# New tyrosine kinase inhibitors in chronic myeloid leukemia

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The deregulated activity of BCR-ABL tyrosine kinase originating from the t(9;22) chromosomal translocation has been shown to be necessary and sufficient for the transformed phenotype of chronic myeloid leukemia (CML) cells. This peculiarity has paved the way for the development of novel therapies specifically targeting the BCR-ABL gene product. The first BCR-ABL inhibitor to come into use in clinical practice, imatinib mesylate, is now the first-choice treatment for all newly diagnosed CML patients, but the initial striking efficacy of this drug has been overshadowed by the development of clinical resistance. The most common mechanisms of resistance include (i) BCR-ABL overexpression, and (ii) BCR-ABL kinase domain mutations disrupting critical contact points between imatinib and BCR-ABL or inducing a transition to a conformation to which imatinib is unable to bind. Several approaches to overcoming resistance have been studied both *in vitro* and *in vivo*. They include dose escalation of imatinib, the combination of imatinib with chemotherapeutic drugs, alternative BCR-ABL inhibitors, and inhibitors of kinases acting downstream of BCR-ABL such as Src kinases. Various novel tyrosine kinase inhibitors (TKI) have been synthesized and have now reached the pre-clinical or clinical phase. This review highlights the development of new TKI as specific molecularly targeted therapy and as the principal mechanisms for overcoming imatinib resistance.

Key words: tyrosine kinase, CML.

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Chronic myeloid leukemia (CML) is a hematopoietic disorder characterized by the malignant expansion of bone marrow stem cells. CML is almost unique among human cancers since a single genetic defect is responsible for the transformed phenotype. The cytogenetic hallmark of more than 90% of CML cases is a reciprocal t(9;22)(q34;q11) chromosomal translocation[1] that creates a derivative 9q+ and a small 22q-, most commonly referred to as the Philadelphia (Ph) chromosome.[2] As a result of this translocation, the latter harbors a *BCR-ABL* fusion gene encoding a chimeric BCR-ABL protein with deregulated tyrosine kinase activity, the expression of which has been shown to be necessary and sufficient for the transformed phenotype of CML cells.[3,4] Thanks to the contributions of numerous researchers, the past twenty years have witnessed considerable advances in our knowledge of the molecular and cell biology of CML, creating the essential platform for engineering targeted molecular therapies. It soon became clear that the BCR-ABL oncoprotein itself is the ideal target, since it plays a central role in CML pathogenesis, and it is not expressed by normal cells. Furthermore, the dissection of the signal transduction pathways affected by the deregulated kinase activity of BCR-ABL has provided information on additional or alternative signaling steps that could be interrupted in an attempt to block the leukemogenic process. BCR-ABL exerts its oncogenic effects in CML cells essentially by stimulating cell proliferation, inhibiting apoptosis and altering cell adhesion to bone marrow stroma. The signal transduction cascades involved in these cellular processes and activated by BCR-ABL include, among others a) Ras;[5] b) mitogen-activated protein kinase (MAPK)[6] and its downstream effectors MEK and Erk; c) phosphatidylinositol-3 kinase (PI3K)[7,8] and its downstream effector Akt.

With the sole exception of Ras, all these proteins share an intriguing feature with BCR-ABL, i.e. they all are tyrosine kinases.

Specific tyrosine kinase inhibitors (TKI) are drugs used to inhibit malignant cell growth and metastasis formation and are currently undergoing a rapid phase of development. Imatinib mesylate (formerly STI571; Gleevec™ or Glivec®, Novartis Pharmaceuticals, Basel, Switzerland) is the first successful example of a TKI for the therapy of CML. It is a small-molecule

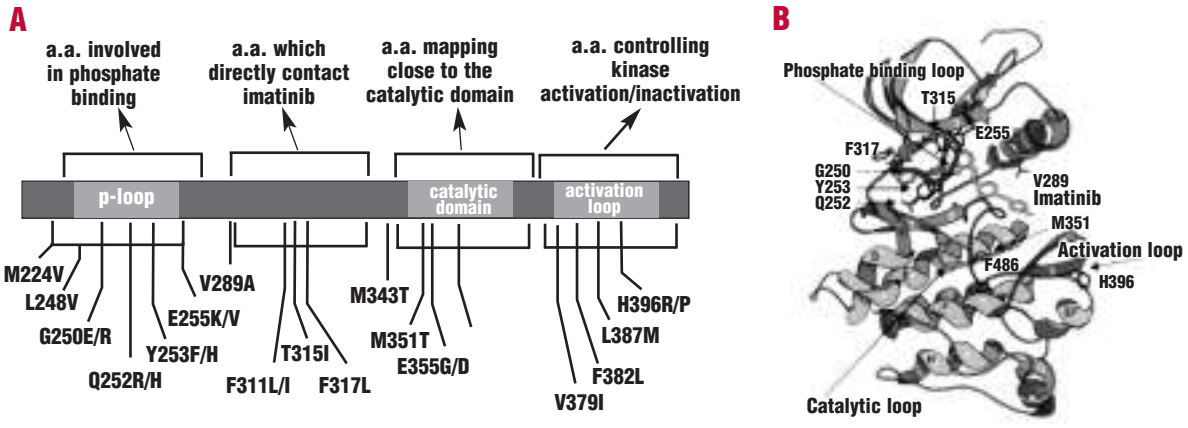


**Figure 1. A.** Distribution of reported point mutations within the ABL kinase domain. Codons most frequently reported as being affected by amino acid (a.a.) substitutions are shown. Mutations cluster in four main regions. One group of mutations (G250E, Q252R, Y253F/H, E255K/V) includes amino acids that form the phosphate-binding loop for ATP (also known as the P-loop). A second group (V289A, F311L, T315I, F317L) can be found in the imatinib binding site; these mutations interact directly with the inhibitor via hydrogen bonds or Van der Waals' interactions. The third group of mutations (M351T, E355G) clusters within the catalytic domain. The fourth group of mutations (H396R/P) is located in the activation loop, whose conformation is the molecular switch controlling kinase activation/inactivation. **B.** Three-dimensional representation of ABL complexed with imatinib. Position of mutations is highlighted, along with the activation loop, P-loop and catalytic loop. β-strands are numbered and α-helices are lettered according to the nomenclature used for insulin-receptor tyrosine kinase (adapted from Gambacorti-Passerini *et al.*).[31]



inhibitor of the BCR-ABL tyrosine kinase, as well as of a limited number of other kinases (c-Kit, PDGF-R, ARG)[9,10] Preclinical and clinical studies[11–18] have confirmed the remarkable efficacy and high tolerability of this drug. Imatinib mesylate is now the first-choice treatment for all newly diagnosed CML patients, but the initial striking efficacy of this drug has been overshadowed by the development of clinical resistance. The emergence of resistance to imatinib has prompted researchers to focus on strategies aimed at preventing or overcoming this phenomenon. Since resistance often coincides with the reactivation of kinase activity within the leukemic clone, either BCR-ABL itself or BCR-ABL-triggered downstream signaling pathways continue to be good targets for molecular therapy. Several approaches have been studied both *in vitro* and *in vivo*. They include dose escalation of imatinib, the combination of imatinib with chemotherapeutic drugs, alternative BCR-ABL inhibitors including Src kinase inhibitors, and inhibitors of kinases downstream of BCR-ABL. Further investigations into the molecular mechanisms of disease and how to specifically target the abnormal cellular processes will serve as guides in the design of new treatment modalities in future clinical trials.

### *BCR-ABL gene amplification and mutation are the primary mechanisms of imatinib resistance*

Despite high rates of hematologic and cytogenetic responses, primary refractoriness and acquired resistance to imatinib are observed in a growing number of patients, especially those at more advanced stages of the disease. Resistance has been traced to two main mechanisms: (i) the overexpression of BCR-ABL, mainly due to gene amplification,[19,20] and, more frequently, (ii) the acquisition or the selection of specific point mutations within several critical regions of the ABL kinase domain (Figures 1A,B).[19–27]

Crystallographic studies have shown that the high selectivity and efficacy of imatinib are due to its ability to bind and lock BCR-ABL in its inactive, auto-inhibited conformation.[28–29] Mutations seem to act by disrupting critical contact points between imatinib and BCR-ABL or, more often, by inducing a transition from the inactive to the active state, i.e. to a conformation to which imatinib is unable to bind. From analyses of clinical samples, the repertoire of mutations found in association with the resistant phenotype has been increasing slowly but inexorably over time (Table 1). Mutations seem to cluster in four main regions. One group of mutations (G250E, Q252R, Y253F/H, E255K/V) includes amino acids that form the phosphate-binding loop for ATP (also known as the P-loop). A second group (V289A, F311L, T315I, F317L) can be found in the imatinib binding site and interacts directly with the inhibitor via hydrogen bonds or Van der Waals' interactions. The third group of mutations (M351T, E355G) clusters in close proximity to the catalytic domain. The fourth group of mutations (H396R/P) is located in the activation loop, whose conformation is the molecular switch controlling kinase activation/inactivation. Conflicting data are still reported regarding the relative as well as the overall frequency of resistance-associated mutations. It is also currently unclear whether ABL mutations, or a specific subset

**Table 1.** BCR-ABL point mutations associated with imatinib resistance detected in CML or ALL patients.

| Nucleotide change[a] | Amino acid change[b] | No. of cases (detected/tested) | References |
|---|---|---|---|
| A1094G | M244V | 6/165 | 20,24,26,c |
| C1106G | L248V | 2/29 | 26,b |
| G1113A | G250E | 7/127 | 21,24,26,c |
| G1112A | G250R | 1/117 | a |
| G1120C | Q252R | 1/32 | 24 |
| G1120T | Q252H | 12/125 | 20,24,26 |
| T1121C | Y253H | 11/194 | 20,21,24,25,c |
| A1122T | Y253F | 8/165 | 20,24,26,c |
| G1127A | E255K | 30/222 | 20,21,24,25,26,57,c |
| A1128T | E255V | 4/141 | 20,21,26,58,c |
| A1191G | D276G | 1/33 | 60,b |
| A1194G | T277A | 1/117 | a |
| T1230C | V289A | | 59 |
| T1494C | F311L | 2/64 | 22,c |
| C1308T | T315I | 27/194 | 20,21,25,26,60 |
| C1308A | T315N | 1/33 | 60 |
| C1315G | F317L | 5/100 | 21,24,c |
| T1392C | M343T | 1/32 | 24 |
| T1416C | M351T | 25/244 | 20,21,22,24,26,c |
| T1428G | E355G | 5/65 | 20,24,26,c |
| T1439G | F359V | 5/99 | 24,26 |
| T1440G | F359A | 2/150 | 60,a |
| G1499A | V379I | 1/32 | 24 |
| T1508C | F382L | 1/32 | 24 |
| T1523A | L387M | 2/149 | 24 |
| G1523C | L387F | 3/117 | a |
| A1551C | H396P | | 25,59 |
| A1551G | H396R | 7/52 | 20,24,26,c |
| A1558C | A397P | 1/117 | a |
| C1614A | S417Y | 1/27 | 26 |
| G1739A | E459K | 1/27 | 26 |
| T1821C | F486S | 1/27 | 26 |

[a]*Nucleotide positions according to GenBank accession number M14752.*
[b]*Amino acid positions, indicated by the single letter code, are those for the GenBank sequence (accession number AAB60394) and correspond to ABL type 1a. Additional mutations: a) Kreil et al., ASH 2003 (Blood 2003;102(Suppl.1):71a); b) Chu et al., ASH 2003 (Blood 2003;102(Suppl 1):70a); c) Soverini et al., ASH 2004 (Blood 2004;104 (Suppl 1)287a).*

**Table 2.** $IC_{50}$ values for wild-type (WT) ABL and BCR-ABL and for the main BCR-ABL mutants.

| BCR-ABL type | $IC_{50}$ ($\mu$M imatinib) Corbin[29] (2003) | Azam[56] (2003) | Hochhaus[20] (2002) | Shah[24] (2002) | Von Bubnoff[25] (2002) | Benefit from dose escalation? |
|---|---|---|---|---|---|---|
| WT ABL | – | – | 0.025 | – | – | – |
| WT BCR-ABL | 0.5 | 0.6 | – | 0.6 | >0.1 >0.5 | – |
| M244V | 1.6 | 3.1 | – | – | – | Y |
| M244I | – | 1.4 | – | – | – | Y |
| G250E | 4.5 | >20 | – | >10 | – | N |
| Q252H | 2.6 | 2.9 | | | | Y |
| Y253H | >17.7 | 17.7 | 3.7 | – | >10 | N |
| Y253F | 5.0 | – | 1.8 | | | N |
| E255V | >17.7 | – | >5 | – | >10 | N |
| E255K | 7.5 | 12.1 | >5 | >10 | >10 | N |
| F311L | 0.7 | 1.3 | – | – | – | N |
| T315I | >17.7 | >20 | – | >10 | >10 | N |
| T315S | – | 3.8 | – | – | – | N |
| F317L | 1.3 | 2.3 | – | 7.5 | – | N |
| M351T | 1.5 | 4.9 | – | 4.4 | – | Y |
| M351I | – | 1.6 | – | – | – | Y |
| E355G | 2.0 | – | – | 2.4 | – | Y |
| F359V | 1.4 | – | – | – | – | Y |
| V379I | 1.0 | – | – | – | – | Y |
| L387M | 1.1 | – | – | – | | Y |
| H396P | 4.3 | – | – | – | >0.1 >0.5 | N |
| H396R | 5.4 | – | – | – | – | N |

**Table 3.** New tyrosine kinase inhibitors.

| Agent | Company | Target | Class | Phase | Principal indications |
|---|---|---|---|---|---|
| SKI-606 | Wyeth-Ayerst | Abl, Src | TKI | I | Imatinib-refractory CML |
| BMS354825 | Bristol-Myers | Abl, Src | TKI | II | Imatinib-refractory CML |
| AZD0530 | Astra Zeneca | Abl, Src | TKI | Pre-clinical | Imatinib-refractory CML |
| AP23464 | Ariad | Abl, Src | TKI | Pre-clinical | Imatinib-refractory CML |
| CGP76030 | Pfizer | Src | TKI | Pre-clinical | Imatinib-refractory CML |
| AMN107 | Novartis | PDGF, Abl, kit | TKI | I-II | Imatinib-refractory CML |

of them, may be of prognostic significance in terms of time to progression and/or survival.[20,24,26] Shah *et al.*[24] screened 13 cytogenetic non-responders for mutations and found that 3 out of 4 patients with mutations suffered disease progression within 18 months of detection of the mutation. In contrast, only 1 out of 9 patients with no detectable mutations developed disease progression. Hochhaus *et al.*[20] examined 43 patients with hematologic relapse and found no difference between patients with and without mutations as far as time to progression was concerned. Branford *et al.*[26] recently showed that in late-chronic phase (CP) and accelerated phase (AP) CML patients, a specific subgroup of mutations – i.e., those in the ATP phosphate-binding loop or P-loop – is significantly associated with a poor prognosis in terms of survival. Clonal cytogenetic evolution, i.e. occurrence of novel cytogenetic abnormalities in addition to the Philadelphia chromosome, has also been reported as contributing to relapse in patients receiving imatinib treatment.[20] Aneuploidy is frequent, most often including the emergence of a second Ph chromosome or trisomy 8. Trisomy 6, 9, 12 and 18 and deletion/monosomy 7 and 16 have also been reported in association with relapse on imatinib treatment.[20] In addition to numerical chromosomal changes, structural changes may also be present, with alteration of the short arm of chromosome 17 (leading to the loss of one p53 allele) being the change most frequently observed.

### Higher dosage of imatinib or alternative ABL inhibitors?

Different ABL mutants appear to have different degrees of resistance to imatinib (Table 2). *In vitro* data

indicate that while some mutations seem to confer a highly resistant phenotype, thereby suggesting withdrawal of imatinib in favor of alternative therapeutic strategies, others might be simply overcome by dose escalation.[30,31] Routine testing for emerging mutations should be adopted in clinical practice in order to ensure rational therapeutic management of CML patients. For those patients who fail to benefit from dose escalation, novel inhibitors would be a welcome addition to the drug armamentarium (Table 3). It is conceivable that several targeted inhibitors may be necessary - this would allow clinicians to combine different compounds or to switch from one to another, individualizing therapy on the basis of molecular surveillance of the BCR-ABL sequences present in the tumor load.

### "Dual" SRC and ABL inhibitors

BCR-ABL activates multiple signaling pathways, including members of the Src kinase family such as Lyn and Hck. Previous studies have demonstrated that multiple domains of BCR-ABL interact with and activate Src kinases independently of BCR-ABL kinase activity, and studies with dominant-negative mutants and Src inhibitors suggest that Src kinases may contribute to the proliferation and survival of myeloid cell lines expressing BCR-ABL *in vitro*.[32]

Hck and Lyn are expressed and activated in CML blast-crisis patients and their increased expression correlates with disease progression or imatinib resistance in some CML patients. This insight suggests the possibility that small molecules with "dual" kinase inhibitory activity against either ABL or Src, might prove active in CML and ALL $Ph^+$ patients. Several compounds originally described as Src kinase inhibitors were subsequently found to inhibit ABL at nanomolar concentrations. Indeed, promising *in vitro* activity against a limited number of imatinib-resistant BCR-ABL isoforms has been seen for two compounds from the pyrido[2,3-d]pyrimidine class of dual Src-ABL inhibitors (PD166326 and PD180970).[33-35]

*PD180970 and PD173955 as ABL and Src inhibitors*. Important alternative ABL inhibitors are PD180970 and PD173955. The crystal structure of PD173955 in complex with imatinib has recently been solved. These studies indicate that the most important difference between the binding of imatinib and PD173955 is the fact that the latter binds both the active and inactive conformations of ABL. Although PD173955 contacts far fewer amino acid residues than does imatinib, it inhibits tyrosine kinase at approximately 100-fold lower concentrations. One study has shown that PD180970 is active against ABL mutations affecting the P-loop and A-loop of ABL.[36] In contrast, there is no activity against the T315I mutant. Although the pharmacological properties of this compound make it unsuitable for clinical use, it was these data that first suggested the possibility of targeting imatinib-resistant ABL mutants.

*SKI-606 and 4-anilino-3-quinolinecarbonitrile Src kinase inhibitors*. Recently, a new class of compounds, the 4-anilino-3-quinolinecarbonitrile Src kinase inhibitors, has been synthesized. One member of this class, SKI-606, is a dual-specificity inhibitor of both the Src kinase family and the ABL kinases. The *in vitro* effects of SKI-606 have been analyzed on human CML cell lines (K562, MK2, Lama-84) using a wide range of concentrations (0.01 μM, 0.1 μM, 1 μM, 10 μM) of this novel agent.[37] Cell cycle analysis of the cell lines showed that one major effect of SKI-606 is that it alters cell cycle progression, producing $G_1$/S arrest. SKI-606 induces dose-dependent inhibition of proliferation with an $IC_{50}$ of 1 μM at 24 hours. Flow cytometry analysis with annexin-V showed that SKI-606 induced apoptosis of 50% of cells at 48 hours. Western blotting and immunoblotting analyses showed reduced phosphorylation of BCR-ABL and also of Lyn and Hck. Activation of caspase-9, a cysteine-protease effector, was also reported after exposure to SKI-606. This study thus showed the potential therapeutic usefulness of the drug in the treatment of CML, particularly in the blast crisis phase.

*A new dual Src and ABL inhibitor entered in clinical trials: BMS-354825*. BMS-354825 is an orally bioavailable ABL kinase inhibitor with two-log greater potency than imatinib. It retained activity against 14 of 15 imatinib-resistant BCR-ABL mutants in pre-clinical studies.[38] The only imatinib-resistant BCR-ABL isoform that was clearly resistant to BMS-354825 was the T315I mutant, which retained kinase activity even in the presence of micromolar concentrations of the compound. BMS-354825 prolonged the survival of mice with BCR-ABL-driven disease and inhibited proliferation of BCR-ABL-positive bone marrow progenitor cells from patients with imatinib-sensitive and imatinib-resistant CML.[38] Recently, preliminary results have been reported from a phase I clinical trial of BMS-354825 in $Ph^+$ CML patients in CP with hematologic progression or intolerance while being treated with imatinib.[39] Twenty-nine patients were treated in 9 cohorts with doses ranging from 15 to 180 mg of BMS-354825 per day given in single or divided doses for 5-7 days a week, over a period of up to 9 months. Like imatinib, BMS-354825 was well tolerated in all patients. Serum levels well above the concentration required to block CML cell proliferation *in vitro* were readily achieved without side effects. Pharmacodynamic studies demonstrated more than 50% inhibition of phosphorylation of the BCR-ABL substrate CRKL and the Src kinase Lyn. To date, 26 patients (22 with imatinib resistance, 4 with imatinib intolerance; average CML duration: 6.1 years) have been followed for more than 4 weeks and are eligible for assessment

of hematologic benefit.[39] Twenty-two patients had detectable BCR-ABL kinase domain mutations prior to starting BMS-354825. All 26 patients were treated with doses of 35 mg per day or more and obtained clinical benefit, including 19 with complete hematologic responses (73%). Of the other 7 partial responders, two subsequently developed disease progression, one of whom with expansion of a CML subclone containing the imatinib-resistant T315I mutation in BCR-ABL. The other 5 partial responders are now being treated with higher doses to attempt conversion to a complete hematologic response. Eleven of 21 patients (52%) treated for more than 3 months obtained cytogenetic benefit, including 6 major (1-35% $Ph^+$), 1 minor (36-65% $Ph^+$) and 4 minimal (66-95% $Ph^+$) cytogenetic responses. One patient achieved a complete cytogenetic response. Dose escalation continues. Phase I clinical results for BMS-354825 in $Ph^+$ and blast crisis (BC) CML patients who had hematologic progression or intolerance while being treated with imatinib are also available.[40] Seventeen patients (6 with AP; 11 with BC) were treated in 3 cohorts with doses of BMS-354825 ranging from 35 mg to 70 mg. Of the 11 BC patients, 7 had a hematologic response: 3 a complete hematologic response (CHR), 2 no evidence of leukemia (NEL), and 2 a return to the chronic phase (RTC). Three additional patients showed significant hematologic improvement despite being on treatment for only a short period (10-23 days). One patient with extramedullary disease was stable. Cytogenetic data were available for 8 of the 11 BC patients. Four of these patients had a major cytogenetic response, 2 patients a minor cytogenetic response and 2 patients no response. BCR-ABL mutation data were available for 2 patients: one patient had no mutation and one who had a non-sustained CHR was found to have an E355G mutation. Three of the 6 AP patients had a hematologic response: 2 CHR and 1 NEL. One patient demonstrated resistance to BMS-354825 due to a T315I mutation in BCR-ABL found in 8 out of 10 clones. BCR-ABL mutation status was available for 3 additional AP patients: no mutations were identified in 2 patients, while 1 patient in CHR had M351T/A imatinib-resistant mutations. Of 3 patients for whom early cytogenetic data were available, 1 had a minor cytogenetic response (40% $Ph^+$). Dose escalation continues also for the AP and BC CML patients. Phase II studies in CP, AP and BC CML are currently being initiated.

*Other dual Src and ABL kinase inhibitors: AZD0530 and AP23464*. Other Src kinase inhibitors seem to be effective in pre-clinical models of solid tumors. This is the case with AZD0530. However, little information has been reported about this drug and clinical applications or pre-clinical studies are not yet available. Another dual Src inhibitor is AP23464. Very recently, O'Hare *et al.*[41] reported that AP23464 is a potent ATP-based inhibitor of Src and ABL kinases. The drug has been shown to display antiproliferative activity against a human CML cell line and BCR-ABL-transduced Ba/F3 cells ($IC_{50}$=14 nM; imatinib $IC_{50}$=350 nM).[41] AP23464 ablates BCR-ABL tyrosine phosphorylation, blocks cell cycle progression, and promotes apoptosis of BCR-ABL-expressing cells. Biochemical assays with the purified GST-ABL kinase domain have confirmed that AP23464 directly inhibits ABL activity. Importantly, the low nanomolar cellular and biochemical inhibitory properties of AP23464 extend to frequently observed imatinib-resistant BCR-ABL mutants, including the nucleotide binding P-loop mutants Q252H, Y253F, E255K, the C-terminal loop mutant M351T, and the activation loop mutant H396P. Like similar compounds, AP23464 is ineffective against the T315I mutant.

*CGP76030, a Src inhibitor, plus an ABL inhibitor show better results in $Ph^+$ acute lymphoblastic leukemia*. A recent study has shown that certain Src kinases are necessary for the induction of B-cell acute lymphoblastic leukemia (B-ALL) but not CML.[32] This evidence has provided a rationale for the use of Src kinase inhibitors to treat $Ph^+$ B-ALL. The kinase inhibitor CGP76030 impairs the proliferation of B-lymphoid cells expressing BCR-ABL *in vitro* and prolongs the survival of mice with B-ALL but not CML. The combination of CGP76030 and imatinib has been found to be superior to imatinib alone in this regard. The biochemical target of CGP76030 in leukemia cells is Src kinases, not BCR-ABL. These results suggest that the Src kinase family could be therapeutic targets in $Ph^+$ B-ALL. Simultaneous inhibition of Src and BCR-ABL kinases with a *dual* inhibitor or with a combined TKI approach may be of benefit to individuals with $Ph^+$ acute leukemia.

## Agents that target pathways downstream of BCR-ABL

TKI or other compounds may be used to inhibit BCR-ABL downstream signal transduction pathways. A multitude of signaling pathways are activated by BCR-ABL. These pathways are potential targets for intervention in cases in which inhibition of BCR-ABL itself is not complete. It is also conceivable that oncogenic stimuli other than BCR-ABL activate such pathways.

### *Farnesyl transferase inhibitors*

Farnesylation, i.e., the transfer of an isoprenoid (farnesyl) moiety to the C terminus of the protein, is required for Ras to localize to the cell membrane. This subcellular localization is necessary for Ras to activate Raf1 and the MAP kinase pathway.[42] Numerous cellu-

lar proteins are farnesylated and the precise mechanism of growth inhibition by farnesyl transferase inhibitors (FTI) is unknown. Since Ras is a major pathway activated by BCR-ABL, this pathway is presumed to be the target of FTI in these cells. FTI inhibit the proliferation of BCR-ABL-positive cells, including those that are resistant to imatinib[43] but do not induce apoptosis. Simultaneous treatment of BCR-ABL expressing cells with imatinib and FTI results in apoptosis only if some inhibition of BCR-ABL is achieved, as in cell lines with increased expression of BCR-ABL. In contrast, apoptosis is not induced in cell lines that express BCR-ABL with the T315I mutation, which has an extremely high $IC_{50}$. This suggests that BCR-ABL tyrosine kinase activity must be reduced below a certain threshold in order for apoptosis to be induced.

### *Mitogen-activated protein kinase inhibitors*

Activation of Ras by BCR-ABL is thought to activate the MAP kinase pathway. Synergism of MAP kinase inhibitors with imatinib has been demonstrated in BCR-ABL-positive cell lines, whereas this drug combination does not affect the proliferation of normal mononuclear cells.[44]

### *Phosphatidylinositol-3 kinase inhibitors*

Phosphatidylinositol-3 kinase activity is another major downstream target of BCR-ABL that has been shown to be required for BCR-ABL to induce leukemia in mice. In cell lines, phosphatidylinositol-3 kinase inhibitors are synergistic with imatinib. The situation is less clear in primary cells, where there is great variability between individual patients.[45] One general caveat for all agents that target pathways downstream of ABL is that inhibition of individual pathways may not be sufficient to shut down the entire system.

## Novel anti-BCR-ABL agents

### *AMN107 is a new anilino-pyrimidine derivative structurally related to imatinib*

AMN107 was tested in three human BCR-ABL positive lines (K562, KCL-22, and Lama-84) and in primary cells derived from two BCR-ABL-positive CML patients who were resistant to imatinib, as well as from one newly diagnosed chronic phase patient.[46] In all patients, sequencing of the BCR-ABL kinase domain excluded any point mutations, but cytogenetic analysis of the bone marrow revealed clonal evolution in the resistant patients, including t(1;5) and t(3;21) translocations, trisomy of chromosome 8 and monosomy of chromosome 7. Determination of the proliferative activity by XTT-assay in cell lines demonstrated a decrease in the $IC_{50}$ in imatinib- versus AMN107-treated samples from 0.08 μM to 0.0075 μM in Lama 84, from 0.25 μM to 0.08 μM in K562 and from 0.45 μM to 0.03 in KCL-22 cells. In primary cells from imatinib-resistant patients, a decrease in the $IC_{50}$ in imatinib- versus AMN107-treated peripheral blood cells from 0.75 μM to 0.1 μM and from 4 to 0.4 μM was detected. In addition, in primary cells from one newly diagnosed CML patient the $IC_{50}$ of AMN107 (2.5 μM) was lower than that of imatinib (5 μM). Immunoblotting showed that, in Lama-84 cells, a concentration of 0.01 μM AMN107 completely inhibited the tyrosine kinase activity as detected by the use of an anti-phosphotyrosine antibody; this contrasted with the almost 5 μM of imatinib necessary. After 48 hours of incubation with either 0.25 μM imatinib or 0.005 μM AMN107, induction of early apoptosis was detected in 8.8% of imatinib-treated and in 26% of AMN107-treated cells. In addition, in MDR1 over-expressing CCRF cells co-cultured with either AMN107 or imatinib revealed elevated AMN107 levels (3.7-fold), indicating that this substance is less susceptible to MDR1-driven resistance than imatinib.

### *Other anti-BCR-ABL agents*

Novel anti-BCR-ABL agents, with mechanisms of antileukemic activity distinct from those described for imatinib, are an attractive alternative. Despite being functionally inhibited, the BCR-ABL oncoprotein continues to be expressed by the leukemic cells. This becomes problematic when the selection of clones with mutated amino acids or with BCR-ABL overexpression leads to the emergence of resistance. Hence, the development of therapies aimed at interfering with BCR-ABL expression is particularly desirable. Geldanamycin and its less toxic analog 17-allylamino-17-demethoxygeldanamycin (17-AAG) inhibit heat shock protein 90 (Hsp90), a molecular chaperone that stabilizes the BCR-ABL protein among others. Treatment of BCR-ABL-expressing cell lines with these agents suppresses growth and induces apoptosis.[47] In their presence, BCR-ABL is degraded via the proteasome pathway. Interestingly, BCR-ABL proteins with mutations of the kinase domain may be even more sensitive to geldanamycin than is the wild-type protein.[47] This observation makes these agents particularly attractive for therapy in patients carrying a mutated BCR-ABL protein. The same marked efficacy has been observed in cells rendered resistant to imatinib via BCR-ABL overexpression. Like geldanamycin and 17-AAG, radicicol, a macrocyclic antifungal antibiotic, binds to the N-terminal Hsp90 and destabilizes Hsp90-associated proteins such as Raf-1. A recent study[48] investigated the effects of radicicol, novel oxime derivatives of radicicol (KF25706 and KF58333), and herbimycin A (HA), a benzoquinoid ansamycin antibiotic, on the growth and differentiation of human K562 CML cells. Although KF25706 and KF58333 induced

the expression of glycophorin A in K562 cells, radicicol and HA caused transient erythroid differentiation. Cell cycle analysis showed that $G_1$ phase accumulation was observed in K562 cells treated with KF58333. KF58333 treatment depleted $p210^{BCR-ABL}$, Raf-1, and cellular tyrosine phosphorylated proteins in K562 cells, whereas radicicol and HA showed only transient depletion of these proteins. KF58333 also down-regulated the level of cell cycle-dependent kinases 4 and 6 and up-regulated the cell cycle-dependent kinase inhibitor p27 protein without any effect on the levels of the Erk and Hsp90 proteins. Immunoprecipitation analysis showed that $p210^{BCR-ABL}$ formed multiple complexes with Hsp90, some containing p23 and others Hsp70; KF58333 treatment dissociated $p210^{BCR-ABL}$ from Hsp90/p23 chaperone complexes. Furthermore, KF58333 induced apoptosis in K562 cells and administration of KF58333 prolonged the survival of SCID mice inoculated with K562 cells. These results suggest that KF58333 may have therapeutic potential for the treatment of CML involving abnormal cellular proliferation induced by $p210^{BCR-ABL}$. Arsenic trioxide ($As_2O_3$) down-regulates BCR-ABL protein levels by translational modulation. Various groups have shown that it enhances the selective cytotoxic effect of imatinib on CML cells. Like geldanamycin and 17-AAG, it is also effective in inhibiting the growth of cell lines resistant to imatinib.[49-53] Finally, those agents that not only enhance the antileukemic effects of imatinib but also exert cytotoxic effects against imatinib-refractory CML BC cells are also particularly attractive. One such agent is LAQ824, a cinnamic acid hydroxamate, which acts as a potent histone deacetylase inhibitor. It has been demonstrated that LAQ824 depletes the mRNA and protein expression of BCR-ABL in human CML BC cells. Exposure to LAQ824 causes cell cycle $G_1$-phase accumulation and apoptosis of CML blasts. LAQ824 also induces acetylation of Hsp90. This inhibits the chaperone association of BCR-ABL with Hsp90, thereby also promoting the proteasomal degradation of BCR-ABL. Additionally, LAQ824 is capable of down-regulating the levels of the T315I BCR-ABL mutant.[54,55]

## Conclusions

Knowledge of the unique genetic lesion responsible for the pathogenesis of CML has paved the way for the development of the first successful example of targeted therapy. The BCR-ABL inhibitor imatinib mesylate is now the first-choice treatment for all newly diagnosed CML patients, but the initial striking efficacy of this drug has been overshadowed by the development of clinical resistance. In the meantime, research has made considerable progress in the dissection of the signal transduction pathways affected by the deregulated kinase activity of BCR-ABL, providing information on additional or alternative signaling steps that could be interrupted in an attempt to block the leukemogenic process. Several promising inhibitors acting on BCR-ABL or at different levels of the BCR-ABL signaling cascade have been synthesized and are now in phases of pre-clinical or clinical testing. The drug armamentarium against CML may soon be expected to include novel weapons.

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