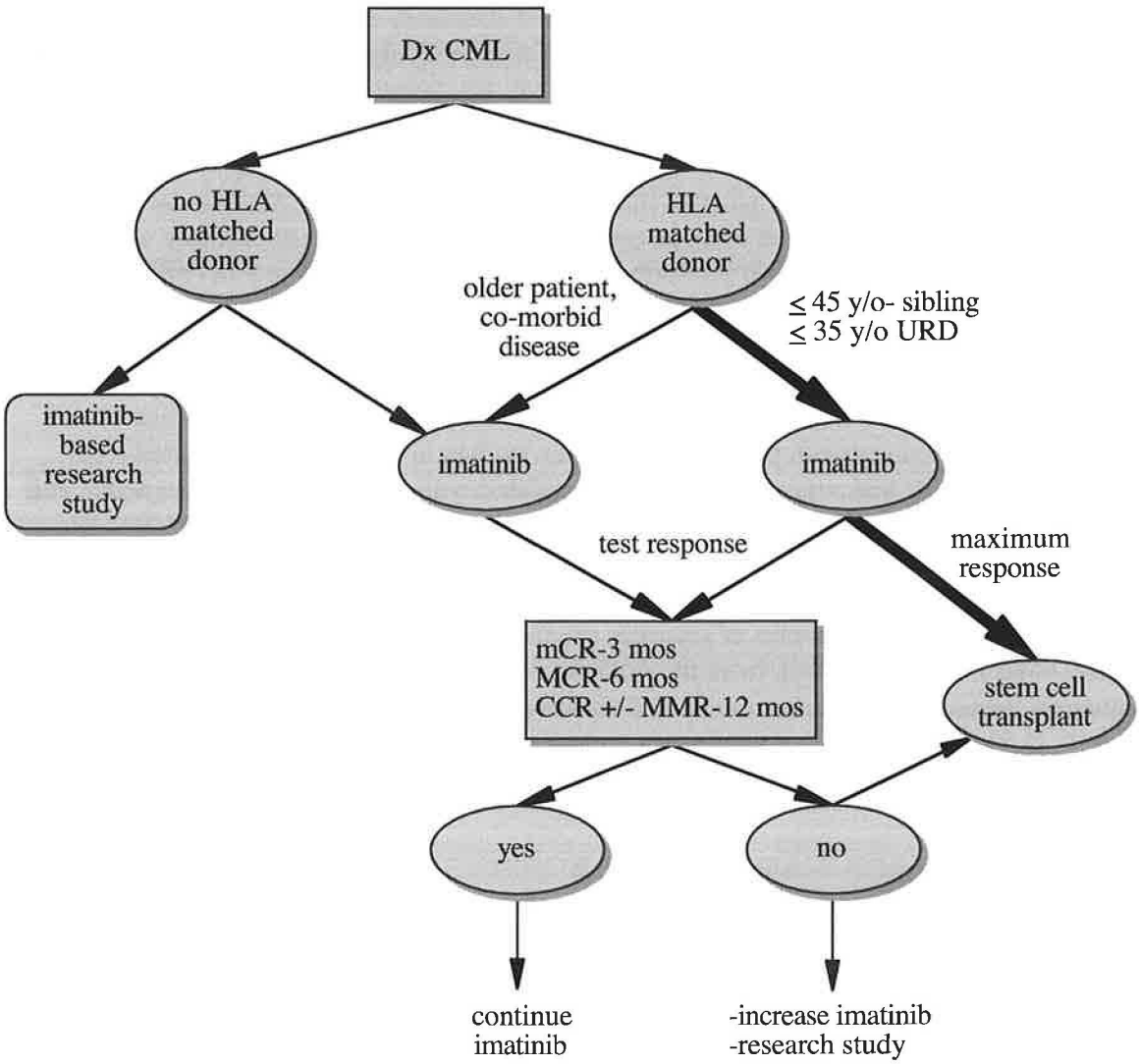


Figure 9. One potential treatment algorithm for newly diagnosed CML patients. Path in bold represents those patients or doctors who chose to emphasize allogeneic stem cell transplant. The goals for imatinib response are that patients must achieve at least a minor cytogenetic response (mCR) by 3 months, a major cytogenetic response (MCR) by 6 months, and a complete cytogenetic response (CCR) with or without a major molecular response (MMR) at 12 months. The treatment plan for patients failing to meet these targets should be re-assessed to determine whether increased dose of imatinib, a research study, or allogeneic stem cell transplant should be considered.

The option of a non-myeloablative stem cell transplant may lower the threshold for transplant for some CML patients. These reduced conditioning regimen transplants have lower acute morbidity and mortality than standard myeloablative transplant regimens, but further study will be required to assess the long term morbidity and overall success rate of non-myeloablative transplants in CML [97-99]. The availability of donor leukocyte infusions to salvage transplant relapse has also been an important advance in improving transplant outcome [100-102], but challenges still remain for carefully balancing graft versus leukemia and graft versus host disease in CML [103-105]. In the future, improvements in

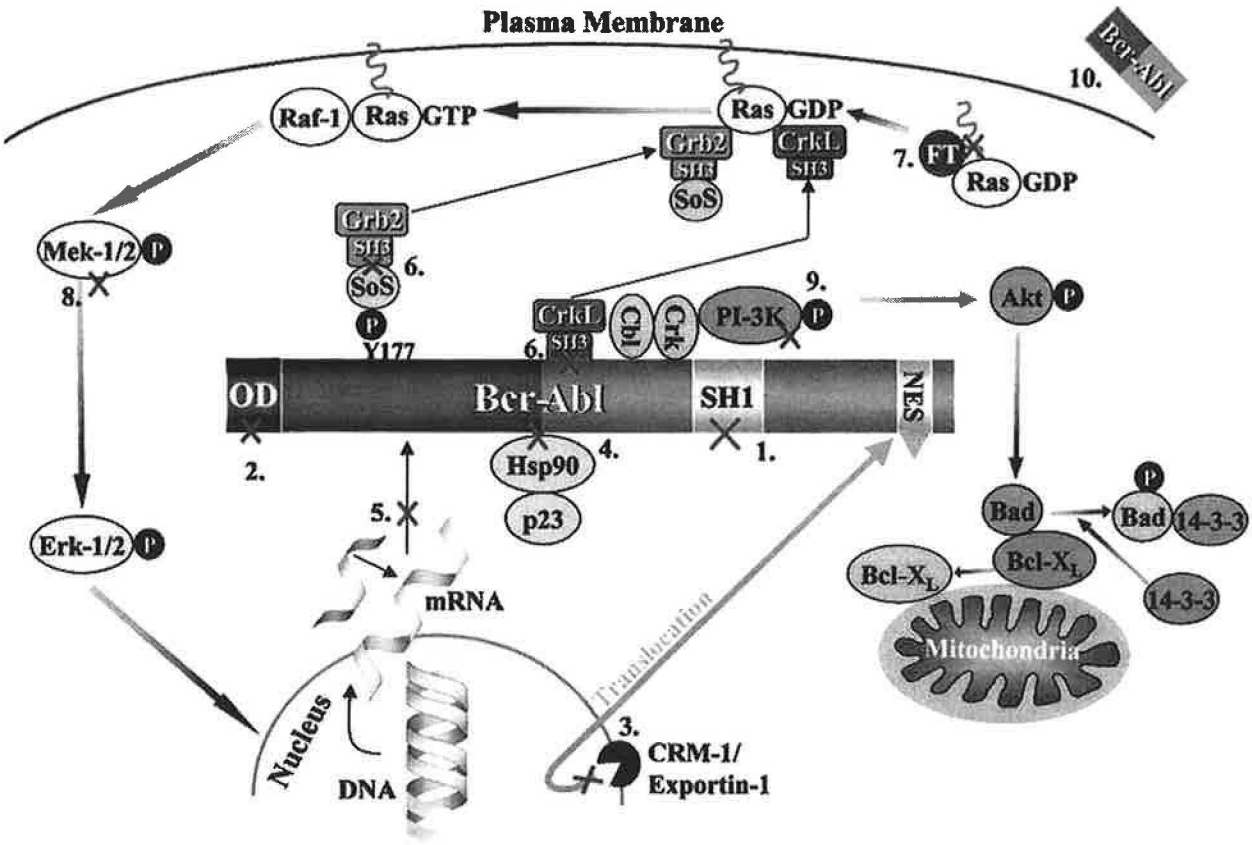
transplantation may facilitate multifaceted CML treatment approaches in which imatinib could be used to improve the functional status of advanced stage patients pre-transplant, and salvage or prevent relapse in patients post-transplant [106-110].

Another important goal for CML therapeutics is the development of a schema to identify and treat patients who show early signs of losing an imatinib response (reviewed in [111, 112]). Although treatment guidelines vary somewhat by treatment center, imatinib-treated patients should be monitored by peripheral blood cytogenetics, *BCR/ABL* FISH, and quantitative BCR/ABL transcript studies at diagnosis and every three months thereafter for at least the first year, or until a maximum imatinib response is achieved. In patients who achieve a complete cytogenetic response, cytogenetic and molecular studies may be spread out to six-month intervals if peripheral blood counts remain normal. Patients whose response reaches a plateau short of complete cytogenetic response should probably be monitored every three months to allow sufficient warning for consideration of alternative approaches. BM studies are generally more sensitive for confirmation of complete cytogenetic or major molecular responses, and are often periodically useful to validate peripheral blood responses. Loss of a complete hematologic response (unrelated to imatinib hematological toxicity) should correlate with alterations in cytogenetic or molecular response, or evidence of clonal evolution (acquisition of additional chromosomal abnormalities in Ph chromosome positive cells by cytogenetic analysis). It should be noted that some patients may experience significant myelosuppression on imatinib. Although BM suppression during imatinib therapy is an adverse risk factor in advanced CML patients [113], it does not necessarily reflect imatinib failure in early stage patients. Thus, it is important to minimize interruptions in imatinib therapy and support patients as needed with cytokines [114-116].

Overall, a loss of a complete hematologic or cytogenetic response, evidence of clonal evolution [117-119], increase in Ph chromosome positive cells by 30% [111], or a consistent increase in BCR/ABL transcripts are indications that alternative therapeutic approaches may need to be considered. Although increased dosage of imatinib may improve the hematologic and cytogenetic parameters in some patients starting to fail imatinib, or in whom the response has reached a plateau [120], the durability of such responses is often brief [121, 122], so attention should be directed to formulating alternative approaches. In selected cases, patient samples may be sent for *BCR/ABL* kinase domain sequence analysis to identify Bcr/Abl mutant clones that might respond to the cessation of imatinib therapy.

Novel treatment approaches for the treatment of patients with de novo and imatinib-resistant CML

The downstream targets of Bcr/Abl and the molecular pathogenesis of Bcr/Abl cellular transformation have been extensively studied (reviewed in [123-125]. The fruit of this effort has been the identification of numerous other molecular targets with potential therapeutic utility for CML patients (figure 10). Therapeutic agents have not been developed for all potential Bcr/Abl targets depicted in the figure on the following page, but several are in pre-clinical and early clinical development. Besides their potential use in treating or preventing imatinib resistance, some may be partnered with autologous BMT, a historically ineffective treatment for CML. There has also been considerable interest in combining imatinib with a variety of other conventional anti-CML drugs such as interferon-α (recently reviewed in [34, 126]), but cumulative myelotoxicity and the relatively rarity of CML will make this a complex and lengthy endeavor.



Adapted from [123].

Figure 10. Potential molecular targets for CML therapy. Agents targeting Ras (items numbered 6-8), the Abl kinase domain (SH1), PI-3 kinase (items 6, 9), and Bcr/Abl protein stability (item 4) are in various stages of pre-clinical and clinical development. Agents targeting the Bcr/Abl oligomerization domain (OD; important for Bcr/Abl activation) or BCR/ABL transcripts (item 5), or attempts at trapping Bcr/Abl in the nucleus (item 3), or eliciting a Bcr/Abl-specific immune response by junction-specific peptide vaccines (item 10) may prove more challenging.

The Abl kinase domain (SH1)

Apart from imatinib, other small molecule tyrosine kinase inhibitors are in various stages of pre-clinical and early clinical development. PD166326, a member of the pyridopyrimidine class, has been demonstrated to be approximately 100 times more potent than imatinib in inhibiting Bcr/Abl kinase activity *in vitro* [127], perhaps because of its ability to inhibit Bcr/Abl in both active and inactive conformations [33]. At least two other tyrosine kinase inhibitors more potent than imatinib are in early pre-clinical/clinical studies. Other agents include Adaphostin (NSC 680410), an analogue of the tyrosine kinase inhibitor tyrphostin AG957, which also targets the Abl kinase domain [128], but appears to act by blocking Bcr/Abl substrate interactions rather than interfering with ATP binding. In pre-clinical studies, adaphostin inhibited Bcr/Abl autophosphorylation and Bcr/Abl protein stability, and had activity against imatinib-resistant cells [129, 130], suggesting that adaphostin and imatinib might be an effective clinical combination.

Bcr/Abl protein stability

The novel anti-cancer drug 17-AAG is an inhibitor of the molecular chaperone protein Hsp90, which is responsible for stabilizing a number of signaling proteins, growth factor receptors, and oncogenic proteins including Bcr/Abl [131-134]. Treatment of Bcr/Abl-expressing cells with 17-AAG has been demonstrated to disturb the interaction of Bcr/Abl with the chaperone protein Hsp90, resulting in a proteosome-dependent decrease in the stability and expression of Bcr/Abl protein, and the apoptosis of Bcr/Abl-expressing cells [132, 135]. 17-AAG also has activity against cells expressing imatinib-resistant *BCR/ABL* mutations *in vitro* [136]. Phase I/II trials of 17-AAG in imatinib-resistant CML are currently in progress, although 17-AAG may prove more effective when administered in combination with imatinib in newly diagnosed CML patients.

Targeting the Ras signal transduction pathway

Generally, the Ras signal transduction pathway has not been considered to play a major role in Bcr/Abl transformation. However, several downstream Ras effectors appear to play collaborative roles in Bcr/Abl leukemogenesis, and therapeutic agents targeting the Ras signaling cascade have had some encouraging initial success. Despite fairly modest constitutive Ras activation in CML, the farnesyl transferase inhibitor (FTI) SCH66336 (lonafarnib, Schlering Plough) had potent activity in a murine Bcr/Abl lymphoid leukemia model, and also inhibited the growth of primary CML cells in a BM colony assay [137]. In this study, SCH66336 had greater activity against Bcr/Abl cell lines than co-expression of dominant negative ras, suggesting FTIs may also inhibit non-Ras targets. SCH66336 also has been demonstrated to have activity against imatinib-resistant human CML cell lines [138]. Another FTI, R115777 (Zarnestra, Johnson & Johnson) has been demonstrated to have activity in CML blast crisis patients [139], suggesting that imatinib and FTI combinations may be useful in high-risk and imatinib-resistant CML patients.

Other Ras effectors such as Raf-1 [140], Grb2/Sos [141, 142], CrkL [143-146], and Shc [141, 147] have been implicated in Bcr/Abl transformation, providing rationale to investigate MEK1 inhibitors, which target the downstream activation of mitogen activated protein kinase (MAPK)1/2. Several different MEK1 inhibitors have shown activity against human CML cell lines [148], and to act synergistically when combined with imatinib in vitro [149]. Because mutagenesis of the Grb2-binding site in Bcr completely blocked the induction of CML in mouse models, peptide-based therapy targeting Grb2 has also been studied. Peptides targeting the src homology (SH) 3 domain of Grb2 disrupted its interaction with Sos, reduced MAPK1/2 activation, and suppressed the proliferation of CML cell lines and primary patient CML blast samples [150].

Inhibition of the PI-3 kinase/mTOR pathway

Inhibition of the phosphatidylinositol (PI)-3 kinase pathway has been demonstrated to impair the proliferation and survival of Bcr/Abl-expressing cells [151, 152]. Targeting PI-3 kinase and its downstream effector Akt has also been of recent interest because of evidence of cross-talk between PI-3 kinase and the adapter proteins Grb2 and Gab2, both of which are important for Bcr/Abl transformation [142, 153]. A recent study found that the PI-3 kinase inhibitors wortmannin and LY294002 both had activity against CML progenitors *in vitro*, including cells resistant to imatinib [154]. Further investigation in CML patients will require the clinical development of effective PI-3 kinase inhibitors. There has also been recent interest in targeting mammalian target of rapamycin (mTOR), a downstream effector of Akt (reviewed in [155]), in CML. A recent study found that rapamycin, an mTOR inhibitor, was syngergistic with imatinib against Bcr/Abl-expressing cells *in*

vitro, and improved the survival of mice in a CML animal model *in vivo* [156]. Several mTOR inhibitors are in early clinical trials in refractory hematological malignancy and other cancers, suggesting the feasibility of studying mTOR/imatinib combinations in patients with advanced CML in the near future.

Summary and future directions

Advances in basic and translational research made molecularly-targeted therapy for CML a reality, and provided an important paradigm for rational cancer therapeutics. Despite important advances in CML therapy, important challenges and goals lie ahead. These include: (1) Determine if tyrosine kinase inhibitors like imatinib, administered at the highest tolerated doses, are capable of achieving durable, long-term remissions/cures (2) Identify imatinib combination regimens capable of curing *de novo* CML (3) Improve allogeneic stem cell transplantation efficacy and minimize morbidity (4) Determine if advances in molecular CML therapy can render autologous transplantation a viable therapeutic option for CML patients (5) Identify new agents for treating imatinib-resistant CML.

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