Part I: Mechanisms of resistance to imatinib in chronic myeloid leukaemia

Jane F Apperley

The introduction of selective tyrosine-kinase inhibitors (TKIs) for the treatment of chronic myeloid leukaemia has changed patient outcome and, consequently, management of this disease. Imatinib is now the treatment of choice for most newly diagnosed patients. Excellent responses, in terms of symptom control and haematological parameters, are usually obtained. However, failure to completely eradicate leukaemic cells and the escape of these cells from previous control has led to an intensive search for the mechanisms of resistance and subsequent treatments by which to overcome this resistance. Up to now, there has been considerable focus on the role of ABL-kinase-domain mutations as mediators of resistance to imatinib, thereby encouraging the development of a second generation of TKIs capable of inhibiting these mutant proteins. However, studies have increasingly shown that these mutations do not account for all cases of resistance and have a negligible role in the inability of TKIs to eradicate residual disease in patients who are good responders. More recently, attention has turned to the relative roles of drug bioavailability and drug efflux and drug influx proteins in the development of resistance to imatinib. This review is the first of two papers and discusses imatinib resistance and its potential causes. The second paper will focus on the assessment and subsequent management of patients with less than optimum responses to imatinib.

Introduction

Chronic myeloid leukaemia (CML) has long been used as a paradigm for understanding the molecular pathogenesis of malignancy. CML has a pivotal place in oncology due to the fact that more than 95% of affected individuals express the hybrid BCR-ABL protein from the genetic rearrangement formed by reciprocal translocation between chromosomes 9 and 22, which results in a shortened 22q—the Philadelphia (Ph) chromosome.

The subsequent dysregulation of the ABL protein leads to enhanced proliferation, resistance to apoptosis, and altered adhesion—key characteristics of CML cells. The success of imatinib, the first tyrosine-kinase inhibitor (TKI) to be introduced into clinical practice, is due to the ability of the inhibitor to occupy the ATP-binding pocket of the ABL-kinase domain (figure 1), which prevents a change in conformation of the protein to the active form of the molecule, with the subsequent death of target cells.

The potential for imatinib and later generation TKIs to cure CML has been debated extensively. Much of the debate has focused on the fact that residual BCR-ABL-positive cells are present in more than 95% of patients treated with imatinib who achieve complete cytogenetic remission and these cells potentially form a reservoir for future disease progression, because they are resistant to imatinib. Furthermore, some newly diagnosed patients do not achieve a complete haematological response, with 20–25% not reaching complete cytogenetic remission; hence, their disease is also resistant. Additionally, a further 20–25% of those who do achieve complete haematological response or complete cytogenetic remission, or both, will eventually stop responding, and acquire resistance during exposure to the drug. Finally, patients treated with imatinib for the first time in the accelerated or blastic phases are more likely to show either primary refractoriness or acquired resistance to imatinib than patients treated in the chronic phase. Because the frequency and degree of resistance clearly differs between these patient groups described above, the mechanisms of resistance will also probably differ.

Definitions of response and resistance

Defining resistance in CML is impossible without first defining response. For all patients treated with imatinib, the initial goal is the attainment of complete...
haematological response, defined as a normal peripheral-blood count in association with less than 5% of blasts in the bone marrow, ideally within 4 weeks of starting treatment and certainly by the 12th week of treatment. Response to treatment is then monitored by serial cytogenetic assessments of the bone marrow with the next level of response being absence of the Ph chromosome—ie, complete cytogenetic remission—detected by conventional metaphase analysis. Most patients treated with imatinib from diagnosis have complete cytogenetic remission within 12 months.2

Further monitoring of **BCR-ABL** is then done either by fluorescence in-situ hybridisation, which has a sensitivity of up to $10^{-3}$ (and a false-positivity of 2–5%) or, preferably, by reverse-transcriptase polymerase chain reaction (RT-PCR), with a sensitivity of $10^{-5}$. Molecular monitoring of patients treated with imatinib from diagnosis shows that attainment of a three-log decrease in the concentration of **BCR-ABL** transcripts by RT-PCR from a laboratory standard value obtained for untreated patients is associated with a low risk of disease progression and, by extrapolation, with an increased duration of chronic phase.1 The three-log decrease has now become synonymous with a major molecular response. Up to now, about 5% of patients who achieve complete cytogenetic remission have become RT-PCR negative, but this number might increase with longer exposure to imatinib.1

Now that responses have been defined, an attempt at classifying lack of response (ie, less than optimum response) seems more reasonable. A consensus panel has made recommendations for the definition of failure, suboptimum response, and warning signs (table 1).4 Allogenic stem-cell transplantation remains a curative option for suitable patients who are responding less well to imatinib and, therefore, the physician should try to make the decision for transplant as soon as possible in the disease course. For this reason, time to achievement of complete haematological response, complete cytogenetic remission, and major molecular response has been included in the definition of a response.5 Failure to reach complete cytogenetic remission and major molecular response within these timeframes is not necessarily synonymous with failure on imatinib. For example, a proportion of patients are known to be slower than others in achieving a complete cytogenetic remission. However, slow responders who eventually achieve complete cytogenetic remission will not necessarily have a worse prognosis than fast responders. Therefore, these response parameters should not necessarily be used as an indication to discontinue imatinib treatment unless a useful alternative treatment is available.

By contrast, loss of response to imatinib is clear if the loss of response involves loss of complete haematological response and complete cytogenetic remission. Loss of molecular response is currently more difficult to assess, because RT-PCR assays are not standardised and individual results should be confirmed by serial monitoring until a definite pattern is identified. Efforts to standardise methods of RT-PCR for **BCR-ABL** using internationally agreed upon standardised reference materials are in progress.6

The issue of resistance is more complex than simply lack or loss of some predefined response. Clearly, several patients who do not have any response to imatinib exist in all phases of disease. This absence of response is known as primary resistance or, perhaps more appropriately, primary refractoriness. Patients that achieve a certain level of response—ie, haematological, cytogenetic, or molecular—and that subsequently lose that response, can be described as having acquired resistance. Rather more difficult to classify are individuals who clearly respond, but do not become RT-PCR negative. In theory, all these patients are resistant. However, the mechanism of resistance is unlikely to be the same in those who achieve a three-log reduction in transcript levels as in those who only obtain complete haematological response and remain 100% Ph-chromosome positive. This is an area in which further clarity is needed, because newer TKIs will probably be initially approved for patients who

<table>
<thead>
<tr>
<th>Months from start of imatinib</th>
<th>Failure of response</th>
<th>Suboptimum response</th>
<th>Warning sign</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>High-risk Sokal score; Del 9q+; additional clonal abnormalities in Ph-chromosome-positive cells</td>
</tr>
<tr>
<td>3</td>
<td>No haematological response (ie, stable disease or progression)</td>
<td>Less than complete haematological response</td>
<td>NA</td>
</tr>
<tr>
<td>6</td>
<td>Less than complete haematological response; no cytogenetic response</td>
<td>Less than partial cytogenetic response (Ph-chromosome-positive cells &gt;35%)</td>
<td>NA</td>
</tr>
<tr>
<td>12</td>
<td>Less than partial cytogenetic response (Ph-positive-chromosome cells &gt;35%)</td>
<td>Less than complete cytogenetic response</td>
<td>Less than major molecular response</td>
</tr>
<tr>
<td>18</td>
<td>Less than complete cytogenetic response</td>
<td>Less than major molecular response</td>
<td>NA</td>
</tr>
<tr>
<td>At any time</td>
<td>Loss of complete haematological response; loss of complete cytogenetic response; mutation with high insensitivity to imatinib</td>
<td>Additional clonal abnormalities in Ph-positive-chromosome cells; loss of major molecular response; mutation with low insensitivity to imatinib</td>
<td>Any rise in BCR-ABL transcripts; additional clonal abnormalities in Ph-chromosome-negative cells</td>
</tr>
</tbody>
</table>

NA—not applicable.

Table 1: Definition of no response to imatinib (adapted from reference 4)
Review

are resistant to imatinib. How patients are defined as resistant is, therefore, important.

**Mechanisms of resistance**

The existence of patients resistant to imatinib was evident soon after the introduction of the drug into clinical practice. Initial responses were lower in patients with advanced-phase disease (ie, acceleration phase, and myeloid and lymphoid blast crises), and responses tended to be transient in most responders with advanced-phase disease. However, rather than being disappointed that imatinib was largely ineffective in advanced disease, researchers should have been surprised that the drug worked at all, because blast crisis is usually attributed to additional genetic changes conferring BCR-ABL-independent pathways of proliferation and maturation arrest.

These findings led to laboratory efforts to model the development of resistance. Resistant cell lines were generated by exposure to gradually increasing concentrations of imatinib. Mechanisms identified at the time included overexpression of BCR-ABL associated with amplification of BCR-ABL, and overexpression of the multidrug-resistant P-glycoprotein (MDR-1; also known as ATP-binding competitor B1 [ABCB1]).

**Figure 2: Mechanisms of imatinib resistance**

**ABCB1**=ATP-binding competitor B1. **OCT1**=organic cation transporter 1. **CYP3A4**=cytochrome P450 isoenzyme 4A. **AGP**=alpha-1 acid glycoprotein.

However, resistance to any particular drug is likely to be a multifactorial process. Imatinib is given orally and, like other oral medications, is therefore subject to variations in gastrointestinal absorption and first-pass metabolism, as well as: plasma-protein binding; cellular drug influx and drug efflux; enzymatic inactivation; changes in expression or mutations of the target molecule; defects in apoptosis, senescence, or repair mechanisms; and the development of alternative pathways of signal transduction (figure 2). These mechanisms and their potential interactions will be reviewed in more detail below.

**Oral bioavailability**

Early studies on the pharmacokinetics of imatinib showed considerable interpatient variability in imatinib concentrations. Oral bioavailability is established by gastrointestinal absorption and first-pass drug metabolism in the liver. Imatinib is largely neutralised by the
cytotoxic drug resistance was first described in the 1970s. Overexpression of the cell-surface transmembrane ATPase ABCB1 seemingly conferred insensitivity to many chemotherapeutic drugs by active (ie, energy-dependent) drug transport. In subsequent years, research not only showed that ABCB1-mediated drug resistance was only one of a variety of mechanisms inducing resistance, but also that the significance of overexpression of ABCB1 was unclear. Cells from patients in blast crisis have a higher expression of ABCB1 compared with those from patients in chronic phase, and upregulation of MDR-1 was deemed a potential cause of the decreased sensitivity to chemotherapy in patients with advanced-phase disease. However, short exposure to drug treatment of primary blast cells in patients with acute myeloid leukaemia showed that MDR-1 was upregulated early, suggesting that this upregulation might be a general response of cells to toxic stimuli, irrespective of whether the drug in question is a substrate for ABCB1.

Overexpression of ABCB1 as a possible mechanism for resistance to imatinib was first recognised by Mahon and colleagues. They developed a resistant cell line, LAMA-84R, by gradual exposure to increasing concentrations of imatinib. Both BCR-ABL and ABCB1 were overexpressed compared with the sensitive parental cell line. Co-culture with verapamil, a protein-pump inhibitor, restored imatinib sensitivity. Overexpression of ABCB1 in resistant cell lines has now been confirmed by other groups. However, overexpression of multidrug-resistance protein 1 (MRP-1)—a key player in another resistance pathway—does not have a role in imatinib resistance, suggesting that imatinib is not a substrate for MRP-1.

Although overexpression of ABCB1 has been suggested as a mechanism for resistance to imatinib, efflux of imatinib from ABCB1-expressing cells is less efficient than efflux of other anticancer drugs, such as doxorubicin, daunomycin, and vincristine. Furthermore, cell-line studies suggest that overexpression of ABCB1 cannot confer resistance to imatinib, and loss of expression in a murine model of CML does not improve response to imatinib.

The role of ABCB1 in clinical resistance is also unclear. A study of 33 patients on imatinib showed that those who did not achieve at least major cytogenetic remission showed overexpression of ABCB1, as did those who developed disease progression. By contrast, Mahon and colleagues were unable to identify ABCB1 overexpression in six patients in blast crisis after imatinib who did not have point mutations or BCR-ABL overexpression, although the addition of PSC833, an alternative pump inhibitor, increased the sensitivity of the primary cells, taken from these patients, to imatinib in a clonogenic assay.

Drug transporters can potentially play a part in resistance to drugs (eg, imatinib), either by transporting the drug out of the target cell or by transporting it out of cells of the gastrointestinal tract. ABCG2, also known as breast-cancer resistance protein, is an alternative mechanism of drug efflux, transporting camptothecin analogues from cells. Both ABCB1 and ABCG2 are expressed at the apical membrane of the small intestine and bile canalicular membranes, and gastrointestinal transport activity could affect oral drug bioavailability. Long-term exposure of intestinal caco-2 cells (an accepted model of intestinal drug transport) to imatinib induces
expression of ABCB1 and ABCG2 at mRNA and protein levels. Chronically exposed caco-2 cells were also shown to have decreased intracellular concentrations of imatinib. The role of ABCG2 in target-cell resistance was identified when the tyrosine-kinase inhibitor, gefitinib, was noted to reverse topotecan resistance, and the combination of gefitinib and topotecan induced cell toxicity. This finding suggested that gefitinib might be acting via inhibition of ABCG2. Because ABCG2 is known to be expressed in primitive normal haemopoietic stem cells, it became a possible candidate for an interaction with imatinib that might result in resistance. Houghton and colleagues confirmed that imatinib reversed ABCG2-mediated resistance of other cytotoxic drugs, but could not confirm that imatinib itself is a substrate for ABCG2. Overexpression of ABCG2 in BCR-ABL-negative cell lines did not affect imatinib sensitivity but imatinib did reverse ABCG2-mediated resistance to campothecin analogues.

Most studies attempting to identify interactions between ABCG2 and imatinib have been done in BCR-ABL-negative cells. However, Nakanishi and co-workers selected BCR-ABL-expressing and ABCG2-expressing K562 cells by exposure to mitoxantrone, a known substrate for ABCG2, to further increase ABCG2 expression. No increase in ABCB1 expression was noted but the K562 cells became slightly more resistant to imatinib. Resistance was abrogated by the ABCG2-specific inhibitor fumitremorgin-C (FTC). These confusing results might be explained if the expression of BCR-ABL itself interfered with the expression or function of ABCG2. ABCG2 membrane localisation (and therefore activity) is regulated by phosphatidylinositol-3-kinase (PI3K)-AKT signalling. Imatinib decreases PI3K activity, so a change in ABCG2 expression by imatinib is entirely possible. Short-term exposure of K562 cells to imatinib reduced surface expression and total cellular concentrations of ABCG2 and decreased phosphorylation of AKT. However, no change in mRNA levels of ABCG2 was noted, suggesting that these changes were post-transcriptional. The changes were of functional importance in that the decreased concentrations of the ABCG2 protein led to K562 cells having increased intracellular concentrations of mitoxantrone when exposed to the drug. When PI3K was directly inhibited by the selective PI3K inhibitor LY294002, ABCG2 concentration decreased in a time-dependent manner. Inhibition of protein degradation using the proteasome inhibitor MG1 32 partially restored the expression of ABCG2, suggesting a post-translational modification.

All these studies were done in cell lines engineered to express ABCG2 and the question remained as to which effect (down-regulation of ABCG2 or ABCG2-mediated resistance) would predominate in primary cells. Jordanides and co-workers have identified increased expression of ABCG2 in primitive CML cells from newly diagnosed patients compared with normal counterparts of the primitive haemopoietic cells. This group also confirmed that imatinib is an inhibitor but not a substrate for ABCG2.

Inhibition of drug-influx activity has now been suggested by several groups as an alternative mechanism affecting intracellular drug availability. The solute carrier superfamily has 12 members, all of which are polyspecific organic cation transporters (OCTs). Polymorphisms of OCT-1 are known to affect substrate transportation and evidence exists for the entry of imatinib into cells by OCT-1. White and colleagues subsequently used the phosphorylation status of the adaptor protein CRKL, a known downstream substrate of BCR-ABL, to calculate the IC₅₀ of imatinib. Variability in the IC₅₀ was wide, identifiable at diagnosis before drug exposure, and predictive of molecular response. Prazosin, a known inhibitor of OCT-1, decreased intracellular imatinib concentrations in primary cells exposed to the drug. However, OCT-1 cannot be the only mechanism of drug influx, because when high concentrations of prazosin were used, uptake of imatinib was not completely prevented. Differences in OCT-1 activity might, however, explain interpatient variability. A role for OCT-1 in clinical resistance is less clear. Thomas and co-workers studied OCT-1 expression in six patients, but could not identify any link with outcome. Furthermore, OCT-1 mRNA has also been shown to be increased at the time of failure to achieve complete cytogenetic remission.

The study of drug influx and drug efflux might have a therapeutically important role in overcoming drug resistance, and might provide a rationale for use of a combination of TKIs that have overlapping molecular targets, but that do not necessarily share the same transport mechanisms.

**Increased expression of BCR-ABL**

In a study by Mahon and co-workers, overexpression of BCR-ABL was shown to be the most frequent cause of resistance identified in cell lines that were engineered to develop resistance. The fact that this finding is not more frequent in clinical practice is surprising. Amplification of BCR-ABL was first reported in three of 11 patients with acquired resistance, and in one individual it coexisted with the presence of a point mutation in the ABL-kinase domain. Several case reports have since described clinical resistance to imatinib in association with BCR-ABL amplification or multiple copies of the Ph chromosome, or both. However, in a large study of 66 patients with primary or acquired resistance to imatinib, only two patients showed BCR-ABL genomic amplification.

The chance of gene amplification is likely to be related to genetic instability and is thought to occur at a rate of 10⁻⁴ per cell division, whereas point mutations occur much less frequently at 10⁻⁹ per cell division. In practice, however, clinical resistance is much more likely to be due to a point mutation than to BCR-ABL amplification.
One explanation for this could be that overexpression of BCR-ABL might itself be harmful to the cell. Cell lines that overexpressed BCR-ABL were noted to have a sudden loss of viability and decreased proliferation when imatinib was withdrawn. Additionally, cell lines that expressed varying amounts of BCR-ABL were noted to have dose-dependent differences in growth-factor dependence, clonogenicity, and migration. Cells with high expression of BCR-ABL were much less sensitive to imatinib and, more importantly, took a substantially shorter time to produce a mutant subclone resistant to the inhibitor than cells with low expression levels. Because BCR-ABL transcript levels are known to correlate with disease phase, the high levels of oncoprotein in advanced-phase disease might underlie the rapid development of resistance seen in these patients.

**Clonal evolution**

Disease progression beyond chronic phase has long been associated with clonal evolution—ie, the acquisition of additional chromosomal abnormalities in the Ph-chromosome-positive cell population. The presence of clonal evolution correlates with a decreased response to imatinib in terms of cytogenetic response, haematological response, and overall survival. In a comprehensive review of 300 patients in various disease phases who were treated with imatinib, Lahaye and colleagues showed that failure to achieve, or loss of
Table 2: Incidence of individual ABL-kinase mutations by disease phase

<table>
<thead>
<tr>
<th>Mutation type at amino acid</th>
<th>Mutation, n</th>
<th>Chronic phase, n (%)*</th>
<th>Accelerated phase, n (%)*</th>
<th>Blast crisis, n (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>M244</td>
<td>47</td>
<td>33 (70)</td>
<td>1 (2)</td>
<td>13 (28)</td>
</tr>
<tr>
<td>L248</td>
<td>13</td>
<td>10 (77)</td>
<td>2 (15)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>G250</td>
<td>63</td>
<td>23 (49)</td>
<td>6 (10)</td>
<td>26 (41)</td>
</tr>
<tr>
<td>Q252</td>
<td>14</td>
<td>3 (21)</td>
<td>3 (21)</td>
<td>8 (58)</td>
</tr>
<tr>
<td>Y253</td>
<td>68</td>
<td>23 (34)</td>
<td>9 (13)</td>
<td>36 (53)</td>
</tr>
<tr>
<td>E255</td>
<td>63</td>
<td>17 (27)</td>
<td>12 (19)</td>
<td>34 (54)</td>
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<td>D276</td>
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<td>2 (17)</td>
<td>4 (33)</td>
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<td>F311</td>
<td>5</td>
<td>2 (40)</td>
<td>1 (20)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>T315</td>
<td>56</td>
<td>9 (16)</td>
<td>12 (23)</td>
<td>35 (63)</td>
</tr>
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<td>F317</td>
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<td>E355</td>
<td>22</td>
<td>13 (59)</td>
<td>4 (18)</td>
<td>5 (23)</td>
</tr>
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<td>F359</td>
<td>35</td>
<td>21 (60)</td>
<td>5 (14)</td>
<td>9 (26)</td>
</tr>
<tr>
<td>H396</td>
<td>29</td>
<td>21 (72)</td>
<td>2 (7)</td>
<td>6 (21)</td>
</tr>
<tr>
<td>S417</td>
<td>3</td>
<td>2 (67)</td>
<td>1 (33)</td>
<td>0 (72)</td>
</tr>
<tr>
<td>E459</td>
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<td>0 (72)</td>
<td>4 (67)</td>
</tr>
<tr>
<td>F486</td>
<td>8</td>
<td>0 (0)</td>
<td>1 (13)</td>
<td>7 (88)</td>
</tr>
</tbody>
</table>


Table 2: Incidence of individual ABL-kinase mutations by disease phase

previous, complete haematological response was more likely to be associated with clonal evolution than with ABL-kinase domain mutations. This finding was most obvious in blast crisis, with 73% of patients having clonal evolution compared with 30% with ABL-kinase domain mutations. Jabbour and colleagues studied 171 patients in all phases of the disease who did not achieve complete haematological response by 3 months, who remained >35% Ph-chromosome positive at 12 months, or who lost a previous complete haematological response or cytogenetic response. Clonal evolution was present at the time of imatinib failure in 36 of 153 (24%) assessable patients and BCR-ABL-kinase mutations were more likely to occur in patients with clonal evolution than in those without (58% vs 28%, respectively). Most patients in both these series had previously been exposed to interferon in their disease course, and whether clonal evolution will be a frequent finding in patients who receive TKI from diagnosis is unclear.

Mutations in the ABL-kinase domain

In 2001, Gorre and co-workers described 11 patients treated with imatinib for CML blast crisis or Ph-chromosome-positive acute lymphoblastic leukaemia (ALL) who relapsed on treatment. BCR-ABL gene amplification was only detected in three patients. Sequencing of the ATP-binding pocket and the activation loop of the kinase domain showed an identical cytosine to thymidine mutation at ABL nucleotide 944 in six of nine assessable patients (two with Ph-chromosome positive ALL, one with lymphoid blast crisis, and three with myeloid blast crisis). This mutation resulted in a single amino-acid change at position 315, designated T315I. Threonine 315 forms a crucial hydrogen bond with imatinib and the absence of an oxygen atom in the substituted isoleucine prevented bond formation. Additionally, the bulkier isoleucine was predicted to induce a steric clash with imatinib, which led to the designation of the 315 residue as the so-called gatekeeper for imatinib.

Up to now, more than 50 mutants have been described (figure 3), some at a higher frequency than others: 15 amino-acid substitutions account for more than 85% of the mutations, and the mutations responsible for 66% of reported cases occur at seven sites only (G250, Y253, E255, T315, M351, F359, H396). Furthermore, different amino-acid substitutions can occur at the same residue—eg, F317C, F317L, and F317V—and can confer different imatinib sensitivities. Certain mutations seem to occur more often in different disease phases. For example, substitutions at M244, L248, F317, H396, and S417 are more likely to occur in patients with chronic-phase disease, whereas those at Q252, Y253, E255, T315, E459, and F486 are associated with advanced-phase disease (table 2). An intriguing suggestion has also been made that the incidence of the various mutations might vary in different ethnicities. The functional importance of all these findings is, as yet, unclear, especially in terms of whether certain mutations are responsible for disease progression (perhaps by conferring a growth advantage to affected cells) or whether they simply act as a surrogate marker of the increased genetic instability associated with advanced-phase disease. Griswold and colleagues studied the transformation potential, kinase activity, and substrate specificity of five of the most frequent mutations—Y253F, E255K, T315I, M351T, and H396P. Compared with wild-type BCR-ABL, Y253F was noted to produce enhanced kinase activity (gain of function) with E255K producing comparable activity. The remaining three mutations decreased enzymatic function (loss of function). Transforming potency did not always correlate with kinase activity. However, clear differences did exist in the tyrosine phosphorylation patterns of the Ba/F3 cells expressing the various mutants, which would be consistent with differences in substrate use and signalling pathway activation.

The inhibitory action of imatinib is mediated through its recognition of the inactive conformation of ABL (figure 1). This recognition accounts for the selectivity of imatinib for BCR-ABL, because diversity within tyrosine kinases is largely confined to the inactive rather than the active conformations. In the inactive conformation, the activation (A) loop (amino acids 381 to 402 of ABL), which is the major regulatory element of the kinase domain, blocks the catalytic centre. Mutations within the A loop will destabilise the kinase or prevent it from adopting the inactive conformation. Additional mutations have been noted to cluster in the catalytic (C) domain (ABL amino acids 350 to 363).
The ATP binding (P) loop is a highly conserved glycine-rich sequence occupying residues 244–255 of ABL. Binding of the A loop to imatinib through hydrogen and van der Waals bonds causes the P loop to move downwards and fold over the drug. Some groups have found that P loop mutations are associated with a more rapid progression to advanced-phase disease than mutations in other regions of the molecule.49 Jabbour and co-workers46 were unable to confirm this finding, although there were some differences in the criteria used to select patients for mutation screening.46

Despite the plethora of published work that exists, two reasons can be given for why accurately defining the incidence of mutations or identifying the factors that affect the risk of developing a mutation remain difficult. First, the sensitivity of the techniques used to detect mutations has differed in different studies (varying from as high as the identification of one mutation in 10 000 wild-type sequences to as low as one in three [about 30%]). Second, studies have focused on different patient populations, with some looking for mutations only in acquired resistance and others concentrating on patients with stable and responsive disease (table 3).

Early studies used the method of direct sequencing for mutation detection (sensitivity in the order of 20–30%) where the resistant clone can only be identified when it forms a considerable proportion of the total. Sequencing after subcloning of PCR products improves sensitivity, but is time consuming and laborious. Alternative, more sensitive, methods include allele-specific oligonucleotide PCR, restriction-fragment-length polymorphism-based assays, and peptide-nucleic-acid-based clamping techniques, but these are not suited for routine screening of the wide range of known mutations. Denaturing high-performance liquid chromatography has been described by several groups,52,53,58 and is deemed to be better suited to large-scale screening. Sensitivities are improved to 1–5% for certain mutations (eg, G250L, Y253R, E255K, T315J, and H369R) and to 5–10% for M351T. More recently, pyrosequencing has been a useful addition to the technical armamentarium, with a sensitivity of about 5% but also the ability to quantify the mutant clone.68

Many of the studies that describe mutations are from studies of patients with acquired resistance, which is more common in patients treated for advanced-phase disease. Furthermore, many of these patients received imatinib only after failure of previous treatments, including interferon. Their overall duration of disease, and, therefore, the duration of time during which the leukaemic cell was at risk of acquiring mutations was, therefore, greater than in patients treated with imatinib from diagnosis. In patients treated with imatinib for late chronic-phase or advanced-phase disease after previous interferon, the presence of a mutation at the time of loss of response occurred in 44–100% (table 3).11,45–49,51–54,57–59,62,64–67,69

Up to now, mutations seem to be less frequent in patients treated with imatinib from diagnosis, but they have been described, usually at the time of acquiring resistance.46,49,55–57,64

Several studies have adopted a rather different approach and have looked for mutations in patients with stable chronic-phase disease, with or without complete cytogenetic remission (table 4).51–53 Chu and colleagues54 studied CD34+ cells from 13 patients with complete cytogenetic remission: seven mutations were identified in five patients. Two of the five patients subsequently lost their response to imatinib, whereas none of the eight patients without mutations experienced loss of response or disease progression. Later, five of these eight patients showed increases in their BCR-ABL transcript levels, and four had mutations at this time.67 A more recent study64 discovered mutations in eight of 42 patients with complete cytogenetic remission. A concomitant rise in BCR-ABL transcript levels was noted in four of these individuals, although only two had disease progression at the time.
of the report. The remaining four patients had stable transcript levels.

Khorashad and co-workers” used pyrosequencing to quantitate mutated and non-mutated alleles over time in 12 patients and described three different patterns. In the first, BCR-ABL transcript levels remained high with the mutant allele predominating, presumably indicating resistance to imatinib; in the second, transcript levels dropped to low levels but the mutant clones persisted and, in fact, formed most of the residual disease, suggesting that the mutated cells remained at least partially sensitive to imatinib; in the third pattern, the transcript levels either fell or remained high but the mutated clones persisted at a very low level. The findings in this third group are difficult to explain, but suggest that mutated clones do not necessarily lead to a proliferative advantage or always account for imatinib resistance.44

Because mutations occur at predictable frequencies, one might expect mutations to be present even before the tumour is detectable. An elegant mathematical model31 has shown that the pretreatment phase is more important in the generation of mutations than the treatment phase. Once a particular mutation has been detected in an individual patient, highly sensitive assays, specific for that mutation, can be used for studying patient samples retrospectively, to establish the first occurrence of the mutated clone. Several groups have shown that the presence of mutations can antedate treatment with imatinib,61,70,77,82–84 a finding that is more commonly described in patients receiving imatinib for advanced-phase disease. This finding would suggest that the mutation occurs in a proliferating stem-cell early in the disease course, but that the mutation offers no survival advantage until exposure in-vivo to imatinib. This model fits with the finding that the time from initiation of treatment to loss of response and the detection of a mutation is often rather short, making the idea that the mutant has arisen secondary to the use of imatinib less likely. The rather surprising finding from the study by Willis and colleagues61 was that, in some patients, the introduction of imatinib did not lead to selection of the mutated clone, which, in some cases, became undetectable. Perhaps predictably, mutations conferring a gain of function (Y253F) were identified more often than those associated with a loss of function (M351T). The latter are more likely to be selected on drug exposure.

The significance of developing a mutation is now very unclear and, consequently, the value of regular screening for the development of mutations is also equivocal. If a mutation occurs in a patient treated for advanced-phase disease who loses their response, the likelihood is that the proportion of cells derived from the mutated clone will increase and be associated with disease progression. By contrast, the development of a mutation in a patient with otherwise stable disease might not be associated, at least in the short term, with any change in disease status or response to treatment.

The incidence of point mutations in BCR-ABL might be affected by the expression of BCR-ABL itself. Findings suggest that BCR-ABL increases the amount of reactive oxygen species in the cell, which predisposes the cell to the development of mutations through oxidative DNA damage.77 This effect of BCR-ABL might be exploitable by drugs such as adaphostin, which induces oxidative stress.

Quiescent stem cells

Although the development of point mutations is generally accepted to be the feature most commonly associated with acquired resistance to imatinib, we do not have an explanation as to why imatinib does not eradicate all leukemic cells even in the best responses. Some groups have suggested that the leukemic stem cell remains resistant to imatinib by virtue of its quiescent or dormant status. These quiescent cells, which form 0.5% of the CD34+ population (estimated total number of 5×10⁹ assuming 1×10² tumour cells at diagnosis) then form a reservoir of disease with the capacity to develop resistance.

The concept of the leukemic stem cell is not new. A population of highly primitive and quiescent stem cells present in the blood of patients with CML and capable of engrafting non-obese diabetic-severe combined immuno-deficient mice have been identified by several groups.73,79

These primitive lineage-negative cells also seem to be inherently insensitive to imatinib,80–82 Several possible explanations exist for this insensitivity, including inherent differences in the balance of drug influx or drug efflux in primitive cells compared with their more mature counterparts, over-expression of BCR-ABL by gene amplification or other mechanisms, decreased protein degradation, or inaccessibility or insensitivity of the target protein to the inhibitor, perhaps by favouring the active conformation or the development of ABL-kinase mutations. Several groups have tested these various hypotheses, but the mechanism of resistance remains elusive. BCR-ABL concentrations have been shown to be identical in the proliferative and non-proliferative cell compartments.81 Jiang and co-workers84 showed that BCR-ABL is over-expressed in primitive CML cells, and this finding has been confirmed and further elaborated by Copland and colleagues,85 who showed that only one copy of BCR-ABL exists in these cells—i.e., no gene amplification, but higher transcript levels. These investigators also showed that no differences in intracellular drug concentrations exist between the most primitive and more mature cellular subpopulations, but that phosphorylation of downstream targets of BCR-ABL, as exemplified by CRKL, is not inhibited in the presence of imatinib or dasatinib, one of the second generation TKIs.

The importance of the quiescent state of leukemic stem cells has been further challenged. Cell-line models
of decreased proliferative activity were established by inhibiting key molecules at the G1/S boundary. Cell-cycle status did not correlate with the ability of imatinib to induce apoptosis.43 Although attractive as an explanation for the failure to eradicate residual leukaemic cells even in the best responders, some difficulties remain with this hypothesis. If the cells are quiescent, then they are present in insufficient numbers to account for the easily detectable residual disease in most patients. Furthermore, they are unlikely to develop point mutations (which occur during proliferation) and should be sensitive to imatinib when they exit G0. Finally, quiescence cannot explain the instances of rapid progression to advanced phase after molecular response. However, eradication of this residual population would seem a desirable target and the most promising approach would seem to be the combination of a TKI with another agent. Strategies include, combining a TKI with a signal-transduction inhibitor (eg, a farnesyl transferase inhibitor), inducing the cell cycle (eg, by growth factors), or immunological methods, such as antigen-specific T cells.

Conclusion
Imatinib has undoubtedly revolutionised the management and outcome of CML, but several patients do not derive the maximum benefit from this drug. Although considerable progress has been made in the elucidation of the mechanisms of resistance, we are far from understanding the cause of this resistance, which is probably multifactorial. Furthermore, the clinical consequences of resistance are unclear. As a result, the management of resistant patients is a complex process. In the second part of this review, I will discuss available treatment options and provide suggestions for the assessment and treatment of individuals with resistant disease.

Conflicts of interest
The author declared no conflicts of interest.

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Review


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