Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia

Ellen Weisberg, Paul W. Manley‡, Sandra W. Cowan-Jacob§, Andreas Hochhaus‖ and James D. Griffin¶*

Abstract | Imatinib, a small-molecule ABL kinase inhibitor, is a highly effective therapy for early-phase chronic myeloid leukaemia (CML), which has constitutively active ABL kinase activity owing to the expression of the BCR-ABL fusion protein. However, there is a high relapse rate among advanced- and blast-crisis-phase patients owing to the development of mutations in the ABL kinase domain that cause drug resistance. Several second-generation ABL kinase inhibitors have been or are being developed for the treatment of imatinib-resistant CML. Here, we describe the mechanism of action of imatinib in CML, the structural basis of imatinib resistance, and the potential of second-generation BCR-ABL inhibitors to circumvent resistance.

**Dana Farber Cancer Institute, Mayer 540, 44 Binney St, Boston, Massachusetts 02115, USA.*
‡Novartis Institutes for BioMedical Research, WKL-136.4.86, Basel, CH-4002, Switzerland.
§Novartis Institutes for BioMedical Research, WSJ-088.9.08A, Basel, CH-4056, Switzerland.
‖Medizinische Fakultat Mannheim der Universitat Heidelberg III. Medizinische Klinik, Theodor-Kutzer-Ufer 1-3, 68167, Mannheim, Germany.
¶Dana-Farber Cancer Institute, Department of Medical Oncology, 44 Binney Street, Boston, Massachusetts 02115, USA.
Correspondence to J.D.G.
e-mail: james_griffin@dfci.harvard.edu
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The *BCR-ABL* oncogene, which is the product of Philadelphia chromosome (Ph) 22q, encodes a chimeric BCR-ABL protein that has constitutively activated ABL tyrosine kinase activity; it is the underlying cause of chronic myeloid leukaemia (CML)[1–3]. Whereas the 210 kDa BCR-ABL protein is expressed in patients with CML, a 190 kDa BCR-ABL protein, resulting from an alternative breakpoint in the *BCR* gene, is expressed in patients with Ph positive (Ph^+) acute lymphoblastic leukaemia (ALL)[4]. The impact of imatinib, an ABL kinase inhibitor that also inhibits KIT and platelet-derived growth factor receptor (PDGFR) at physiologically-relevant concentrations, on the field of cancer therapy has been dramatic. This targeted therapy has not only changed how newly diagnosed patients with CML are treated and greatly improved their prognosis, it has altered the natural history of the disease.

Some patients develop imatinib resistance, particularly in the advanced phases of CML and Ph^+ ALL. This resistance is usually caused by point mutations in the kinase domain of the BCR-ABL enzyme that reduce sensitivity towards imatinib, although there are also BCR-ABL-independent mechanisms of imatinib resistance that can occur. Strategies developed to overcome imatinib resistance in second generation inhibitors include targeting the integrity and/or stability of the BCR-ABL protein itself, as well as signalling pathways downstream of BCR-ABL that are necessary for transformation (FIG. 1). Structural biology studies have facilitated the design of new drugs to circumvent resistance, and several new agents have been developed specifically for this purpose. These compounds have been well characterized for efficacy against the mutant enzymes in preclinical studies, and impressive therapeutic activity has now been reported for two second generation drugs in phase I and II clinical trials in patients with imatinib-resistant CML.

Imatinib resistance

Mutations that cause imatinib resistance are usually those that lead to a BCR-ABL protein with a functional ABL tyrosine kinase domain, but that totally abrogate or impair drug binding. On the molecular level, point mutations in BCR-ABL reduce the binding of imatinib to the protein by either a direct or an indirect mechanism. In the case of direct mechanisms, mutations are clustered around the imatinib binding site, which partially overlaps that of ATP, and reduce imatinib binding either as a result of changes to amino-acid side-chains, which contribute favourable lipophilic contacts or hydrogen-bond (H-bond) interactions, or as a result of topographical changes that sterically hinder imatinib binding. Examples of residues that inhibit imatinib binding when they are mutated are Thr315 and Phe317 (REF. 5) (TABLE 1).

Mutations that inhibit imatinib binding through an indirect mechanism exploit the particular binding mode of the drug to its target protein. Imatinib binds to a catalytically inactive conformation of the ABL kinase

At a glance

- The structural basis for imatinib resistance in chronic myeloid leukaemia (CML) involves the emergence of imatinib-resistant BCR-ABL point mutations; mutations are usually those that impair drug binding.
- More than 50 different BCR-ABL mutations have been identified in patients with imatinib-resistant CML and through random mutagenesis assays.
- Different imatinib-resistant BCR-ABL point mutants can have different transforming potentials in cells and different prognostic outcomes.
- Methods to predict imatinib-resistant BCR-ABL mutants include PCR-based screening assays, such as the highly sensitive allele-specific oligonucleoside (ASO)-PCR method, and the denaturing high-performance liquid chromatography (D-HPLC)-based assay.
- Imatinib-resistant BCR-ABL point mutations have been found to pre-exist in newly diagnosed patients with CML, as well as be acquired owing to selective pressure of imatinib. Furthermore, imatinib fails to deplete leukaemic stem cells.
- New BCR-ABL inhibitors in clinical trials include ABL inhibitors (nilotinib), dual Src family and ABL kinase inhibitors (bosutinib, INNO-404 and AZD0530), non-ATP competitive inhibitors of BCR-ABL (ON012380) and Aurora kinase inhibitors (MK-0457 and PHA-739358). The dual Src and ABL inhibitor dasatinib has recently been approved by the US Food and Drug Administration for the treatment of patients with CML or Philadelphia chromosome positive acute lymphoblastic leukaemia resistant or intolerant to imatinib.
- BCR-ABL point mutants resistant to the second generation inhibitors nilotinib and dasatinib have been identified through cell-based resistance screens.
- Strategies to circumvent the emergence of resistance include combination therapy using inhibitors of BCR-ABL and other targets.

domain, often referred to as the 'DFG-out' conformation, in which the highly conserved Asp–Phe–Gly (DFG) triad is flipped out of its usual position in active kinase conformations. This makes a channel beyond the Thr315 gatekeeper residue that opens up an auxiliary binding site, which is occupied by the piperazinyl-substituted benzamide moiety of imatinib. This inactive conformation of the DFG motif has not been observed in crystal structures of ABL not bound to inhibitors, and it is therefore unclear as to whether it has a physiologically relevant role in the autoregulation of ABL, or whether it is purely drug-induced. In addition, the nucleotide-binding loop (P-loop) of the kinase domain, which usually adopts an extended conformation in active kinases allowing it to interact with the phosphate group of ATP, is found to fold down over imatinib, forming a cage-like structure around the pyridine and pyrimidine groups. Point mutations in the ABL kinase domain that destabilize the inactive conformations of the P-loop and the DFG motif with respect to the catalytically active conformation increase the free energy of the imatinib–ABL complex, and therefore reduce the imatinib binding affinity. This leads to a shift in the equilibrium between the inactive and active states and a restoration of BCR-ABL kinase activity. Examples of imatinib-resistant mutations that destabilize the inactive conformation are those that affect residues Glu255, Tyr253 and Gly250 in the P-loop of the ABL kinase domain (TABLE 1). Therefore, for example, the mutation of Tyr253 to Phe or His results in the loss of an H-bond between the OH of Tyr253 and Asn322 thought to be important in stabilizing the inactive P-loop conformation[6,7].

Gatekeeper residue
The gatekeeper is a residue located at the back of the ATP-binding site, the properties of which (size, charge and hydrophobicity) regulate the binding of inhibitors.

Cap region
A region at the N terminus of wild-type ABL, which has a role in keeping the kinase in an inactive state.

Imatinib-induced BCR-ABL mutations

Early investigations into the underlying mechanism of imatinib resistance in cell lines revealed *BCR-ABL* gene amplification, and the overexpression of *BCR-ABL* mRNA and protein[8–10]. However, the most frequent mechanism of resistance in patients is now known to be point mutations in the *BCR-ABL* gene, resulting in amino-acid changes in the catalytic domain of the BCR-ABL protein that impair imatinib binding, thereby reducing the ability of imatinib to inhibit the tyrosine kinase activity of the enzyme. A *BCR-ABL* point mutation conferring resistance to imatinib was first detected in patients by Sawyers and colleagues as a result of the analysis of nine patients that were resistant to treatment with imatinib[11]. In this study, *BCR-ABL* gene amplification was found to occur in three out of nine patients, but six out of nine patients harboured a point mutation that resulted in an isoleucine substitution (T315I) in the kinase domain. Based on a crystal structure of an analogue of imatinib bound to the ABL kinase domain[12], Druker and co-workers[13] had previously recognized this residue as being crucial for the interaction of imatinib with ABL, and found that mutation to valine (T315V) resulted in a catalytically active kinase domain that was substantially less sensitive to imatinib compared with the wild-type enzyme. The Thr315 residue is located at the gatekeeper position at the periphery of the nucleotide-binding site of the protein, and participates, through the hydroxymethylene side-chain, in a crucial H-bond interaction between imatinib and ABL[12], as well as BCR-ABL[14,15]. Mutation to isoleucine abrogates the possibility of this H-bond interaction, which, combined with the additional bulk of the isoleucine side-chain, sterically hinders imatinib binding and leads to imatinib insensitivity and consequently resistance.

Further *BCR-ABL* mutations associated with imatinib resistance were then rapidly identified, and more than 50 different point mutations have been described[6,16–18]. However, many of these mutants are relatively rare, and the most common, affecting residues Gly250, Tyr253, Glu255, Thr315, Met351 and Phe359, account for 60–70% of all mutations.

As well as being detected in patients with imatinib-resistant CML, additional mutants have been generated by the random mutagenesis of BCR-ABL *in vitro* and selection for imatinib resistance[17,18]. In both patient samples and laboratory-generated mutants, imatinib resistance is associated with mutations throughout the kinase domain, including the activation loop, phosphate binding P-loop, and the hinge-region that links the C- and N-terminal lobes of the kinase domain to form the ATP-binding cleft. In the laboratory-generated mutants, mutations were also identified in regions outside the kinase domain (in the N-terminal cap region, the SRC homology 3 (SH3) domain, the SH2 domain and the linker between the SH2 and kinase domains), many of them in positions required to maintain the inactive state of the enzyme[18–20].

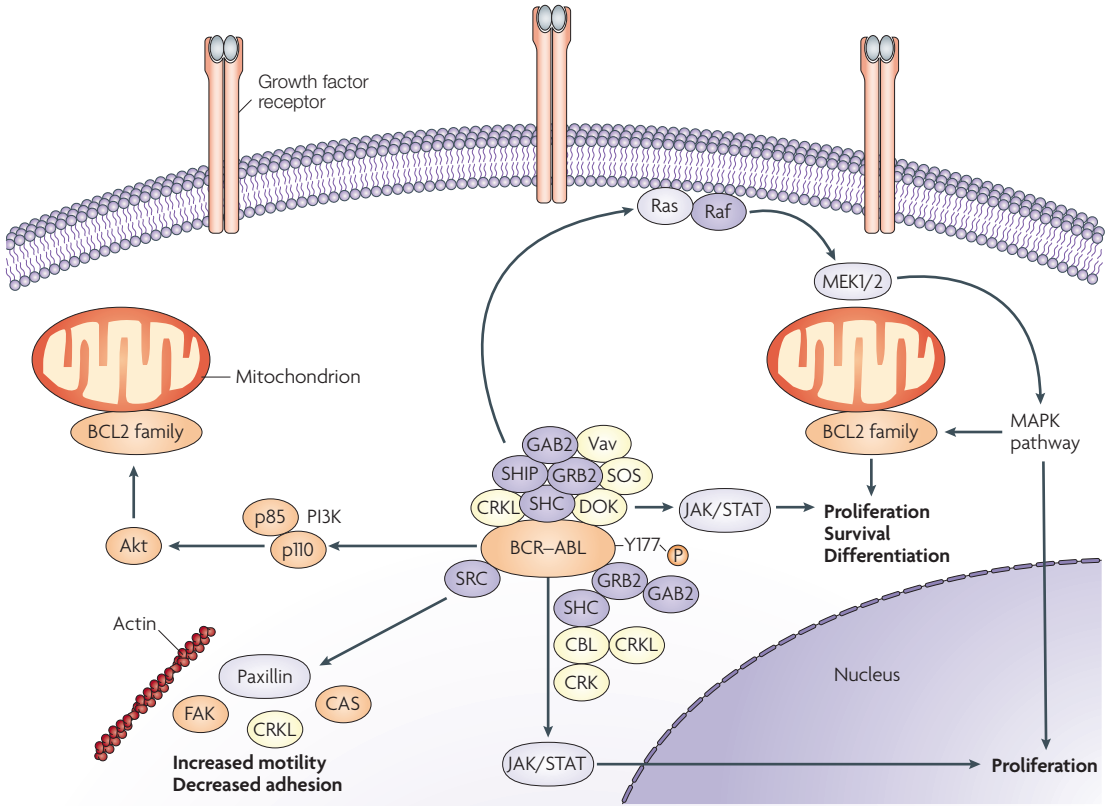

Figure 1 | **BCR-ABL signalling in chronic myeloid leukaemia.** With the aid of several mediator proteins, BCR-ABL associates with Ras and stimulates its activation. The adaptor protein, growth factor receptor-bound protein 2 (GRB2), interacts with BCR-ABL through the proximal SRC homology 2 (SH2)-binding site that develops when the tyrosine 177 (Y177) residue of BCR-ABL is autophosphorylated. GRB2, when bound to BCR-ABL, interacts with the son of sevenless (SOS) protein. The resulting BCR–ABL–GRB2–SOS protein complex activates Ras. The adaptor proteins CRKL (CRK-like) and SHC (SH2-containing protein) can also mediate the BCR-ABL activation of Ras. Ras and the mitogen activated protein kinase (MAPK) pathway are coupled by Raf (a serine/threonine kinase). Raf catalyses the phosphorylation of the mitogen-activated and extracellular-signal regulated kinase kinases 1 and 2 (MEK1 and MEK2); this results in their activation. Through the stimulation of the Ras–Raf pathway, BCR-ABL increases growth factor-independent cell growth. BCR-ABL also associates with and activates the phosphatidylinositol-3 kinase (PI3K) pathway, suppressing programmed cell death and increasing cell survival. BCR-ABL is associated with components of the focal adhesion (that is, actin, paxillin and focal adhesion kinase, or FAK); the activation of CRKL–FAK–PYK2 leads to a decrease in cell adhesion. BCR-ABL also associates with the Janus kinase and signal transducer and activator of transcription (JAK–STAT) pathway. Finally, BCR-ABL activates pathways that lead to atypical responses to chemotactic factors, which leads to an increase in cell migration. BCR-ABL also associates with survival proteins that interact with the mitochondrial-based BCL2 family. CAS, p130 CRK-associated substrate; GAB2, GRB2-associated binding protein 2; SHIP, SH2-containing inositol-5-phosphatase

Oncogenic potential of BCR-ABL mutants

The expansion of mutant clones in imatinib-treated patients is often prognostic for relapse and disease progression. However, studies with full-length BCR-ABL mutant proteins in cells indicate that the degree of inhibition of BCR-ABL autophosphorylation and phosphorylation of its substrates does not always correlate with the antiproliferative activity of imatinib[21], and therefore different mutants can have different transforming potency in cells[22,23]. Two of the more frequently detected mutants seem to have the greatest transforming potential, with a rank order being Y253F, E255K>native BCR-ABL>T315I>H396P>M351T. These *in vitro* findings are consistent with clinical findings in patients with CML and ALL treated with imatinib. It has also been shown in imatinib-treated patients that a mutant clone does not necessarily have a proliferative advantage, and the presence of such a mutant does not always account for resistance to imatinib[24]. The high *in vitro* transforming potential of the Y253H and E255K mutants (P-loop mutations) is also consistent with P-loop mutations being associated with a poor prognosis in terms of time to disease progression and overall survival in imatinib-treated patients[25]. Similarly, in retrospective studies, P-loop and T315I mutations, generally found in patients with advanced disease, translated into significantly worse overall survival compared with other mutations in patients who continued imatinib therapy[26,27]. When patients that express a particular mutant clone respond to drug treatment, but subsequently relapse with a further mutation, the new mutant does not normally emerge on the background of the first mutant, but on

PI3K
PI3K is a heterodimer that is made up of a regulatory (p85) subunit (which BCR-ABL interacts with), and a catalytic (p110) subunit.

Focal adhesion
A cell-to-substrate adhesion structure that anchors the ends of actin microfilaments (stress fibres) and mediates strong attachment to the extracellular matrix.

Table 1 | **Characterization and analysis of some imatinib-resistant mutant forms of BCR-ABL**

Mutation	Imatinib sensitivity* (IC_{50}, nM)		Frequency in patients	Molecular mechanism of resistance
	Autophosphorylation‡	Cell proliferation‡		
Wild type	221 ± 31	678 ± 39	NA	NA
Met244Val	937	2,036	Low or medium	Indirect: possibly causes an increase in entropy of ABL less favourable for inhibitor binding
Leu248Val	1,011	2,081	High	Direct: poorer topological fit with imatinib, and destabilization of the inactive state
Gly250Ala	313	1,269	High	Mechanism unclear
Gly250Glu	2,287 ± 826	3,329 ± 826	Low or medium	Indirect: stabilization of the active or other conformational state to which imatinib does not bind
Gln252His	1,080 ± 119	851 ± 436	Low or medium	Indirect: destabilization of the inactive state
Gln252Arg	ND	ND	Low or medium	Indirect: destabilization of the inactive state
Tyr253His	> 10,000	> 10,000	High	Direct: loss of π–π interaction with imatinib and destabilization of the inactive state
Tyr253Phe	ND	ND	High	Indirect: destabilization of the inactive state
Glu255Lys	4,856 ± 482	5,567	Low or medium	Indirect: destabilization of the inactive state
Glu255Val	6,353 ± 636	7,161 ± 970	Low or medium	Indirect: destabilization of the inactive state
Glu292Lys	275 ± 81	1,552	Low or medium	No obvious reason for resistance
Phe311Ile	ND	ND	Low or medium	Indirect: destabilization of the inactive state
Phe311Leu	ND	ND	Low or medium	Indirect: destabilization of the inactive state
Thr315Ile	> 10,000	> 10,000	High	Direct: steric hindrance and loss of H-bond to imatinib
Phe317Leu	797 ± 92	1,528 ± 227	High	Direct: poorer topological fit with imatinib
Phe317Val	544 ± 47	549 ± 173	Low or medium	Direct: poorer topological fit with imatinib
Met343Thr	ND	ND	Low or medium	Indirect: possibly causes increase in entropy of ABL less favourable for inhibitor binding
Met351Thr	593 ± 57	1,682 ± 233	High	Indirect: possibly causes increase in entropy of ABL less favourable for inhibitor binding
Glu355Gly	601	1,149	High	No obvious reason for resistance
Phe359Ala		ND	Low or medium	Direct: poorer topological fit with the inhibitor
Phe359Val	1,528	595	High	Direct: poorer topological fit with the inhibitor
Val379Ile	ND	ND	Low or medium	Indirect: possibly causes increase in entropy of ABL less favourable for inhibitor binding
Met388Leu	ND	ND	Low or medium	Indirect: possibly causes increase in entropy of ABL less favourable for inhibitor binding
His396Arg	ND	ND	High	Indirect: destabilization of the inactive state
His396Pro	ND	ND	Low or medium	Indirect: destabilization of the inactive state
Phe486Ser	1,238 ± 110	3,050 ± 597	Low or medium	Indirect: possibly causes increase in entropy of ABL less favourable for inhibitor binding

* See Box 1 and references 21 and 47 for methods used to determine sensitivity. ‡Where indicated, ± standard error of the mean (SEM). Where not indicated, only two measurements were available and were averaged. H-bond, hydrogen bond; IC_{50}, half-maximal inhibitory concentration; NA, not applicable; ND, not determined.

Table 2 | **New tyrosine kinase inhibitors in clinical trials for CML**

Inhibitor	Company	Targets	Route of administration	Developmental status	Refs
Nilotinib (AMN 107)	Novartis	ABL, PDGFR, KIT, EPHB4	Oral	Phase II	51, 79, 81
Dasatinib (BMS-354825)	Bristol-Myers Squibb	ABL, PDGFR, KIT, FGR, FYN, HCK, LCK, LYN, SRC, YES, EPHB4	Oral	Approved	60, 61, 81, 85
Bosutinib (SKI-606)	Wyeth	ABL, FGR, LYN, SRC	Oral	Phase II	65
INNO-406 (NS-187)	Innovive	ABL, LYN, PDGFR, KIT	Oral	Phase I	NA
AZD0530	AstraZeneca	Src family kinases	Oral	Phase II (solid tumours)	NA
MK-0457 (VX-680)	Merck	Aurora kinases, FLT3, JAK2, ABL (including T315I)	Intravenous	Phase II	73
PHA-739358	Nerviano	Aurora A, B and C	Oral	Phase II	NA

CML, chronic myeloid leukaemia; FLT3, FMS-related tyrosine kinase 3; JAK2, janus kinase 2; NA, not applicable; PDGFR, platelet-derived growth factor receptor.

the native BCR-ABL. This suggests that either all of the mutant BCR-ABL clones usually pre-exist before BCR-ABL inhibitor therapy, or that double-mutants are rarely enzymatically competent.

Methods to predict resistance mutants

The development of highly sensitive, PCR-based screening assays has greatly facilitated the detection and identification of point mutations in imatinib-resistant patients, and could be useful for predicting the best course of treatment for these patients, as well as patients resistant to the second generation BCR-ABL inhibitors[25,28–30]. The highly sensitive allele-specific oligonucleoside (ASO)-PCR method was used to detect the first set of BCR-ABL point mutations shown to simultaneously exist in a patient with imatinib-resistant CML[17,28]. A denaturing high-performance liquid chromatography (D-HPLC)-based assay has been successful in identifying point mutations in patients with CML who had cytogenetic resistance to imatinib, or a deficiency of partial or complete cytogenetic remissions [25,29].

Therapy surveillance by molecular methods has become a crucial part of the clinical management of patients with CML[31]. The goal of detection and quantification of residual disease by reverse transcription PCR and the early detection of mutations is to enable timely therapeutic interventions to optimize therapy. International standardization of the methodology is required to establish a sound basis for therapeutic decisions[32]. The prognostic value of early detection of imminent resistance to kinase inhibitors by sensitive methods (such as D-HPLC and ASO-PCR) to allow early therapeutic interventions needs to be evaluated.

Origin of resistance

Imatinib resistance might arise through a low-to-undetectable background level of BCR-ABL mutations (less than 1% of tumour cells) that exist before imatinib treatment, and which grow in prevalence during therapy owing to the selective pressure of imatinib[33,34]. Several studies also suggest that drug-resistant BCR-ABL point mutations can arise during imatinib treatment. Such acquired resistance usually involves the re-emergence of BCR-ABL tyrosine kinase activity, which suggests that the mutant BCR-ABL protein is still a putative target for inhibition in imatinib-resistant patients[26,35–37].

Although imatinib potently inhibits the production of differentiated leukaemic cells, leading to high rates of cytogenetic and haematological remissions, it fails to deplete leukaemic stem cells[38,39]. This is true despite the presence of higher levels of BCR-ABL transcripts and protein in these cells compared with more differentiated CML cells[40]. This seems to be because these quiescent leukaemic stem cells do not appear to be dependent on BCR-ABL. Despite the complete inhibition of BCR-ABL-mediated CRKL phosphorylation in these leukaemic stem cells with imatinib and the second generation ABL inhibitor nilotinib, the cells remain viable[41]. Therefore, a population of BCR-ABL-positive, quiescent stem cells remain after imatinib therapy, even in patients who have achieved complete responses, and the insensitivity of these cells to imatinib is believed to contribute to the relapse observed in some patients following the termination of imatinib treatment.

Recent studies suggest the intriguing possibility that BCR-ABL directly alters both the amount of DNA damage in leukaemic cells and important pathways of DNA repair, and that both activities are BCR-ABL kinase dependent (reviewed in REF. 42). Increased DNA damage, including the occurrence of point mutations, has been linked to the increase in production of reactive oxygen species caused by BCR-ABL[43,44]. Equally important are observations that DNA repair pathways are affected by BCR-ABL. These observations lead to the hypothesis that residual leukaemic stem cells are accumulating DNA damage that, in some cases, will be manifested by imatinib resistance and progressive disease. Importantly, as kinase activity is required to increase DNA damage, the more effectively that kinase inhibitors block activity

Cytogenetic remission
Complete cytogenetic remission is the absence of metaphase cells positive for the BCR-ABL rearrangement (or Philadelphia chromosome positive cells). Partial cytogenetic remission is the presence of ≤35% of metaphase cells positive for the BCR-ABL rearrangement (or Philadelphia chromosome positive cells).

Haematological remission
Complete haematological remission is the achievement of a normal white blood cell (WBC) and platelet count, and no signs and symptoms of CML. Partial haematological remission is a decrease in the WBC count to less than 50% of pretreatment levels.

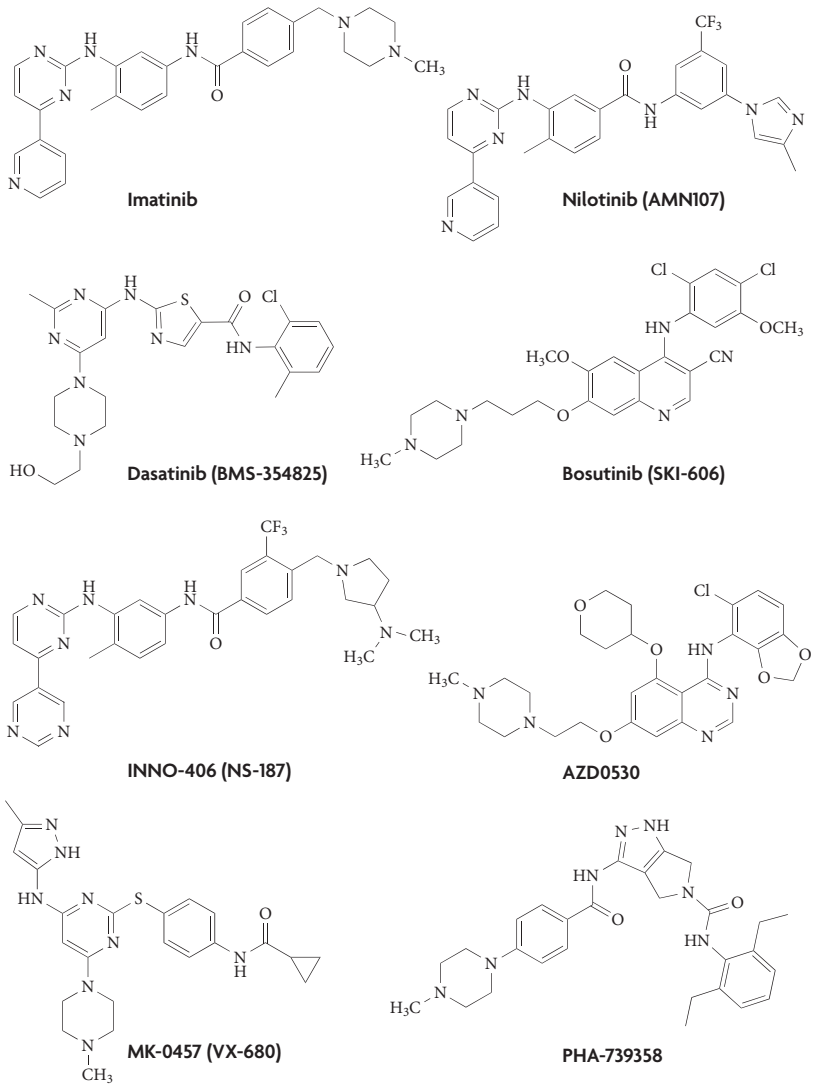

Figure 2 | **Molecular structures of the tyrosine kinase inhibitors in clinical trials for CML.** Clinical data on these compounds are detailed in TABLE 2. These are novel tyrosine kinase inhibitors for chronic myeloid leukaemia (CML) that are currently being investigated in clinical trials.

Chronic phase
An early phase of CML characterized by variable duration. Patients often lack symptoms, or are mildly symptomatic; if left untreated, this will progress to an accelerated phase.

Accelerated phase
Occurs between chronic phase and blast crisis. Characterized by 10–19% myeloblasts and >20% basophils in blood or bone marrow and cytogenetic evolution; increasing splenomegaly, platelet count or white blood cell count also occurs in patients who are unresponsive to treatment.

in stem cells and the more rapidly the stem cell pool is reduced, it would be predicted that the rate of resistance and progression should be further decreased.

Characteristics of new BCR-ABL inhibitors

The discovery of resistance mechanisms, such as the emergence of BCR-ABL point mutations and the overexpression of the BCR-ABL protein in response to imatinib therapy, spurred the development of alternative therapies designed to override resistance to imatinib. These inhibitors are summarized in TABLE 2, molecular structures of these inhibitors are shown in FIG. 2, and methods used to assess the sensitivity of imatinib-resistant BCR-ABL point mutants to new compounds are summarized in BOX 1. Classes of these new inhibitors include selective ABL inhibitors, inhibitors of both ABL and Src-family kinases, Aurora kinase inhibitors, and non-ATP competitive inhibitors of BCR-ABL.

Nilotinib. The development of the phenylaminopyrimidine derivative nilotinib (AMN107; Novartis) followed rational drug design, based on the crystal structures of inhibitors in complexes with ABL[7]. It is about 30-fold more potent than imatinib as an ABL inhibitor (half-maximal inhibitory concentration (IC_{50}) <30 nM), but is much more selective, having only similar activity to imatinib against the receptor tyrosine kinases KIT (IC_{50} = 90 nM)[45,46] and PDGFRβ (IC_{50} = 72 nM)[45,47]. Although it also inhibits the ABL-related kinase ARG and has some activity against the ephrin receptor EPHB4 (REF. 48), nilotinib is devoid of activity against the related Src-family of tyrosine kinases. Most importantly for the treatment of imatinib-resistant disease, nilotinib inhibited 32 of 33 mutant BCR-ABL forms resistant to imatinib *in vitro* at physiologically relevant concentrations that have been approved for and tested in patients[21,49]. Furthermore, nilotinib has been shown to have efficacy at well-tolerated oral doses in mouse models of CML, driven by either parental or mutant BCR-ABL, following once- or twice-daily administration (Jensen, M. R., Brüggen, J. DiLea, C., Mestan, J. and P.W.M., unpublished data). It should be noted that some BCR-ABL mutants require higher concentrations of nilotinib than others to be inhibited.

Results of a phase I study of nilotinib in 97 patients with imatinib-resistant CML in chronic, accelerated, and blast phase, and 9 patients with Ph^+ ALL treated at doses ranging from 50 mg to 1,200 mg daily, have been reported[50]. Significant clinical responses were obtained in all phases of CML (TABLE 3).

In phase II trials in all phases of CML and Ph^+ ALL after imatinib treatment had failed, results of treatment of 494 patients with nilotinib[50–52] have been reported and are listed in TABLE 3. Preliminary analyses demonstrate good efficacy and tolerability of nilotinib when it is administered twice a day at a dose of 400 mg (plasma half-life of 15–24 hours; C_{max} of 3,600 nM).

Fluid retention is a well-characterized adverse event associated with imatinib therapy, which is frequently manifested as periorbital oedema and believed to be a consequence of PDGFR kinase inhibition. However, although nilotinib inhibits PDGFR with a similar potency to that of imatinib, and tissue concentrations of this drug in patients treated with 400 mg twice a day are significantly above those necessary to inhibit this receptor tyrosine kinase, the incidence of oedema in these patients is relatively rare.

ABL and Src-family inhibitors

The Src family of tyrosine kinases modulates multiple intracellular signal transduction pathways involved in cell growth, differentiation, migration and survival, many of which are involved in oncogenesis, tumour metastasis and angiogenesis. The family includes SRC, FYN and YES, which are ubiquitously expressed, as well as HCK, LYN, FGR, LCK and BLK, the expression of which is mainly restricted to haematopoietic cells[53]. It has been shown that BCR-ABL activates Src kinases both through phosphorylation and direct binding. Furthermore, LYN is overexpressed and activated in the BCR-ABL-positive but

Box 1 | **Methods to assess the sensitivity of mutants to imatinib and prospective new drugs**

Biochemical assays used to evaluate potential ABL kinase inhibitors generally use a truncated recombinant ABL kinase domain, lacking the autoregulatory domains, to phosphorylate an artificial substrate. However, because of possibly altered kinase domain plasticity and substrate selectivity, combined with a different phosphorylation state and non-physiological ATP concentrations, such biochemical assays may be misleading. Consequently, assays measuring compound effects on the native kinase in BCR-ABL-expressing cell lines have clear advantages.

BCR-ABL autophosphorylation in cell lysates is best quantified with an ELISA (enzyme-linked immunosorbant assay) using an ABL-specific capture antibody, together with an enzyme-labelled anti-phosphotyrosine antibody and a luminescent substrate. Although ABL is expressed in most cells, it is tightly regulated with negligible background tyrosine kinase activity, so that only BCR-ABL autophosphorylation is detected in the cell lines investigated. Drug effects can be evaluated using human leukaemia cell lines that naturally express BCR-ABL (for example, K562 and KU812F), as well as with mouse haematopoietic cells (32D and Ba/F3) transfected to express BCR-ABL or BCR-ABL mutants, analogous to those detected in imatinib-resistant patients. Using this assay, imatinib inhibits BCR-ABL autophosphorylation in K562, KU812, 32D and Ba/F3 cells with mean half-maximal inhibitory concentration (IC_{50}) values of 498 ± 59, 457 ± 69, 230 ± 43 and 221 ± 31 nM, respectively. Following transfection with *BCR-ABL*, Ba/F3 cells lose their interleukin 3 dependency and become BCR-ABL dependent. Consequently, BCR-ABL inhibition in these cells affects their survival and proliferation, allowing the potencies of BCR-ABL inhibitors to be assessed with cell proliferation assays. The use of transfected Ba/F3 cells is particularly advantageous, as the effects of inhibitors on BCR-ABL autophosphorylation can be directly compared with effects on BCR-ABL-dependent proliferation. For selective BCR-ABL inhibitors, the inhibition of both BCR-ABL autophosphorylation and cell proliferation is highly correlated in cells that express either native BCR-ABL or imatinib-resistant mutant forms of this enzyme.

imatinib-resistant K562R leukaemia cell line (derived from imatinib-sensitive K562 cells) (BOX 1), and the inhibition of LYN reduced the proliferation and survival of K562R cells but had limited effects on imatinib-sensitive K562 cells[54]. Analyses of patient samples taken before and after imatinib treatment failure showed that the activation of Src-family kinases such as LYN and HCK can occur during disease progression, suggesting that the overexpression of these tyrosine kinases might mediate BCR-ABL-independent imatinib resistance in some patients. Therefore, simultaneously targeting BCR-ABL and Src kinases could overcome imatinib-mediated resistance.

Dasatinib. Dasatinib (BMS-354825; Bristol-Myers Squibb) is a highly potent, orally active inhibitor of SRC and Src-family kinases including FGR, FYN, HCK, LCK, LYN and YES[55]. However, it is also a potent BCR-ABL kinase inhibitor, and has additional activity against the KIT, PDGFR and ephrin receptor tyrosine kinases[48]. The lack of selectivity over the Src-family kinases is probably related to the fact that dasatinib, in contrast to both imatinib and nilotinib, inhibits BCR-ABL by binding to the active conformation of the ABL kinase domain, which is similar for ABL and the Src kinases[56]. However, Src kinase inhibition might be advantageous in imatinib-resistant disease, where dasatinib has been shown to directly inhibit 21 out of 22 mutant forms of BCR-ABL resistant to imatinib[57–59].

Based on positive data in phase I and phase II trials[60,61] (TABLE 3), dasatinib received accelerated approval by the US Food and Drug Administration (FDA) in June 2006, and the European Medicines Agency (EMEA) in November 2006, for the treatment of adults in all phases of CML with resistance or intolerance to imatinib therapy. Full approval was also granted for the treatment of adults with Ph^+ ALL with resistance or intolerance to previous therapy. Dasatinib is administered twice a day at a recommended dose of 70 mg (plasma half-life of 3–4 hours; C_{max} of 90 nM).

A category of adverse events reported in patients treated with dasatinib relates to fluid retention, which, in addition to the superficial peripheral oedema observed with imatinib, frequently occurs in the form of pleural effusion, pulmonary oedema and pericardial effusion. These differences in occurrence might relate to different underlying mechanisms. One such mechanism might involve the inhibition of PDGFRβ, which is believed to have a role in mediating the fluid retention observed in patients treated with tyrosine kinase inhibitors[62].

Bosutinib. Bosutinib (SKI-606; Wyeth) has been developed as an inhibitor of Src-family kinases for the treatment of solid tumours, although, like dasatinib, it also targets BCR-ABL[63]. However, unlike dasatinib, bosutinib does not inhibit KIT or PDGFR[63]. It inhibits the phosphorylation of cellular proteins, including STAT5, and the proliferation of CML cells. The phosphorylation of the autoactivation site of the Src-family kinases LYN and/or HCK is also reduced by treatment with bosutinib. This compound inhibits SRC and ABL in an enzyme assay with IC_{50}s of 1.2 nM and 1 nM, respectively[64]. Bosutinib showed *in vitro* activity against all imatinib-resistant mutants except T315I. The oral administration of this compound once a day at 100 mg per kg (body weight) for 5 days causes the complete regression of large xenografts of the leukaemia cell line K562 (BOX 1) in nude mice. In a phase I/II clinical trial in imatinib-resistant CML and Ph^+ ALL, bosutinib has shown evidence of efficacy at well-tolerated doses[65].

INNO-404. INNO-406 (Innovive; originally developed by Nippon Shinyaku as NS-187) is a potent BCR-ABL and LYN dual tyrosine kinase inhibitor, structurally related to imatinib and nilotinib, in clinical development as a potential treatment for patients with CML[66]. INNO-406 is more than 20-fold more potent than imatinib against the BCR-ABL-positive leukaemia cell lines K562 and KU812, and against Ba/F3 mouse haematopoietic cells engineered

Blast phase
The final phase of CML (also known as blast crisis), which is similar to an acute leukaemia (poor prognosis). Characterized by > 20% myeloblasts or lymphoblasts in blood or bone marrow.

Oedema
Swelling of tissues that results from the accumulation of excess lymph fluid.

Pleural effusion
Accumulation of excess fluid in the fluid-filled space that surrounds the lungs.

Pulmonary oedema
Accumulation of fluid in the alveoli and interstitial spaces of the lungs.

Pericardial effusion
Accumulation of fluid inside the sac covering the heart.

Autoactivation site
Region within the kinase domain activation loop that, when phosphorylated by the kinase itself, results in the activation of the protein.

Table 3 | **Responses to nilotinib and dasatinib in BCR-ABL-positive leukaemias after imatinib failure***

Agent	CML phase	Number of patients	Response (%)				Refs
			Haematological		Cytogenetic		
			Partial	*Complete*	*Partial (Ph^+ ≤35%)*	*Complete (Ph^+ = 0%)*	
Phase I							
Nilotinib	CP	17	11/12 = 92‡	11/12=92‡	35	35	50
	AP	56	38/51 = 74‡	26/51=51‡	27	14	
	My BP	24	42	8	21	4	
	Ly BP	9	33	0	11	11	
Dasatinib	CP	40	92	92	45	35	60
	AP	11	82	45	27	18	
	My BP	23	61	35	35	26	
	Ly BP and Ph^+ ALL	10	80	70	80	30	
Phase II							
Nilotinib	CP	279	137/185 = 74‡	137/185 = 74‡	52	34	51
	Im resistant	193					
	Im intolerant	86					
	AP	64	36	23	36	22	79
	Im resistant	52					
	Im intolerant	12					
	My BP	87	27	21	ND	ND	52
	Ly BP	27	30	26	ND	ND	
	Ph+ ALL (active)	37	24	24	ND	ND	
Dasatinib	CP	387	91	91	59	49	81,86
	Im resistant	288					
	Im intolerant	99					
	AP	174	64	45	39	32	61,85
	Im resistant	161					
	Im intolerant	13					
	My BP	109	34	27	33	26	61,85,87
	Ly BP	48	35	29	52	46	
	Ph+ ALL	46	41	33	57	54	88

*Care should be exercised when drawing comparisons between the different agents in different trials owing to differences in recruitment criteria, response criteria and length of treatment. ‡Response calculation for patients with active disease only. AP, accelerated phase; ALL, acute lymphoblastic leukaemia; BP, blast phase; CML, chronic myeloid leukaemia; CP, chronic phase; Im, imatinib; Ly, lymphoid; My,myeloid; ND, not determined; Ph^+, Philadelphia chromosome positive.

to express parental p210 BCR-ABL (BOX 1). INNO-406 inhibits several imatinib-resistant mutants, but not T315I. INNO-406 also suppresses the autophosphorylation of PDGFR and KIT with a similar level of potency to that of imatinib. In a solid-tumour KU812 subcutaneous xenograft model, INNO-406 inhibited tumour growth at an oral dose of 0.2 mg per kg (body weight) a day[66]. INNO-406 is also a potent inhibitor of LYN kinase, and acquired imatinib resistance might be mediated in part through the overexpression of LYN, but it has no effect on SRC kinase activity[55,66]. The fact that INNO-406 is a selective inhibitor of LYN kinase and not a broad Src-family kinase inhibitor suggests that it might show less toxicity compared with the broad Src-family inhibitors, but clinical data on this inhibitor have not yet been reported.

AZD0530. AZD0530 (AstraZeneca) is an orally available inhibitor of ABL and the Src-family kinases, developed as an anti-metastatic and anti-invasive agent for solid tumours, although it could be useful for leukaemias as well. AZD0530 has nanomolar activity against Src-family kinases and weakly inhibits BCR-ABL, with high selectivity against a panel of tyrosine and serine/threonine protein kinases in isolated enzyme assays[67,68]. It is currently unclear as to whether or not this drug will be developed for either CML or ALL.

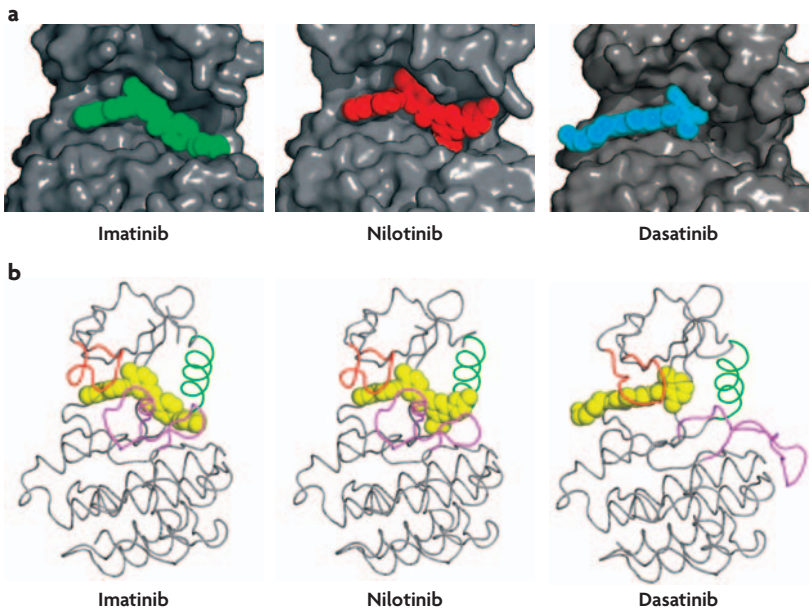

Figure 3 | **Structure of ABL in complex with imatinib, nilotinib and dasatinib.** **a** | Surface representations of crystal structures of ABL kinase in complex with imatinib (green), nilotinib (red) and dasatinib (blue). Residues from the nucleotide-binding loop (P-loop) and activation loop (A-loop) are omitted from the surface calculation for clarity. **b** | Comparison of the different binding modes of three ABL inhibitors: imatinib (left), nilotinib (middle) and dasatinib (right). The positions of the P-loop (red) and A-loop (magenta) vary according to whether the kinase is in an active conformation, in which the P-loop adopts an extended conformation and the N-terminal end of the activation loop adopts a 'DFG-in' conformation (right) or an inactive conformation, in which the P-loop is bent over the inhibitor and the N-terminal end of the activation loop adopts a 'DFG-out' conformation (left and middle). Imatinib and nilotinib block the kinase in an inactive conformation. The green helix is helix C, which often moves between the active and inactive states of kinases.

Non-ATP-competitive inhibitors of BCR-ABL

A potential alternative approach to ATP-competitive BCR-ABL inhibition is to use molecules that inhibit the kinase activity either by a non-ATP competitive allosteric mechanism or by preventing the binding of substrates to the kinase. This strategy has the advantage that the imatinib-resistant mutants are unlikely to be resistant to such inhibitors, owing to the different binding sites.

ON012380 (Onconova Therapeutics) inhibits the proliferation of BCR-ABL and mutant BCR-ABL-expressing cells with an IC_{50} in the nanomolar range[69]. It also inhibits the proliferation of a range of other cells dependent on other protein kinases. It has been speculated that the compound interacts with the substrate-binding sites of these kinases, perhaps through a covalent interaction. However, the drug has not yet entered clinical trials.

High-throughput screening for inhibitors of BCR-ABL-dependent cell proliferation resulted in the identification of a lead compound that was subsequently modified to give 3-[6-[[4-(trifluoromethoxy)phenyl]amino]-4-pyrimidinyl]benzamide (GNF-2) as a prototype inhibitor, which bound to the myristoyl binding site of BCR-ABL, resulting in the allosteric inhibition of ABL tyrosine kinase activity[70]. GNF-2 inhibits the proliferation of Ba/F3 cells transfected with p210 non-mutated BCR-ABL (BOX 1), as well as with the E255V and M351T mutant forms of the enzyme, and presents an intriguing lead molecule for the design of allosteric inhibitors of BCR-ABL, which could conceivably inhibit the activity of imatinib-resistant BCR-ABL kinase-domain mutants.

Aurora kinase inhibitors

The Aurora family of serine/threonine kinases is essential for mitotic progression. Aurora A has a crucial role in mitotic spindle formation and centrosome maturation, such that the inhibition of Aurora A kinase activity disrupts cell-cycle progression. Aurora B is a chromosomal passenger protein essential for chromosomal congression and cytokinesis. It is associated with centromeres during prometaphase and with the spindle midzone during anaphase and telophase. In normal tissues, the expression of Aurora C kinase is predominantly restricted to germ cells, although the function of this enzyme remains unclear[71].

MK-0457. MK-0457 (Merck; originally developed by Vertex Pharmaceuticals as VX-680) is an Aurora kinase inhibitor in clinical development for the treatment of cancer. In addition to being a potent inhibitor of all three Aurora kinases and FLT3 in the nanomolar range, MK-0457 is also a moderate to strong inhibitor of other kinases, including ABL and JAK2, which are relevant targets for a range of myeloproliferative disorders[72,73]. MK-0457 also inhibits the autophosphorylation of T315I mutant BCR-ABL in transformed Ba/F3 cells with an IC_{50} of ~5 μM, although it inhibits cell proliferation at submicromolar concentrations[74]. In phase I clinical trials, MK-0457 has been evaluated as a 5-day intravenous infusion (20 mg per m^2 per hour, delivering plasma levels of 1–3 μM), administered every 2–3 weeks to patients with a broad range of relapsed or refractory leukaemias, including patients with CML and Ph^+ ALL[73]. This treatment regimen is well-tolerated, with mucositis being one of the few reported side-effects, and has shown efficacy in patients with highly refractory CML, including some who express BCR-ABL with the T315I mutation. Efficacy seems to correlate with the level of phosphorylation of CRKL, a downstream element in the BCR-ABL signalling pathway. A phase II study has been initiated to evaluate the efficacy of MK-0457 in patients with CML and Ph^+ ALL who carry the T315I mutation, and in patients resistant or intolerant to second generation BCR-ABL inhibitors.

PHA-739358. PHA-739358 (Nerviano Medical Sciences) is an orally bioavailable inhibitor of Aurora kinases A, B and C that has potent anti-proliferative activity on a wide range of cancer cell lines, and significantly inhibits tumour growth in different animal tumour models at well-tolerated doses[75]. Following successful phase I clinical trials, this compound is currently being studied in a phase II clinical trial in patients with CML who have relapsed after imatinib therapy.

Resistance mutants detected with new agents

In patients with CML and Ph^+ ALL, drug resistance can develop to new BCR-ABL inhibitors, in the same way as for imatinib, through the evolution of point mutations that disrupt drug binding to the ABL kinase domain.

Therefore, as it is expected that resistance to second generation inhibitors will eventually present a challenge in the treatment of imatinib-resistant patients, investigators have begun to search for and characterize BCR-ABL point mutations that confer resistance to the new inhibitors.

Nilotinib. Nilotinib-resistant mutations that had some overlap with imatinib-resistant point mutants were identified in a cell-based resistance screen[76]. In contrast to imatinib, with which 26 changes were observed affecting 21 amino acid residues at concentrations of up to 4,000 nM, resistance to nilotinib at concentrations of up to 400 nM was associated with a limited spectrum of nine BCR-ABL kinase mutations (G250E, Y253H, E255K/V, E292V, T315I, F359C, L384M and L387F) affecting eight residues. With the exception of T315I, all of the mutations that were identified were effectively suppressed when the nilotinib concentration was increased to 2,000 nM, which is usually achieved in patients treated with nilotinib at 400 mg twice a day. Results of other mutagenesis screens similarly uncovered BCR-ABL point mutations resistant to nilotinib[77,78]. Therefore, in an *N*-ethyl-*N*-nitrosourea (ENU)-induced cell-based mutagenesis study, ten mutants emerged (L248V, G250E, Y253H, E255K/V, E292V, T315I, F359C, L384M and L387F) affecting nine residues[77]. In phase I trials for nilotinib, 20 different ABL mutations were observed in 37 out of 91 patients who underwent baseline assessment for mutational status. However, nilotinib was active in patients with and without mutations, and there were no significant differences in the response rates between the two groups[51,79]. Drug-resistant mutations can be detected at low levels in patients, but for some reason these do not always expand to drive the disease, and the patients continue to respond to the drug.

Dasatinib. Preclinical results of mutagenesis screens have uncovered BCR-ABL point mutations resistant to dasatinib that show some overlap with imatinib- and nilotinib-resistance profiles[60,77,78,80]. In the same ENU-induced cell-based mutagenesis study cited above, nine dasatinib-resistant mutants were identified (L248V, Q252H, E255K, V299L, T315I, F317I/C/L/V) affecting just six residues[77]. In phase I clinical trials for dasatinib, 60 of 84 study patients (71%) had BCR-ABL mutations detected at baseline. Haematological and cytogenetic responses were observed broadly across all BCR-ABL genotypes, with the exception of carriers of T315I, the single mutation predicted to confer cross-resistance to dasatinib and imatinib in preclinical studies. In two patients who did not respond to treatment, this mutation existed before treatment[61]. In chronic phase patients enrolled in phase II clinical trials for dasatinib, the proportions of haematological and cytogenetic responses were not different for patients with or without BCR-ABL mutations. However, there was a trend for better heamatological and cytogenetic responses for patients who harboured mutations that showed a relatively low IC_{50} for dasatinib in preclinical testing. As expected, the three patients with T315I did not show any response to dasatinib[80,81]. The fact that leukaemic cells expressing BCR-ABL mutations, such as V299L and T315I, can emerge to drive disease during dasatinib therapy indicates that the inhibition of the Src-family kinases is not sufficient to stop the proliferation of these cells. This notion is further supported by the fact that dual ABL and Src-family kinase inhibitors are inactive *in vitro* against cells that express T315I BCR-ABL, and therefore the inhibition of the Src family might not contribute towards efficacy in CML.

Potential strategies to circumvent resistance

The different binding properties of second generation ABL inhibitors, coupled with their distinct mutagenicity profiles, suggest significant potential for combinations of these inhibitors to prevent or suppress the emergence of drug-resistant clones in patients with CML. Saturation mutagenesis has shown BCR-ABL point mutations both unique to dasatinib and cross-resistant to imatinib[60]. The combination of imatinib and dasatinib was found to significantly reduce the occurrence of drug-resistant mutants, and highlights the benefit of combining two inhibitors with distinct binding properties to ABL: dasatinib binds to both the active and the inactive conformation of BCR-ABL, in contrast to imatinib, which preferentially binds to the inactive conformation (FIG. 3). A screening assay involving ENU-exposed BCR-ABL-expressing Ba/F3 cells showed 20 different mutations associated with imatinib, ten associated with nilotinib and nine associated with dasatinib[77]. Drug combinations proved to be effective in suppressing the expansion of drug-resistant clones, although cross-resistance was observed among the compounds for the T315I mutant[77]. Other studies focusing on the combination of imatinib with dual Src and ABL inhibitors, such as dasatinib, indicate that these compounds can improve the effects of imatinib against imatinib-resistant BCR-ABL point mutants (including Y253F, E255K and M351T); however, these combinations are inactive against the T315I mutant[58]. A cell-culture-based screen used to identify BCR-ABL mutations arising in response to the novel ABL inhibitor, PD166326, yielded mutations that conferred resistance to both PD166326 and imatinib; these mutant proteins were able to be inhibited by increasing the concentration of PD166326 (REF. 82). However, increasing the concentration of imatinib was not as effective in suppressing the mutants. The BCR-ABL and LYN dual tyrosine kinase inhibitor INNO-406 was found to inhibit six out of seven imatinib-resistant BCR-ABL mutants (with the exception of the T315I mutant)[83].

The future of therapeutics in CML

The discovery of the nature and prevalence of BCR-ABL mutations represents a true milestone, as does the impact of second-generation kinase inhibitors on imatinib-resistant disease by suppressing or preventing the emergence of these mutations. The advances in our understanding of resistance against BCR-ABL-targeted therapy could also have important implications for the development of new targeted treatments of other malignancies, such as lung cancer, in which the mutation of the epidermal growth factor receptor (EGFR) kinase imparts resistance to erlotinib.

Although we predict that protein tyrosine kinase inhibitors such as the second generation inhibitors nilotinib and dasatinib will significantly inhibit the progression of disease in patients with imatinib-resistant CML, the development of drug resistance presents a challenge. Although progress is being made towards the development of a 'global' pan-BCR-ABL inhibitor that inhibits the full spectrum of identified imatinib-resistant BCR-ABL point mutants (including T315I), the potential for the evolution of new drug-resistant point mutations in BCR-ABL at crucial points that influence drug binding, protein expression or protein activity continues to exist. This prediction justifies the continued development of more potent BCR-ABL inhibitors with their own unique mutagenicity profiles, as well as the continued use of more than one BCR-ABL inhibitor in combination. It also warrants the use of specific signal transduction inhibitors in combination with BCR-ABL inhibitors to achieve highly efficacious therapy with a reduced potential for the development of drug resistance.

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Competing interests statement
The authors declare competing financial interests: see web version for details.

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Ellen Weisberg received her Masters and Ph.D. in pharmacology from Albany Medical College, in Albany, USA. She did postdoctoral training at the Dana Farber Cancer Institute and Harvard Medical School, and is now an Instructor of Medicine at Harvard Medical School. She presently works in the laboratory of J. Griffin, and is actively involved in the development of drug therapies designed to target chronic myeloid leukaemia and acute myeloid leukaemia.

Paul W. Manley is the head of a Leukaemia Research Programme Team at the Novartis Institutes for BioMedical Research, Basel, Switzerland. Before joining Novartis he worked at Sandoz Pharmaceuticals, Basel, Switzerland, and Searle Research & Development, UK. He received his doctorate in organic chemistry from University of Liverpool, UK, in 1979. He has published over 80 papers on leukaemia and oncology drugs, medicinal and organic chemistry, and is co-inventor of over 50 patents. He is a member of the American Society of Hematology, the Swiss and American Medicinal Chemistry Societies and the Royal Society of Chemistry.

Sandra W. Cowan-Jacob**,** head of biomolecular structure, has been providing structural data to drive drug discovery at Novartis since 1993, lately specializing in kinase inhibitors for diverse indications. Before joining Novartis she did protein crystallography at Uppsala University in Sweden and then at Basel University, where she solved structures of integral membrane proteins. She was born in Australia and obtained her BSc and Ph.D. degrees in chemistry and biochemistry at the University of Melbourne and St Vincents Institute of Medical Research. She has published more than 50 scientific articles, mainly in the areas of structural biology and chemistry.

Andreas Hochhaus is professor of internal medicine, haematology and oncology at the Medical Faculty Mannheim of Heidelberg University, Germany. He has been involved in randomized studies of the German CML Study Group for more than 17 years, and has been an investigator for studies with imatinib, novel tyrosine kinase inhibitors, interferon-α and downstream signal transduction inhibitors. His special interests are the molecular monitoring of minimal residual disease and mechanisms of resistance in chronic myeloid leukaemia. He is a member of the the American Society of Hematology, the American Society of Clinical Oncology, the European Haematology Association, the International Association for Comparative Research on Leukemia and Related Disease and the German Society for Hematology and Oncology, and has published over 175 peer-reviewed papers.

James D. Griffin received his MD from Harvard Medical School, USA, in 1974. After residency training in internal medicine at Johns Hopkins Hospital, he completed a haematology fellowship at Massachusetts General Hospital and a medical oncology fellowship at the Dana Farber Cancer Institute (DFCI). In 1981, he joined the staff of DFCI, where he is now director of the Leukemia Program and chair of the Department of Medical Oncology. He also serves on the scientific advisory boards of the Lombardi Cancer Center at Georgetown University and the Johns Hopkins Cancer Center.

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TOC Blurb
Imatinib is a highly effective treatment for chronic myeloid leukaemia. However, patients often develop resistance to this ABL kinase inhibitor. This Review discusses second generation inhibitors of ABL and other signalling pathways that might help circumvent imatinib resistance.

Competing Interests
Paul W. Manley and Sandra W. Cowan-Jacob are employees of Novartis pharma AG, Switzerland. Andreas Hochhaus receives research funding from Novartis, Bristol-Myers Squibb, Wyeth, Merck and Innovive.