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Novel targeted therapies to overcome imatinib mesylate resistance in chronic myeloid leukemia (CML)

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Abstract

Imatinib mesylate (Gleevec) was developed as the first molecularly targeted therapy that specifically inhibits the BCR–ABL tyrosine kinase activity in patients with Philadelphia chromosome positive (Ph+) chronic myeloid leukemia (CML). Due to its excellent hematologic and cytogenetic responses, particularly in patients with chronic phase CML, imatinib has moved towards first-line treatment for newly diagnosed CML. Nevertheless, resistance to the drug has been frequently reported and is attributed to the fact that transformation of hematopoietic stem cells by BCR–ABL is associated with genomic instability. Point mutations within the ABL tyrosine kinase of the BCR–ABL oncoprotein are the major cause of resistance, though overexpression of the BCR–ABL protein and novel acquired cytogenetic aberrations have also been reported. A variety of strategies derived from structural studies of the ABL–imatinib complex have been developed, resulting in the design of novel ABL inhibitors, including AMN107, BMS-354825, ON012380 and others. The major goal of these efforts is to create new drugs that are more potent than imatinib and/or more effective against imatinib-resistant BCR–ABL clones. Some of these drugs have already been successfully tested in preclinical studies where they show promising results. Additional approaches are geared towards targeting the expression or stability of the BCR–ABL kinase itself or targeting signaling pathways that are chronically activated and required for transformation. In this review, we will discuss the underlying mechanisms of resistance to imatinib and novel targeted approaches to overcome imatinib resistance in CML.

Keywords: Tyrosine kinase inhibitor; Resistance; Imatinib mesylate; BCR–ABL; Chronic myeloid leukemia

1. Introduction

1.1. The BCR–ABL oncogene

The Philadelphia chromosome (Ph) was originally described as a chromosomal abnormality in 1960 by Nowell and Hungerford [1]. In 1973, Rowley reported that this abnormal chromosome, found in most patients with chronic myeloid leukemia (CML), has an apparent loss of the long arm of chromosome number 22 and is the result of reciprocal translocation involving the long arms of chromosomes 9 and 22, t(9;22) [2]. The molecular genetics of the Ph chromosome showed the *ABL* gene to be on the segment of chromosome 9 that is translocated to chromosome 22 [3]. Breakpoints in chromosome 22 were found to occur over a very short stretch of DNA (5–6 kb), termed the *breakpoint cluster region* (*BCR*) gene. The native c-ABL tyrosine kinase is located partially in the nucleus and has tightly regulated kinase activity. The *BCR–ABL* fusion results in the production of a constitutively active cytoplasmic tyrosine kinase that does not block differentiation, but enhances proliferation and viability of myeloid lineage cells. BCR–ABL is likely sufficient to cause CML, but over time other genetic events occur and the disease progresses to an acute leukemia. CML is typically characterized by phases of variable duration, starting with an initial chronic phase (CP), followed by progression to accelerated phase (AP) and finally resulting in blast crisis (BC) [4]. In CML, the classic fusion is b2a2 or b3a2 fusing exon 13 (b2) or exon 14 (b3) of BCR to exon 2 (a2) of ABL, leading to an oncoprotein of 210 kDa molecular weight. In the case of Ph positive acute lymphoblastic leukemia (ALL), there is fusion with the production of an oncoprotein of 190 kDa molecular weight. A third fusion gene (e19a2) was associated with the rare chronic neutrophilic leukemia, encoding a 230 kDa BCR–ABL protein. Each of these oncoproteins contains the same segment of ABL and differs in the amount of BCR present in the oncogene. In ALL, the p210BCR–ABL fusion protein is found in every third patient, p190BCR–ABL is found in two out of three patients [5], whereas p230BCR–ABL is associated with CMML [6,7]. Currently, it is not known how these three forms of BCR–ABL differ from each other in terms of signaling but additional genetic changes seem necessary to reach full disease activity [8]. In addition, increased length of the BCR portion in the fusion protein has been shown to correlate with reduced ABL kinase activity in vitro. BCR releases inhibitory constraints in the ABL kinase domain, leading to greatly increased ABL tyrosine kinase activity [9–11]. Cells expressing the chimeric BCR–ABL oncoprotein show signs typical of malignant transformation, including excessive cell growth of immature myeloid cells with lack of differentiation and inhibition of apoptosis [12]. The mechanisms through which BCR–ABL contributes to malignant transformation have been extensively studied by using BCR–ABL expressing murine or human cell models. Many of the activated signaling pathways are normally regulated by hematopoietic growth factors, such as c-kit ligand, thrombopoietin, interleukin-3, or granulocyte/macrophage-colony stimulating factor. Not surprisingly, it has been demonstrated that BCR–ABL activates a variety of signaling pathways and downstream targets like RAS, STATs, phosphatidylinositol-3′-kinase, production of reactive oxygen species (ROS) and others that can also be activated by hematopoietic growth factors. A major difference between activated growth factor receptors in normal signaling and BCR–ABL is the dysregulated activation of signaling pathways by BCR–ABL, inducing abnormal proliferation and neoplastic expansion. In addition, the BCR–ABL oncoprotein decreases apoptotic reaction to mutagenic stimuli, which results in a survival advantage to the neoplastic clones [13]. In many model systems, BCR–ABL completely abrogates growth factor dependence, and has been associated with reduced requirement for growth factors in primary hematopoietic cells. It is clear, however, that there are other activities of BCR–ABL that remain

poorly understood, in particular, the propensity for CML to evolve into blast crisis.

1.2. Targeting ABL with the small molecule drug imatinib mesylate

An improved understanding of the molecular mechanism involved in the development of CML has led the way for the development of targeted therapies. BCR–ABL is unique in the sense that it is both required and sufficient for transformation of hematopoietic stem cells, making it an ideal target for drug development. In 1996, Druker and colleagues in collaboration with Novartis Pharmaceuticals (formerly Ciba-Geigy, Basel, Switzerland) developed the experimental drug CGP57148B, which is today known as imatinib mesylate (imatinib, STI571, Gleevec®/Glivec®). Imatinib is an ABL tyrosine kinase inhibitor of the 2-phenylamino pyrimidine class that was created using the structure of the ATP binding site of the ABL protein kinase [14–16]. Imatinib binds to and stabilizes the inactive form of BCR–ABL rather than occupying the whole ATP-binding pocket as previously supposed [17]. Functionally, imatinib acts by revoking the effects of the BCR–ABL oncoprotein through inhibition of BCR–ABL autophosphorylation and substrate phosphorylation, inhibition of proliferation, and induction of apoptosis [14,18,19]. Initial preclinical studies indicated that imatinib inhibits the proliferation of CML progenitor cells in BCR–ABL positive, but not BCR–ABL negative cell lines. Targeted therapy with imatinib has led to a revolution in the treatment of CML. Druker et al. conducted a phase I clinical trial of this drug in 1998, whereby using doses of imatinib of 300 mg, 98% of patients with interferon-resistant CML in chronic phase achieved a complete hematologic response and 31% of these patients achieved a complete or major cytogenetic response. The following phase II study, involving a relative large number of patients, confirmed these impressive results. Further, a multicenter randomized trial showed that imatinib is superior to the combination of interferon-α plus low-dose cytarabine (Ara-C) as first-line treatment. With this treatment, 95% of patients in chronic phase can achieve a complete hematologic remission and 60% can achieve a major cytogenetic response. However, most patients in blast crisis either do not respond or relapse shortly after an initial response. In these cases, only 7% of patients treated with imatinib achieve a complete cytogenetic response [20]. Unfortunately, no survival benefit has been found for this patient group. Currently, imatinib is tested in large multicenter phase IV trials in combination with interferon-α or arabinosyl–cytosin or with an increased dose of 600 or 800 mg as monotherapy [21].

Generally, imatinib has been well tolerated in patients with CML, as well as in patients with other BCR–ABL positive leukemias [22]. Its toxicity profile is as favorable as that of hydroxyurea and superior to that of interferon-α [23]. Imatinib is well absorbed after oral administration with a mean absolute bioavailability for the capsule formulation of 98%. Following oral administration, the elimination half-lives of imatinib and its major active metabolite, the *N*-desmethyl derivative, are approximately 18 and 40 h, respectively [24]. The major human P450 cytochrome enzyme involved in the microsomal biotransformation of imatinib is CYP3A4 [24]. In addition, imatinib is a competitive inhibitor of CYP2C9, CYP2D6 and CYP3A5 in human liver microsomes in vitro. Imatinib may reduce CYP2D6- and/or CYP3A4/5-mediated clearance of co-administered drugs that are cleared through these pathways [25]. Plasma levels of drugs that are substrates for these cytochromes may increase if administered in conjunction with imatinib, due to competition for biotransformation pathways. Those that are enzyme inhibitors or inducers may increase or decrease the plasma levels of imatinib, respectively.

1.3. Additional activities of imatinib mesylate

Despite the apparent specific anti-leukemic effect of imatinib in CML through inhibition of the ABL protein kinase, imatinib also possesses inhibitory properties against other members of the tyrosine kinase family including PDGFRα/β and c-kit [15]. The inhibitory activity of imatinib is stronger against these kinases with an IC_{50} to inhibit proliferation of 30 and 100 nM, respectively, compared to the ABL IC_{50} value of 630 nM [8,26]. In addition, imatinib is a good inhibitor of the ABL related kinase ARG. The activation of tyrosine kinases has been found to be the causative event in the pathogenesis of a variety of BCR–ABL negative chronic myeloproliferative disorders, a heterogeneous spectrum of conditions for which the molecular pathogenesis is not completely understood. Coincidentally, PDGFR kinases are frequently found to be fused to multiple partner genes in reciprocal translocations, resulting in fusion proteins that are sensitive to imatinib treatment (Table 1). For example, FIP1L1-PDGFRα, a recently described fusion protein that is generated by a cytogenetically invisible interstitial deletion on chromosome 4q12, was identified in patients with hypereosinophilic syndrome (HES) and systemic mastocytosis (SMC) [27]. Consistent with the effect of imatinib on c-ABL and c-PDGFR, the IC_{50} of imatinib required to inhibit cells transformed by BCR–ABL was 582 nM [28] and cells expressing FIP1L1-PDGFRα fusion protein needed a lower concentration of only 3 nM [27]. There are also activating mutations within c-kit in systemic mastocytosis. The most frequent mutations occur in the phosphotransferase domain (e.g. D816V) or in the intracellular juxtamembrane coding region (e.g. V560G) which is in general more common for gastrointestinal stromal tumors (GIST) [16,29,30]. Of interest, imatinib has shown to be effective against wild-type and juxtamembrane mutant c-kit kinase, but has shown no effect on the activity of the D816V mutant [31]. Therefore, imatinib is unlikely to be a good candidate for the majority of patients with aggressive forms of systemic mastocytosis.

The hypothesis that deregulated tyrosine kinases are fundamental to the pathogenesis of chronic myeloproliferative disorders has been supported by the finding of a single acti-

Table 1
Tyrosine kinase fusion genes in chronic myeloproliferative disorders and their susceptibility to imatinib

Karyotype	Fusion gene	Imatinib-sensitive	References
t(9;22)(q34;q11)	*BCR–ABL*	+	–
t(9;12)(q34;p13)	*ETV6-ABL*	+	[178]
t(8;13)(p11;q12)	*ZNF198-FGFR1*	–	[179]
t(6;8)(q27;p11)	*FGFR1OP-FGFR1*	–	[180]
t(8;9)(p11;q33)	*CEP1-FGFR1*	–	[181]
t(8;22)(p11;q22)	*BCR-FGFR1*	–	[182]
t(7;8)(q34;q11)	*TIF1-FGFR1*	–	[183]
ins(12;8)(p11;p11p21)	*FGFR1OP2-FGFR1*	–	[184]
t(8;17)(p11;q25)	*MYO18A-FGFR1*	–	[185]
t(8;19)(p12;q13)	*HERVK-FGFR1*	–	[186]
t(9;12)(p24;p13)	*ETV6-JAK2*	–	[187]
t(8;9)(p21;p24)	*PCM1-JAK2*	–	[188]
t(9;12)(q22;p12)	*ETV6-SYK*	–	[189]
del(4q)	*FIP1L1-PDGFRA*	+	[27]
t(4;22)(p11;q11)	*BCR-PDGFRA*	+	[190]
t(5;12)(q33;p13)	*ETV6-PDGFRB*	+	[191]
t(5;7)(q33;q11)	*HIP1-PDGFRB*	+	[192]
t(5;10)(q33;q21)	*H4-PDGFRB*	+	[193]
t(5;17)(q33;p13)	*RABEP1-PDGFRB*	+	[194]
t(1;5)(q23;q33)	*PDE4DIP-PDGFRB*	+	[195]
t(5;17)(q33;p11)	*HCMOGT-PDGFRB*	+	[196]
t(5;14)(q33;q24)	*NIN-PDGFRB*	+	[197]
t(5;15)(q33;q22)	*TP53BP1-PDGFRB*	+	[198]
t(5;14)(q33;q32)	*KIAA1509-PDGFRB*	+	[199]

vating JAK2 point mutation in polycythemia vera, essential thrombocythemia and idiopathic myelofibrosis. Five research groups demonstrated independently and simultaneously a Val617Phe mutation in the pseudokinase domain of JAK2, leading to deregulated tyrosine kinase activity [32–36]. Nevertheless, it still needs to be demonstrated that this mutation is sufficient and/or required for the myeloproliferative phenotype in these patients. In addition, JAK2 does not appear to be sensitive to imatinib and therefore, it is unlikely that patients with this mutation will benefit from imatinib treatment.

To date, the only known curative treatment for CML is allogeneic bone marrow transplantation from matched donors, but the procedure is associated with a notable risk of morbidity and mortality. Imatinib has the potential to be a curative drug and can be used as a first line treatment in the management of CML. However, the long-term effects with imatinib and its ability to cure CML as a single agent are unknown. It is still unclear whether or not imatinib is sufficient to eradicate the disease; in addition, there is well-documented proof of primary or secondary resistance of leukemic cells to imatinib. The primary effect of imatinib on BCR–ABL expressing progenitor cells seems to be inhibition of proliferation rather than induction of apoptosis [37,38]. As imatinib holds the activity of BCR–ABL dormant, it is possible that a single drug may not be sufficient to completely eradicate the tyrosine kinase positive stem cells from the body. Despite successful results in patients with early stages of CML, the unsatisfying activity of imatinib in advanced stages of CML raised the need to identify novel drugs that are more potent against ABL and that are also effective in regard to resistant patients, particularly those with point mutations.

2. Mechanisms of resistance to imatinib

Resistance to imatinib can be defined as the lack of complete hematological response in patients with chronic phase disease or as a lack of return to chronic phase for patients in acute phase, in blast crisis CML, or with Ph positive ALL. Depending on the time of onset, two categories of resistance can be distinguished: If there is no response after initial treatment, resistance is described as primary or extrinsic. In contrast, secondary or intrinsic resistance is present if resistance develops after achieving an objective response. Development of resistance to imatinib in advanced phase CML is frequent, where over 70% of patients with acute leukemia evolve resistance within the first 6 months of treatment [39]. As opposed to patients in chronic phase disease, relapse after a complete cytogenetic response was reported to be exceptional [40]. However, longer follow-up studies of patients who are currently treated with imatinib are required to obtain full assessment of occurrence of resistance in chronic phase.

2.1. Imatinib binds to inactive BCR–ABL

Structurally, c-ABL contains an N-terminal SH3 domain, followed by an SH2 domain and a kinase domain. There is also a nuclear localization signal, a DNA binding domain and an actin binding domain in the C-terminal portion of the protein [41]. The mechanisms of regulation of the c-ABL and BCR–ABL tyrosine kinase activity and the influence of imatinib-binding to ABL have been intensively studied in the recent past (see also for review [42]). These data have offered valuable clues about the mechanisms of imatinib resistance and have given hope for the development of new drugs that might overcome such resistance. The c-ABL SH3 domain exercises autoinhibitory activity through intramolecular binding to a single proline residue between the SH2 and catalytic domains. Mutations of this SH3 domain or its binding site can result in increased catalytic activity of c-ABL [43]. Phosphorylation of Tyr412 in ABL, which is located in the kinase domain activation loop, induces phosphorylation of Tyr242. Phosphorylation at Tyr242 subsequently leads to displacement of the SH3 domain from its binding site, resulting in increased kinase activity [44]. Imatinib takes advantage of these structural requirements. Studies of the ABL–imatinib crystal structure revealed that the Tyr412 residue is not phosphorylated in the presence of imatinib-binding and the kinase is in an inactive conformation [17]. Phosphorylation of Tyr412 is associated with conformational changes, orienting this residue away from the catalytic pocket and leading to the active conformation of ABL [44]. Structural data from ABL in the active state in complex with the alternative ABL inhibitor PD173955 suggest that the active state may not be favorable for imatinib-binding [45]. The BCR–ABL fusion protein is likely to undergo a similar regulatory mechanism,

involving the retained SH3 domain as a regulator of the monomeric fusion protein. In the current model, the BCR portion of BCR–ABL releases inhibitory constraints, thus contributing to oligomerization. This process allows autophosphorylation of different residues including Tyr1127 (Tyr242 in c-ABL), causing the release of the SH3 domain from its binding site, which has been associated with increased catalytic activity. The BCR–ABL protein is likely to be present in equilibrium, changing between the active and the inactive conformation. Therefore, imatinib is expected to bind to the inactive conformation when BCR–ABL is monomeric and unphosphorylated and when the autoinhibitory SH3 domain is in contact with its binding site.

2.2. Point mutations in BCR–ABL decrease imatinib sensitivity

Potentially the most frequent clinically relevant mechanisms that change imatinib sensitivity in BCR–ABL transformed cells are mutations within the ABL kinase, affecting several of its properties. Point mutations can directly influence the proper binding of imatinib to the target molecule, as well as the binding of ATP. Furthermore, mutations can lead to conformational changes of the protein, affecting binding of either imatinib or ATP in an indirect way. Imatinib-resistant mutations are likely to be induced by imatinib itself, due to selection of BCR–ABL expressing clones that harbor the point mutation. In these particular cells, imatinib is unable to efficiently bind and thus permits a growth advantage due to lack of ABL kinase inhibition. This is consistent with the finding that resistance-mediating mutations can be found at very low levels in patients prior to clinical imatinib resistance. The first mutation detected in resistant patients was the exchange of the amino acid threonine for isoleucine at position 315 (T315I) of the ABL protein [46]. To date, more than 30 different point mutations encoding for distinct single amino acid substitutions in the BCR–ABL kinase domain have been identified in 50–90% of relapsed CML patients [46–50]. These mutations are more frequently observed in relapsed patients when compared to primary resistant patients [47,50]. As of now, rare additional alternative forms of imatinib-resistant mutations have been identified, including Y253F/H, G250E/R, E255K/V, Q252H/R, F311L/I and E355G [49,51]. Mutations of the kinase domain can reactivate the kinase activity of the BCR–ABL protein, leading to decreased sensitivity of imatinib by 3- to >100-fold. Mutations in BCR–ABL are more common in patients with BCR–ABL positive lymphoid leukemia (ALL, lymphoid blast-crisis CML) than in patients with myeloid leukemia.

The regions within BCR–ABL in which point mutations occur can be categorized into four groups: First, the P-loop (also known as first-loop) is a highly conserved, flexible, glycine-rich structure that normally acts as a nucleotide-binding loop for the phosphate groups of ATP. P-loop mutations are the most common and patients reported with these mutations have a particularly bad prognosis [50], though the reason for this remains unknown. Frequently affected changes of residues in this group include Y253F, G250E, E255K and Q252H [49,52,53]. Currently, the mechanism of how mutations at this residue cause imatinib resistance is not understood, as most of them seem to not be directly involved in imatinib binding [54]. At least some of these mutations may contribute to resistance by shifting the equilibrium of the active and inactive state of the ABL kinase, causing a push towards the active state [55]. A likely example of this are mutations at Y253, which cause the loss of a hydrogen bond with N322, therefore forcing the balance towards the active conformation [17]. Second, the T315 proximal region where the T315I and F317L mutations can occur contains non-conserved residues that directly regulate imatinib binding without significantly affecting the binding of ATP. Therefore, this region is required for the selective inhibition of the ABL kinase by imatinib [56]. The third region affects the catalytic domain that involves the residues 351–359, which can contain the M351T, E355G and F359V mutations. Interestingly, the M351T mutation results in a significant decrease in kinase activity [52]. The last group of mutations affects the activation loop (also known as A-loop), a region that connects the N- and C-terminal lobes of the kinase domain [57]. Mutations of residues located in this domain, such as H396R, V379I, and L378M, prevent the kinase from achieving the conformation required to bind imatinib. The decrease of imatinib sensitivity is heterogeneous and varies between the distinct mutations; investigators have observed ranges spanning from a minor increase of the median inhibitory concentrations of imatinib to a virtual insensitivity to imatinib.

2.3. Imatinib resistance by BCR–ABL gene amplification

Resistance to imatinib can also be caused by overexpression of the BCR–ABL protein due to gene amplification of the BCR–ABL gene. This mechanism was initially described in the LAMA84R cell line with a 4.6-fold increase in mRNA levels [58]. In contrast to some ABL point mutations that can lead to complete imatinib resistance, higher concentrations of imatinib were able to inhibit the function of the BCR–ABL oncoprotein in overexpressing cells. This mechanism of resistance is observed in a proportionally small number of imatinib-resistant patients [46] and can be detected by interphase fluorescence in situ hybridization using fluorescently labeled probes for BCR and ABL genes [46,47]. Interestingly, there are also some patients overexpressing BCR–ABL in the apparent absence of gene amplification [59], raising the possibility of the existence of additional, yet unknown mechanisms. CML patients with imatinib resistance due to BCR–ABL overexpression are likely to respond to increased concentrations of imatinib (see for review [60]).

2.4. Pharmacological mechanisms

Like most other small molecule drugs, imatinib needs to pass through the plasma membrane to reach its target protein.

In many cases of drug resistance, transmembrane proteins involved in ion transport across the plasma membrane (or pumps) have been implicated in mediating drug resistance. It appears that this mechanism plays a minor role in imatinib related resistance. For example, overexpression of the P-glycoprotein, which is encoded by the multidrug resistance *MDR-1* gene, has been frequently implicated in resistance to various chemotherapeutic drugs [61–63]. The P-glycoprotein acts as a pump that is able to clear the cell of soluble compounds by transporting them through the plasma membrane. The *MDR-1* gene is commonly overexpressed in blast cells of patients in the advanced stage CML. A BCR–ABL transformed cell line resistant to doxorubicin, due to *MDR-1* gene overexpression, was shown to grow continuously in the presence of 1 μM imatinib but died soon after adding the P-glycoprotein pump modulators verapamil or PSC833 at non-toxic concentrations [64,65]. However, the suggestion that *MDR-1* gene overexpression might confer resistance to imatinib mesylate in leukemic cell lines is controversial [66]. Another pharmacological mechanism that could contribute to imatinib resistance involves the A1-acid glycoprotein (AGP), which is present in the plasma and can bind to imatinib, thus possibly reducing its intracellular concentration at high plasma levels. Hence, mice treated with erythromycin, which binds to AGP have been shown to overcome such resistance [67]. However, the significance of resistance in these observations is still controversial and needs to be clarified in further studies. In addition, recent data suggest that imatinib inhibits the function of the ABCG2 (or BCRP protein). ABCG2 transports certain drugs such as the quinoline-based camptothecins out of the cells, thus causing drug resistance in human cancer [68,69]. Imatinib may reverse ABCG2-mediated resistance, but has not been shown to be an ABCG2 substrate for efflux by itself. This important function requires additional consideration when combining imatinib mesylate with other anticancer cytotoxic agents that are putative ABCG2 substrates. Nevertheless, data by Burger et al. suggest that imatinib levels are significantly decreased in ABCG2-overexpressing cells and that ABCG2-mediated efflux can be reversed through a specific ABCG2 inhibitor [70]. Thus, the role of imatinib as an ABCG2 substrate needs further evaluation. The interaction of imatinib with ABCG2 may influence its gastrointestinal absorption and play a role in cellular resistance to imatinib. It seems apparent that ABL point mutations are a more prevalent mechanism of imatinib mediated drug resistance when compared to mechanisms involving drug pumps. The type and incidence of mechanisms causing resistance may become more common with novel targeted therapies geared to overcome imatinib resistance.

2.5. BCR–ABL-independent cytogenetic aberrations in imatinib resistance

BCR–ABL has been associated with genomic instability, which may have particular relevance during disease progression from chronic phase to accelerated and blast phase CML. There is a great body of literature describing additional mutations in Ph+ cells, however there does not appear to be a particular mutation in addition to the t(9;22)(q34;q11) translocation that drives transformation in any specific way. It could be possible that accumulation of additional mutations may be sufficient for transformation by themselves, independent of BCR–ABL. For example, one study involving 36 patients with paired cytogenetic analyses (pretherapy/refractory or resistant disease), 19 patients showed chromosomal aberrations in addition to the Philadelphia chromosome translocation [47]. These alterations included aneuploidy in thirteen patients, alteration of the short arm of chromosome 17 leading to the loss of one p53 allele in seven patients and new reciprocal translocations in two patients. Furthermore, eight patients showed multiple cytogenetic aberrations. Of particular relevance to genomic instability is the fact that transformation of hematopoietic cells with BCR–ABL results in an increase in reactive oxygen species compared to quiescent, untransformed cells [71]. Increased production of reactive oxygen species has the potential to cause a transforming phenotype itself [72] and is likely to support transformation by acting as second messengers to regulate activities of redox-sensitive enzymes. An increase in ROS can have long-term consequences for genetic stability. ROS are quenched not only by enzymes, antioxidants and sulfhydryl groups, but also by reacting with DNA bases [73]. Recent data by Skorski's group suggest that elevated ROS levels are sufficient to induce DNA damage in BCR–ABL transformed cells [74].

3. Novel ABL kinase inhibitors targeting imatinib resistant BCR–ABL

The expanded knowledge of the different mechanisms of imatinib resistance has helped to devise strategies to solve these problems. One goal is to identify new compounds that bind to and inhibit the ABL kinase but are less affected by point mutations through their static conformation. In particular, the crystal structure analysis of the ABL–imatinib complex [17] has proven helpful in identifying potential critical residues that hinder interaction of imatinib with mutated ABL. One might also presume that potency of inhibition could be increased by identifying compounds that can bind to the active and inactive ABL kinase conformation. Another strategy has been to target the substrate binding pocket in ABL. We will summarize recent findings on three novel drugs as well as a variety of dual-specific Src/ABL inhibitors that have been developed using different strategies.

3.1. AMN107, a cousin of imatinib

Recently, several preclinical studies have shown promising results for the second generation ABL inhibitor AMN107, developed by Novartis Pharmaceuticals (Basel, Switzerland). Imatinib was developed through rational drug design, and based on it success, the structurally related anilio-pyrimidine

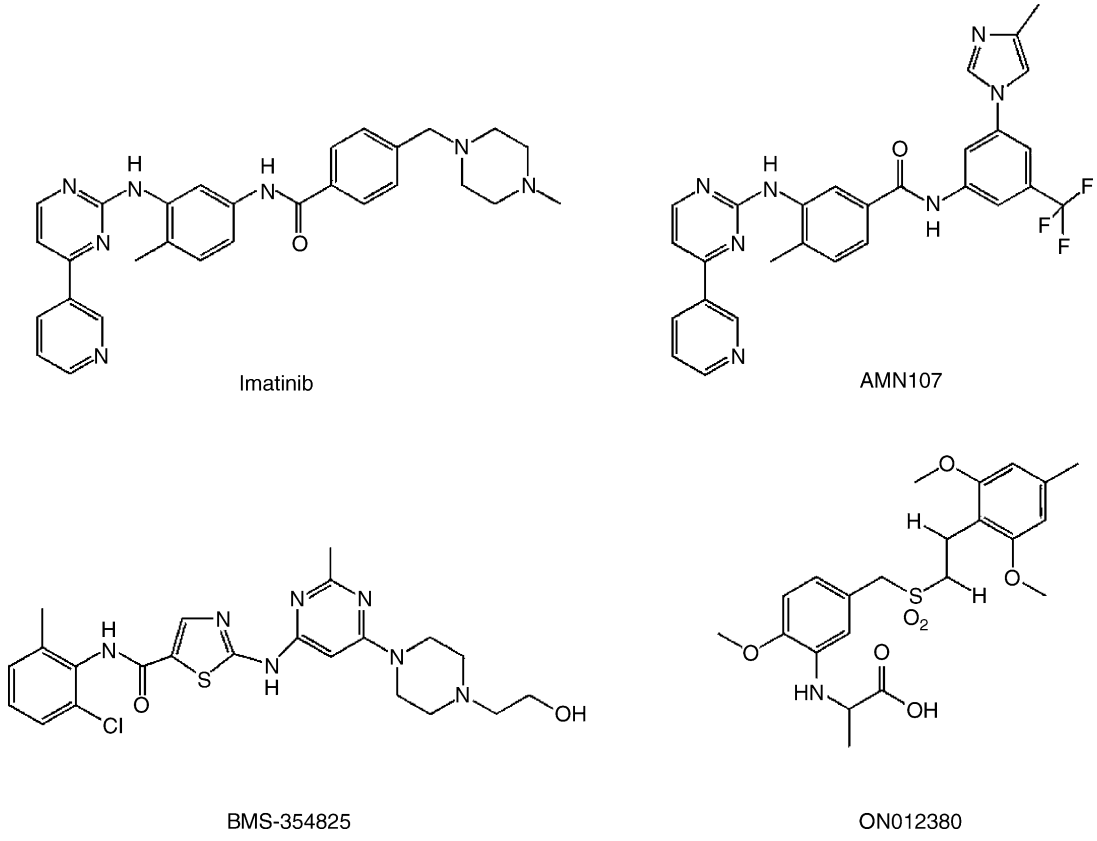

Fig. 1. ABL tyrosine kinase inhibitors. Chemical structure of the ABL tyrosine kinase inhibitors imatinib, AMN107, BMS-354825 and ON012380.

derivative, AMN107, was created (Fig. 1) [75]. Due to this structural similarity between the two compounds, AMN107 also requires the ABL protein to be in the inactive conformation for optimal binding. The pharmacological profile of AMN107 towards wild-type BCR–ABL protein and several imatinib-resistant BCR–ABL proteins with point mutations has already been established (Table 2). Using numerous BCR–ABL transformed hematopoietic cell lines, AMN107 was found to be 10–25-fold more potent compared to imatinib in reduction of both autophosphorylation and proliferation [75]. Inhibition of cell growth was associated with induction of apoptosis. As expected, AMN107 also effectively inhibits autophosphorylation of BCR–ABL on Tyr177, an important binding site for the Grb2 adapter protein. Tyr177 is involved in BCR–ABL pathogenesis through the regulation of diverse signaling pathways, including activation

Table 2
Inhibition of proliferation by imatinib, AMN107, BMS-354825 and ON012380 in cell lines

	AMN107	Imatinib	BMS-354825	Imatinib	ON012380	Imatinib
p210-wt	25	334	1.3	323	9	98
M244V	39	3100	n.t.	n.t.	8	n.t.
L248R	919	>20000	16	>10000	n.t.	n.t.
G250E	219	4800	n.t.	n.t.	6	> 10000
Q252H	16	2900	n.t.	n.t.	7	n.t.
Y253H	751	17700	10	>10000	8	∼10000
E255K	566	>6000	13	8400	8	> 10000
E255V	681	6368	n.t.	n.t.	8	n.t.
V299L	n.t.	n.t.	18	540	n.t.	n.t.
T315A	n.t.	n.t.	125	760	n.t.	n.t.
T315I	>10000	>10000	>1000	10000	8	> 10000
F317L	80	1583	18	810	8	n.t.
F317V	n.t.	n.t.	53	350	n.t.	n.t.
M351T	33	1285	n.t.	n.t.	8	n.t.

The IC_{50} values (ng/mL) are shown for inhibition of cell proliferation in various cell lines transformed by wild-type (wt) BCR–ABL or imatinib-resistant BCR–ABL proteins with a single amino acid substitution (as indicated) in response to imatinib, AMN107 and BMS-354825 [75,82,200]. Some of the cell lines were not tested (n.t.) for a particular drug and there may be partial variability in the sensitivity of the different cell lines generated by various investigators.

of phosphatidylinositol-3 kinase (PI3K) and Ras/Erk [76]. Phosphorylated Tyr177 and its associated proteins were also identified as key lineage determinants and regulators of the severity of BCR–ABL transformation [76]. Treatment of BCR–ABL expressing K562 cells with 10 nM doses of AMN107 resulted in down-regulated phosphorylation of the BCR–ABL autophosphorylation site Tyr177 [77]. It has been suggested that the enhanced potency of AMN107 compared to imatinib is due to its higher affinity to the ABL kinase pocket [75]. This is consistent with the AMN107-ABL crystal structure, indicating a better topographical fit of AMN107 to ABL due to the somewhat increased contact with the binding surface [75].

Cell lines expressing the imatinib-resistant BCR–ABL mutants M351T, F317L and E255V can be inhibited by AMN107. However, higher doses are needed to obtain the same inhibition as observed in their wild-type counterparts. Another study determined intermediate sensitivity for the E255KV, L248R and Y253H BCR–ABL mutants with IC_{50} values over 500 nM [75]. AMN107 was not effective against the T315I and G250E BCR–ABL mutants. Of particular interest is that the T315I mutant of BCR–ABL remained resistant to AMN107 concentrations up to 10 μM [75]. The frequently occurring Y253F, F311L, M359V, and H396P/R mutations have not yet been tested for AMN107-mediated inhibition. At AMN107 concentrations <100 nM there was no significant inhibition of normal myeloid progenitor cell growth in the in vitro models, indicating a lack of general toxicity. Like imatinib, AMN107 is also an inhibitor of the tyrosine kinases PDGFRA, PDGFRB and c-kit as demonstrated in A31 and GIST882 cells. However, in contrast to imatinib, AMN107 is most effective against ABL with an IC_{50} of 25 nM and is therefore approximately 20-fold more potent than imatinib. The increased efficacy of AMN107 towards wild-type BCR–ABL is not carried over to PDGFR (57 nM) or c-kit (60 nM), making AMN107 a similarly effective inhibitor of those kinases compared to imatinib.

Unfortunately, resistance to AMN107 induced inhibition of cell growth and viability in several BCR–ABL expressing cells has already been observed at concentrations of up to 100 nM AMN107. The molecular mechanisms underlying resistance still need to be determined [78]. Additional studies to determine if AMN107 resistance differs from imatinib resistance are currently underway. Other approaches evaluated the aforementioned pharmacological mechanisms of imatinib resistance in AMN107 treated cell lines. MDR1 over-expressing cells that were originally obtained from a 3-year-old child with T-ALL were exposed to either AMN107 or imatinib. Results indicated a 3.7-fold elevation of AMN107 levels compared with imatinib, suggesting that AMN107 might be less affected by MDR1 driven resistance than imatinib [79]. In this context, it is of note that imatinib treatment has been shown to correlate with decreased interstitial fluid pressure (IFP), causing a potentially improved uptake of anti-cancer drugs [80]. Therefore, it would be of great interest to evaluate if AMN107 can cooperate with imatinib in inhibiting its target proteins by promoting the uptake of AMN107. Though both drugs target the same kinases and are structurally related, this does not necessarily mean that they behave in a competitive manner.

In vivo studies using mice with BCR–ABL-transformed bone marrow cells have confirmed the improved efficacy of AMN107 compared to imatinib. AMN107 significantly reduced the accumulation of leukemic cells in marrow, spleen, lymph nodes, and liver. AMN107 also prolonged survival of mice expressing wild-type BCR–ABL and the imatinib-resistant E255V and M351T mutant proteins [75]. Both BCR–ABL mutant expressing mice treated with AMN107 showed shortened survival in regard to the wild-type form, which is consistent with the fact that these mutants are 27- and 2-fold less sensitive to AMN107 than wild-type BCR–ABL. Consistent with the in vitro results, control mice (not expressing the BCR–ABL protein) did not show evidence of hematopoietic toxicity after treatment for 14 days with AMN107.

AMN107 has made its way into clinical testing. Phase I/II studies open to CML patients with accelerated or blastic phase disease and patients with Ph positive acute lymphoblastic leukemia are currently under way [81]. Initial results of these studies indicate hematologic response rates >50% as well as cytogenetic response in patients with acute phase and blast phase. The dose-limiting toxicity has not yet been defined, but AMN107 has been well tolerated at levels up to 1200 mg per day with only moderate side-effects. In conclusion, it seems that AMN107 has superior potency to imatinib as an inhibitor of BCR–ABL in vitro and in vivo. Furthermore, many imatinib-resistant BCR–ABL mutations might be effectively targeted by this new inhibitor, however, clones carrying the Y253H, E255V, and T315I mutations are all markedly resistant, even at very high in vitro doses. It may also be too early to draw conclusions about the safety, tolerability or general toxicity of AMN107.

3.2. BMS-354825, a novel ABL and SRC family tyrosine kinase inhibitor

The pyridol[2,3-d]pyrimidine BMS-354825 is a novel ABL-targeted small-molecule inhibitor (Fig. 1) developed by Bristol–Myers Squibb (Princeton, USA) that also shows activity towards Src kinases. Shah et al. recently demonstrated that BMS-354825 has up to 100-fold increased activity against the ABL kinase compared to imatinib and retains activity against 14 of 15 imatinib-resistant BCR–ABL mutants in vitro (Table 1) [82]. The T315I substitution is the only known BMS-354825 resistant mutant [82] and as discussed above, has been shown to be resistant to AMN107 treatment in preclinical studies. The crystal structure of the BMS-354825-ABL complex has revealed the necessary requirements for the interaction of the inhibitor with the ABL kinase. BMS-354825 binds to the ATP-binding site in a position that is similar to imatinib. The central cores of BMS-354825 and imatinib share overlapping regions, the dif-

ference being that they extend in opposite directions. Unlike imatinib and AMN107, which are only able to bind to the inactive conformation, BMS-354825 is able to bind to the active as well as to the inactive conformation of ABL. Furthermore, BMS-354825 makes fewer contact points with ABL than imatinib or AMN107 and places less stringent conformational requirements on the kinase. The finding that BMS-354825 is able to recognize multiple states of the enzyme and needs less contact points with ABL than imatinib may partially explain why its binding affinity to the ABL kinase is greater than imatinib [83].

BMS-354825 is a potent inhibitor of multiple Src-family members, including Lck, Fyn, Src, and Hck. The proliferation of cells overexpressing these family members can be inhibited at IC_{50} values of 0.5 nM [84]. Thus, BMS-354825 is a more potent inhibitor of Src kinases than of ABL. Furthermore, this drug has significant activity against c-kit and PDGFRβ, with IC_{50} for inhibition of kinase activity at values of 5 and 28 nM, respectively. As previously mentioned, activating mutations of c-kit have been associated with systemic mastocytosis and gastrointestinal stromal tumors. Whereas the wild-type c-kit kinase has been shown to be imatinib-sensitive, the most common mutation in systemic mastocytosis D816V, which is located in the kinase domain, leads to imatinib resistance. The less common V560G mutation in c-kit occurs in the intracellular juxtamembrane region of c-kit and has been reported to be more sensitive to imatinib compared to wild-type c-kit [31]. BMS-354825 was also shown to be effective on these c-kit mutations. The human mastocytosis leukemia cell line HMC-1^{560} containing the c-kit V560G mutant could be inhibited by an IC_{50} of 1–10 nM of BMS-354825 [85]. Autophosphorylation of the imatinib-resistant D816V c-kit mutation was also inhibited with an IC_{50} of about 100 nM BMS-354825. Cell growth could also be completely inhibited at 1 μM BMS-354825 in the HMC-$1^{560,816}$ cell line which harbors the V560G, as well as the D816V c-kit mutation [86]. The IC_{50} ranged from 0.1 to 1 μM in these experiments. In addition, the tyrosine phosphorylation of c-kit was significantly reduced in the presence of nanomolar concentrations of BMS-354825. These findings underline the likelihood that BMS-354825 may also overcome imatinib resistance in myeloproliferative diseases other than CML, including systemic mastocytosis.

The effect of BMS-354825 was also tested in mice that were injected with BaF3 cells expressing BCR–ABL wild-type, as well as the T315I and M351T point-mutants [82]. Only mice injected with BCR–ABL wild-type and M351T cells showed significantly prolonged survival, in contrast to mice injected with T315I cells.

In view of the promising in vitro and in vivo data, phase I trials with BMS-354825 to test the safety and efficacy of the treatment of imatinib-resistant CML are currently under way. These trials include patients with Ph+ CML in chronic phase with hematologic progression or intolerance when treated with imatinib. Primary results from these trials indicate that the drug (15–180 mg of BMS-354825 per day for 5–7 days per week) was well tolerated in 28 patients, except one, who developed a single episode of grade 4 thrombocytopenia [87]. The maximal duration of BMS-354825 treatment was 9 months. BMS-354825 was reported to be absorbed rapidly and easily reaches serum levels above the required in vitro concentration for blocking CML cell proliferation. In another study, a pool of 26 patients including 22 patients with imatinib resistance and 4 with imatinib intolerance with an average disease duration of 6.1 years, were treated with doses of 35 mg per day or greater. All of the patients experience clinical benefit and 19 (73%) had a complete hematologic response. Seven patients responded partially and of those two endured disease progression. Interestingly, one of the patients with disease progression was identified as having the imatinib-resistant T315I mutation in BCR–ABL. These results are in agreement with the in vitro studies, in which cells harboring the T315I mutation in the BCR–ABL oncoprotein could not be inhibited by BMS-354825, imatinib or AMN107 [49,75,82]. Monitoring patients with CML has become an important aspect in disease management to observe treatment response and early detection of relapses. Quantitative real-time PCR correlates well with cytogenetic response in patients treated with imatinib and represents a useful tool for monitoring disease progression or response to treatment [88]. A recent study with 13 patients resistant or intolerant to imatinib with chronic phase disease were found to show a cytogenetic response after BMS-354825 treatment. Their response correlated with a two-log-reduction in BCR–ABL transcript levels as determined by quantitative PCR [89]. Overall, these early findings suggest that BMS-354825 may inhibit ABL at lower concentration than imatinib and has the potential to overcome some forms of imatinib-related drug resistance. However, it still needs to be evaluated if the inhibitory effect of BMS-354825 towards other tyrosine kinases may have detrimental effects in vivo or if this supports its promising efficacy.

3.3. ON012380, a substrate binding site ABL inhibitor

Unlike imatinib, the ABL inhibitor ON012380 (Fig. 1) was specifically designed by Onconova Therapeutics (Princeton, USA) to block the substrate binding site rather than the ATP binding site [90]. This strategy has the advantage in that the previously described imatinib-resistant mutants are unlikely to be resistant to this inhibitor, due to the different binding sites (Table 1). In vitro studies confirmed this assumption and ON012380 was able to inhibit both wild-type and all imatinib-resistant kinase domain mutations, even the problematic T315I mutation with an IC_{50} of less than 10 nM. Interestingly, ON012380 also has activity against the PDGFR kinases and the Src family member Lyn with IC_{50} values for inhibition of proliferation of approximately 80 nM. However, inhibition of the c-kit kinase is more weakly pronounced with an IC_{50} value of 446 nM [90]. ON012380 works synergistically with imatinib in wild-type BCR–ABL inhibition, which is not unexpected since these two drugs bind to different sites on the ABL kinase. ON012380 showed a 10-fold stronger

inhibition of wild-type BCR–ABL compared to imatinib. In addition, ON012380 was similarly effective against all 17 tested imatinib-resistant ABL kinase domain mutants with an IC_{50} of less than 10 nM in the 32Dc13 cell line. These findings were confirmed through in vivo experiments by injecting nude mice with 32Dc13 cells expressing imatinib-resistant BCR–ABL with the T315I mutation [90]. After 7 and 14 days, mice that were treated with ON012380 showed a significantly lower cell count of leukemic cells expressing the T315I mutant than imatinib- or saline-treated mice. Moreover, doses of 300 mg/kg of ON012380 produced no signs of toxicity in mice, suggesting a very desirable safety profile. This new compound shows encouraging results, particularly in its ability to inhibit the BCR–ABL T315I mutant. However, this small molecule drug has not yet entered clinical trials where it must prove its safety in use, efficiency in achieving molecular remission and preventing the appearance of imatinib resistance.

3.4. Additional Src family tyrosine kinase inhibitors with ABL inhibitor activity

The activity of Src tyrosine kinases has long been known to be elevated in the presence of the BCR–ABL oncoprotein [91–93]. However, recent genetic evidence by Hu et al. suggests that Src kinases may play a minor role in early CML. Using mice with disruption of the *Lyn*, *Hck* and/or *Fgr* genes, Hu et al. investigated the role of these Src family kinases in the development of a myeloid or lymphoid leukemia induced by BCR–ABL [94]. Lack of only one kinase had no effect, but deletions of at least two of these three Src genes extended disease latency and survival in these mice, exhibiting the symptoms of an ALL-like disease, but not a CML-like disease. Therefore, Src kinases may be essential to BCR–ABL mediated induction of ALL, but not CML. Although the amino acid sequence in the imatinib binding pocket of ABL closely resembles the homologous sequence in Src family kinases, these kinase are not inhibited by imatinib at clinical doses [14]. It has been hypothesized that this might be due to differences in the conformation of the activation loop in the inactive Src and ABL kinase [17]. Src inhibitors, including AZM475271, PP2 or AP-23236 have been previously tested in various cancers [95–97]. Recently, the pyridopyrimidine PD166326, a dual-specific ABL/Src inhibitor, has been shown to be effective against BCR–ABL kinase activity in vitro and in murine models [98].

PP1 and CGP76030 were originally characterized as Src kinase inhibitors. Later, they were found to also inhibit ABL kinase activity and thus are designated as dual Src/ABL inhibitors [99]. PP1 and CGP76030 induce growth arrest and apoptosis in BCR–ABL positive cell lines. Furthermore, mutations of the residue 315 of the ABL kinase did not completely disrupt the inhibitory effects of PP1 and CGP76030 on cell growth and survival. PP1 and CGP76030 bind to the imatinib binding pocket in close proximity to the ATP-binding pocket of the ABL kinase and share large overlapping binding modes with imatinib [100]. Even though both inhibitors can block ABL kinase activity, they were also shown to induce growth arrest and apoptosis independent of ABL inhibition. These ABL-independent effects may be due to inhibition of the Src kinases. This is reflected by a strong correlation of the effects of PP1 and CGP76030 on the growth of cells expressing imatinib-resistant BCR–ABL mutants with their ability to inactivate Src family kinases. A less complex binding mode of PP1 and CGP76030 may at least partly explain the somewhat broader target profile of these compounds [101,102]. PP1 and CGP76030 may represent valuable compounds for treatment of advanced or imatinib-resistant Ph+ leukemias. However, it has not yet been determined if these compounds are sufficiently specific and safe for clinical treatment.

Similar to PP1 and CGP76030, the pyrido[2,3-d]pyrimidine compound PD166326 was originally described as an inhibitor of the receptors for fibroblastic growth factor (FGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF) as well as a Src kinase inhibitor [103–105]. Additional related compounds, PD180970 and PD173955 have already been identified as potent inhibitors of the ABL and BCR–ABL tyrosine kinases by in vitro assays [106–108]. Recently, anti-leukemic activity of PD166326 against BCR–ABL wild-type and imatinib-resistant forms in cell lines and in murine models has been evaluated [98]. In these studies PD166326 was determined to be almost 100-fold more potent in inhibiting BCR–ABL than imatinib using a BCR–ABL transfected cell line model. In addition, PD166326 was shown to be 6.8 times more potent than imatinib in blocking c-kit mediated proliferation and showed increased activity against Lyn. This may be of potential interest, considering that acquired imatinib resistance may be mediated in part through overexpression of Src family members, in particular Lyn [109,110]. PD166326 is also more potent than imatinib in murine CML models. Oral application of PD166326 was well tolerated and quickly reached concentrations sufficient to inhibit BCR–ABL kinase activity in these models [98]. PD166326 prolonged the survival of mice with a CML-like myeloproliferative disorder and was also superior to imatinib in controlling the peripheral blood granulocytosis and splenomegaly. PD166326 was also more effective against the M351T and H396P mutations in BCR–ABL, but not towards the T315I mutation. However, after up to 4 weeks of treatment there were still BCR–ABL positive leukemic clones detected in all animals including the wild-type form, indicating that not all BCR–ABL expressing clones were eradicated.

4. Targeting BCR–ABL-dependent signaling pathways required for transformation

Despite the identification of many different signaling pathways activated by BCR–ABL, it has been difficult to link any single signaling event to a specific biologic effect. In addition, there may be considerable redundancy in these activities.

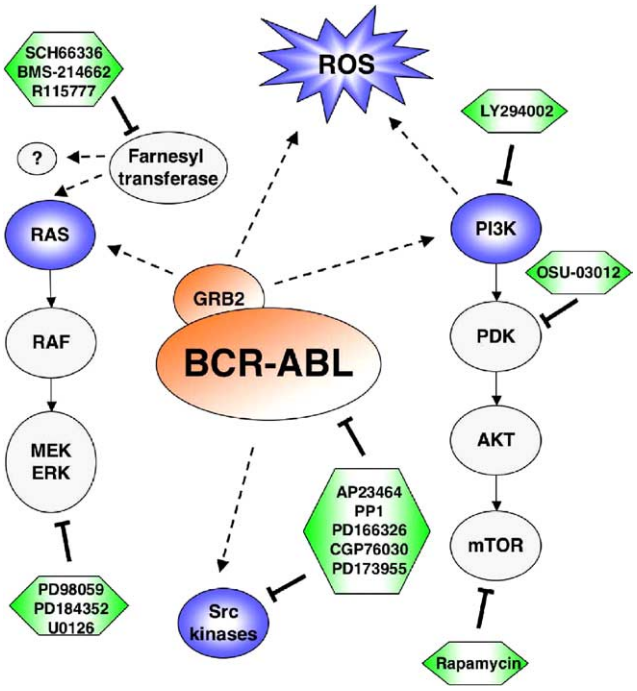

Fig. 2. Targeting signaling pathways of BCR–ABL. The BCR–ABL oncoprotein chronically activates many different downstream signaling pathways to confer malignant transformation in hematopoietic cells. For example, efficient activation of PI3K, Ras and reactive oxygen species requires autophosphorylation on Tyr177, a Grb2 binding site in BCR–ABL. Also, activation of Src family tyrosine kinases have been implicated in the BCR–ABL related disease process. A selection of some of the inhibitors and pathways discussed are depicted. There are additional important signaling pathways and inhibitors that have been shown to overcome imatinib-related drug resistance, which are not shown here.

Many BCR–ABL chronically activated signaling pathways, including the PI3K, STATs and RAS pathway or the induction of reactive oxygen species have been shown to be critical for transformation. Several of the current approaches to overcome imatinib resistance take advantage of this fact by using drugs that inhibit pathways contributing to malignant transformation of neoplastic clones, rather than using ABL inhibitors. Some of these strategies and novel drugs are summarized and discussed (Fig. 2).

4.1. Targeting the Ras pathway

The Ras protein is a well-studied proto-oncogene that is mutated in approximately 30% of all human cancers [111]. Also, hematologic malignancies are frequently affected by this mutations, ranging from 5% in ALL patients to up to 65% of patients with chronic myelomonocytic leukemia [111,112]. The activation of Ras is dependent on SOS and Ras-GAP. In CML cells, evidence has been found for constitutive Ras activation and reduced Ras-GAP activity [113]. A major pathway that leads to activation of Ras in BCR–ABL transformed cells requires autophosphorylation of BCR–ABL on Tyr177 and binding of Grb2 [114,115]. The constitutive activation of Ras in BCR–ABL transformed cells leads to the stimulation of a pathway that is required but insufficient for transformation [116]. Thus, here may not be sufficient pressure to select for clones with activating mutations, such as in lung or breast cancer, due to chronic activation of Ras [115,117]. Inhibition of Ras itself or its downstream intermediates may represent not only an excellent target to overcome imatinib resistance, but also may help in the treatment of other hematologic malignancies or cancers.

4.1.1. Farnesyl transferase inhibitors

Farnesyl transferase inhibitors (FTIs) were initially designed to primarily block the post-translation farnesylation of Ras, which is required for its activation Farnesylation is a crucial step in the post-translational modification of Ras, which contributes to complete Ras protein localization to the inner plasma membrane. This initial farnesylation step is sufficient to confer transforming potential [118]. Consistent with Ras as a frequent target of activating mutations and due to its important role in transformation, FTIs have shown significant anti-tumor activities in a variety of cancers [119,120]. Nevertheless, it became clear that FTIs also act by additional and more complex mechanisms that may extend beyond inhibition of Ras farnelysation, such as inhibiting Rho, lamin B, and other farnesylated proteins [121–124]. To date, the primary substrate of FTIs and its current impact for malignant transformation remain mostly unknown.

Despite these additional effects on multiple Ras-independent pathways, FTIs have been successfully tested in hematologic malignancies. Initially, FTIs were designed as peptidomimetics by containing both natural and non-natural amino acids. Unfortunately, these compounds have a short half-life due to proteosomal degradation. Non-peptidomimetic substances have shown to be less complicated in terms of bioavailability. There are several promising FTIs of this group that are under investigation in hematologic malignancies, including R115777 (Tipifarnib, Zarnestra), SCH66336 (Lonafarnib, Sarasar), and BMS-214662. For example, the orally administrated inhibitor R115777 demonstrated effectiveness against AML in first clinical trials, especially in older patients. R115777 was also active in CML as well as myelodysplastic syndromes [125–128]. Significant adverse effects in these studies included peripheral neuropathy, liver toxicity, and dose-limiting neurotoxicity or myelosuppression [126,129]. Combination treatment of imatinib and R115777 had a synergistic inhibitory effect on growth in several imatinib-resistant cell lines by induction of apoptosis and blockage of the cell cycle [130]. Another compound named SCH66336 has already been tested in phase I and II studies in patients with solid tumors where it was generally well tolerated [131,132]. In a p190BCR–ABL positive transgenic mouse model, SCH66336 was shown to suppress the progression of the disease. SCH66336 is also effective against BCR–ABL transformed cells expressing the T315I mutation in imatinib-resistant cells [133]. The combination of imatinib

with SCH66336 was shown to enhance the inhibitory activity of imatinib by inducing apoptosis of imatinib-resistant cells overexpressing the BCR–ABL protein. Due to gastrointestinal toxicities and in contrast to the two previously mentioned compounds, BMS-214662 is given intravenously and holds the most apoptotic potency of all known FTIs [134]. Currently, phase I and II studies are under way, including the combination of BMS-214662 with cisplatin and paclitaxel in hope for a possible synergistic effect which was suggested by preclinical data [135,136]. Future studies aimed to develop optimal dose schedules and potential combinations with other drugs will help to assess the value of FTIs in imatinib-resistant CML.

4.1.2. MAP kinase inhibitors

The protein serine/threonine kinase Raf is a downstream target of Ras and an important regulator of the MEK/ERK pathway. This pathway is constitutively activated by BCR–ABL. By using specific MEK/ERK inhibitors, a significant role in the regulation of anti-apoptotic mechanisms of this pathway could be established [137]. Previously, it was indicated that the MEK/MAPK activation is associated with apoptosis induction in BCR–ABL expressing cells that were treated with imatinib. Inhibition of the MEK/MAPK pathway in BCR–ABL expressing K562 cells is sufficient to induce apoptosis [138]. In another study, treatment with the specific MEK inhibitor U0126 led to reduction of survival in AML cell lines [137]. It also reduced expansion of CD34 positive progenitor cells in CML. Furthermore, the combination of U0126 with imatinib had a synergistic effect on these progenitor cells. Another specific MEK inhibitor is PD184352, which in combination with imatinib led to increased mitochondrial damage in K562 cells resulting in synergistic induction of apoptosis [139].

4.2. Targeting the PI3K pathway

The BCR–ABL protein leads to the activation of many downstream targets that have been shown to play important roles in malignant transformation. One of the major pathways constitutively activated by BCR–ABL and required for transformation involves PI3K. PI3K can phosphorylate phosphatidylinositol (PI) at the D3 position and in vivo produces mainly PI-(3,4)-bisphosphate and PI-(3,4,5)-trisphosphate, which may function as second messengers. A role for PI3K as a pleiotropic regulator of signal transduction pathways and biological functions includes in part the functional regulation of AKT (survival, growth), Rac (motility, survival), S6kinase (protein synthesis) and others. PI3K also regulates Ras and can be regulated by Ras itself [140,141]. We have shown that BCR–ABL regulates PI3K activity through Gab2, which forms a complex with Grb2. Phosphorylation of Gab2 and activation of PI3K is regulated through the Tyr177 autophosphorylation site in BCR–ABL [76]. Gab2 is a key regulator of PI3K in BCR–ABL transformed cells and is likely to be required for transformation in vivo.

4.2.1. Phosphatidylinositol-3′-kinase inhibitors

PI3K and its downstream targets, including the serine/threonine kinases AKT, mTOR and p70S6kinase, play a key role in the regulation of cell survival and proliferation. Its activity is regulated by BCR–ABL and is required for the growth of CML cells [142–144]. Recently, we showed that activation of the PI3K/mTOR pathway by BCR–ABL contributes to increased production of reactive oxygen species [145], thus linking PI3K/mTOR to mechanisms that have been implicated in genomic instability and imatinib resistance [74]. Signal transduction through this pathway can be blocked by inhibitors such as LY294002 or wortmannin, which specifically target PI3K. The cellular response to PI3K inhibition includes induction of apoptosis and inhibition of proliferation [146]. Imatinib was found to synergize with either wortmannin or LY294002 in the induction of apoptosis in BCR–ABL expressing cells [147]. Wortmannin and LY294002 were also effective in imatinib-resistant K562 by increasing the sensitivity to imatinib, suggesting that resistance to imatinib does not confer resistance to PI3K inhibition in this specific cell line model [148].

4.2.2. PDK1 inhibitor

Another approach focuses on the inhibition of the PI3K downstream target PDK1 [149]. OSU-03012 is a derivate of the cyclooxygenase-2 (COX-2) inhibitor celecoxib, however OSU-03012 is actually devoid of its COX-2 inhibitory activity. Instead, OSU-03012 belongs to the group of PDK1 inhibitors that have been demonstrated to deactivate AKT and induce apoptosis in transformed cells [150]. OSU-03012 was successfully tested on the imatinib-resistant mutants T315I and E255K in BaF3 cells as well as on regular BaF3 and 32Dcl3 cells where it showed no toxic effects with IC_{50} values of 4.8 and 4.4 μM, respectively [149]. In these cell models, OSU-03012 showed an anti-proliferative effect due to induction of apoptosis irrespective of BCR–ABL mutations and at IC_{50} values of 4–5 μM. It was suggested that there is no overlap in the underlying mechanisms of resistance between OSU-03012 and imatinib [149]. Moreover, augmented effects were observed when both mutated and non-mutated cells were treated with the combination of OSU-03012 and imatinib. Even though initial results look promising, this new inhibitor must first prove itself to be efficient and safe, in both animal models and for clinical use.

4.2.3. mTOR inhibitors

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that is located downstream of PI3K/AKT and which is involved in the regulation of cell survival, proliferation, growth and probably additional biological effects [151]. A major normal function of mTOR is to phosphorylate p70S6kinase (p70S6K) and initiation factor 4E-binding protein-1 (4E-BP1), both of which have been demonstrated to regulate mRNA translation. In CML cells, the translational regulators ribosomal protein S6K1 and 4E-BP1 are constitutively phosphorylated through increased

activity of mTOR [152]. The orally bioavailable macrolide rapamycin and its derivatives RAD001 (everolimus), CCI-779 and AP23573 bind to the immunophilin FKBP12, best known for its interaction with the immunosuppressant FK506. This complex inhibits mTOR, resulting in G1 cell cycle arrest. Furthermore, inhibition of mTOR leads to inactivation of ribosomal S6K1 and inhibition of cap-dependent translation initiation through the 4E-BP1/eIF4E pathway [153]. Imatinib has been shown to synergize with rapamycin in the inhibition of BCR–ABL transformed myeloid and lymphoid cells, especially in the inhibition of 4E-BP1 phosphorylation [154]. Rapamycin/imatinib combinations also inhibit imatinib-resistant mutants of BCR–ABL [154]. Remarkably, proliferation of the imatinib-resistant BCR–ABL T315I mutant was inhibited by rapamycin in treated BaF3 cells. The observed inhibition was further enhanced by combining low-dose rapamycin with imatinib in this BCR–ABL mutant as well as in the imatinib-resistant G250E and M351T BCR–ABL. The efficacy of this drug combination has already been tested in CML murine models showing that mice treated with a combination of imatinib and rapamycin exhibited significant prolonged survival when compared to those treated with either imatinib or rapamycin alone [154].

4.3. Additional targets

There are currently alternative targeted therapies being pursued, which may have the potential to be of great clinical importance. One useful approach is to combine the power of tyrosine kinase inhibitors with drugs targeting activated signaling pathways in hopes of obtaining a more effective treatment for drug-sensitive or drug-resistant cancers. For example, we have investigated the effect of combining the small molecule drug 2-methoxyestradiol with imatinib in BCR–ABL transformed cell lines [155]. The anti-angiogenic drug 2-methoxyestradiol binds to and destabilizes microtubules in vitro and in vivo. We found imatinib-resistant cells to be sensitive to 2-methoxyestradiol treatment and that combination with imatinib resulted in reduced cell growth. Another interesting approach to overcome imatinib resistance is to use proteasome inhibitors that obstruct the catalytic 20S core of the proteasome such as bortezomib [156,157]. This interference prevents the elimination of various proteins through proteosomal degradation such as cell-cycle regulatory proteins. Finally, histone deacetylase inhibitors have the potential to overcome imatinib resistance as well. These inhibitors act through hyperacetylation of the NH2-terminal residue of the nucleosomal histones, which subsequently leads to transactivation of multiple transcription factors like $p21^{WAF1/CIP1}$ [158]. For example, the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) markedly downregulates levels of BCR–ABL in the CML cell line BV173 and also induces apoptosis in these cells [159]. Nevertheless, the exact mechanism of how histone deacetylase inhibitors manifest their inhibitory potential against cancer cells remains for the most part unknown. Of interest is also the DNA methylation inhibitor 5-aza-2-deoxycytidine (decitabine) that holds cytotoxic and chromatin-modifying activity. This drug was shown to be beneficial in cells that were imatinib resistant or had residual sensitivity to imatinib monotherapy. However, this effect was dependent on the molecular mechanism of resistance and imatinib appeared to induce decitabine resistance in cells expressing BCR–ABL with the T315I mutation [160]. There are additional signaling-targeted therapies at different stages of development, and in the future it will be interesting to see how these drugs can act either alone or in combination with imatinib to overcome resistance.

5. Targeting BCR–ABL expression

There is broad genetic evidence that the BCR–ABL oncoprotein is not only required but also sufficient for transformation of hematopoietic cells. Therefore, it seems reasonable to target expression of BCR–ABL itself. We have already discussed several promising molecular therapies that target the kinase domain of the BCR–ABL protein. However, despite inhibition of the function of BCR–ABL, the oncoprotein continues to be expressed in leukemic cells. This circumstance may contribute to the selection of imatinib-resistant clones and subsequently to relapse. Therefore, additional efforts have been made with the goal to inhibit expression of the BCR–ABL oncoprotein (Fig. 3).

5.1. Targeting mRNA of BCR–ABL

5.1.1. Anti-sense oligonucleotides

Anti-sense oligonucleotides encode for short DNA sequences that are complimentary to mRNA transcribed from the target gene, such as *BCR–ABL*. They are introduced into cells where they form mRNA/oligonucleotide heteroduplexes. As a result, the anti-sense oligonucleotides prevent the BCR–ABL mRNA from associating with ribosomes, leaving the transcripts open for degradation by RNase H. Chemical modifications of the anti-sense oligos, such as morpholine substitutions in the sugar backbone, increase the anti-sense properties. Additionally, improving the pharmacokinetic properties of oligos by increasing their binding to serum proteins may lead to prolonged in vivo half-lives (see for review [161]). Despite encouraging results with anti-sense oligonucleotide treatment in CML murine models, this technique is difficult to adapt for clinical purposes [162]. A general disadvantage of this method includes the high quantities of anti-sense oligonucleotides that are required to reach an effective dose. As a consequence, there are potential toxic effects due to non-specific binding [163]. An alternative could be to identify targets with a short half-life and a low copy number whose transcripts are required for transformation.

5.1.2. RNA interference

A promising approach is to use RNA interference (RNAi) technology, which silences gene expression by small interfer-

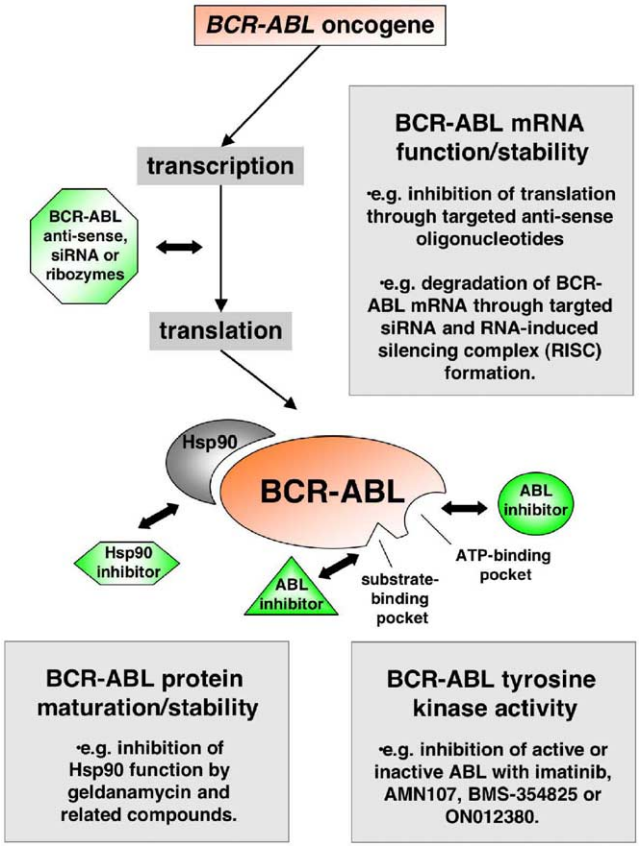

Fig. 3. Current strategies targeting BCR–ABL in chronic myeloid leukemia (CML). Several different approaches to target BCR–ABL are depicted. A seemingly straight forward approach is to target BCR–ABL itself. Current efforts aim to attack BCR–ABL or its generation in several ways: Reducing BCR–ABL RNA levels or blocking translation by RNA interference, anti-sense oligonucelotides or ribozymes that specifically recognize BCR–ABL mRNA. Other approaches try to change the protein maturation and/or stability of through inhibition of binding molecules, such as the Hsp90 inhibitor geldanamycin, leading to proteosomal degradation of BCR–ABL. Blocking the ATP-binding pocket with inhibitors such as imatinib has been demonstrated to be effective in CML patients, but is insufficient in advanced stages or resistance. Novel compounds that target different sites in the ATP binding pocket with higher affinities or compounds that block the substrate binding site may be more effective and help to overcome imatinib resistance.

ing RNAs (siRNA). This method has great potential because RNAi is uniquely suitable to target the expression of a specific gene. The possibility for drug resistance based on genomic mutations in the BCR–ABL gene is low because BCR–ABL siRNA can be used as a pool, targeting different regions in the mRNA of the oncogene while still leading to degradation of the whole transcript. RNAi is also superior to conventional anti-sense strategies because of high stability of siRNAs and the effectiveness of the induced silencing process [159,160]. Scherr et al. demonstrated that *BCR–ABL* gene expression can be successfully repressed using siRNA technology. They showed that *BCR–ABL* mRNA was reduced up to 87% in BCR–ABL positive cell lines and in primary cells from CML patients [164]. The next challenge will be to provide a suitable system to introduce BCR–ABL siRNA into leukemia cells. The RNAi technique relies on the introduction of double-stranded RNA molecules into the cell, usually by transfection. Double stranded siRNA can be simulated by a vector-based shRNA approach. Lentivirus-based vectors may be suitable because they are able to infect target cells with high efficiency and are generally safe to use.

5.1.3. Ribozymes

Another method to inhibit BCR–ABL protein expression/activity is by employing specific ribozymes. Ribozymes are RNA molecules with highly specific intrinsic enzymatic activity against their target RNA sequences [165–167]. Ribozymes can discriminate mutant sequences from their wild-type counterparts, even when they differ by a single nucleotide base. Following binding to the RNA substrate by base-pair complementation, the ribozyme irreversibly cleaves the target RNA and then releases itself for new rounds of cleavage. This results in an improved target:effector stoichiometry compared to antisense oligonucleotides of the same specificity. It has been demonstrated that BCR–ABL expression can be effectively inhibited in EM-2 cells by a transcript-specific ribozyme, indicating that this technology may be of clinical use if delivery of ribozymes can be optimized [168].

5.2. HSP90 inhibition

Altering the protein stability of tyrosine kinases may also represent a novel strategy for treatment of patients with CML. Heat-shock proteins (Hsps) can act as molecular chaperones which help various proteins in folding and subsequent maturation. Hsp90 is an abundant cytosolic protein that gains full function only under the help of partner proteins such as Hsp70, Hsp40 p23, Hip and Hop [169]. Inactivation of Hsp90 using benzoquinone ansamycins such as geldanamycin (GA), or its less toxic analogues such 17-allylamino-17-demethoxygeldanamycin (17-AAG), results in dissociation of Hsp90 from its client proteins and is followed by rapid degradation of proteins that require this chaperone for maturation or stability [170,171]. GA and 17-AAG have been shown to down-regulate intracellular BCR–ABL protein levels by shifting the binding of BCR–ABL from Hsp90 to Hsp70. The proteasome inhibitor PS-341 reduced both, GA and 17-AAG mediated down-regulation of BCR–ABL levels and inhibited apoptosis of HL-60/BCR–ABL and K562 cells [172]. Therefore, it has been suggested that degradation of BCR–ABL is of proteasomal nature. GA and 17-AAG have also been shown to be effective against the imatinib-resistant mutations T315I and E255K of the ABL kinase in vitro. Interestingly, results showed a trend indicating more potent activity against mutant BCR–ABL proteins compare to wild-type [173]. The combination of Imatinib and 17-AAG led to synergistic effects in primary chronic phase CML cells [174]. However, cells over-expressing BCR–ABL as the leading mechanism of resistance to imatinib are also resistant to 17-AAG monotherapy

[175] indicating the importance of identifying the resistance mechanism before starting second-line therapy.

6. Conclusion

With the introduction of imatinib, major advances in the treatment of CML and Ph+ ALL have been achieved. However, similar to many other anti-cancer drugs, clinical resistance to imatinib monotherapy has emerged. The need for alternative or additional treatment has guided the way to design a second generation of targeted therapies, resulting mainly in the development of novel small molecule inhibitors like AMN107, BMS-354825 and ON012380. Despite promising results from preclinical and early clinical studies, the long term safety and efficacy of these drugs has to be determined. The previously mentioned observation that BMS-354825, in contrast to AMN107 or imatinib, also contains Src kinase family inhibiting activity demands additional attention. A broader spectrum of protein kinase inhibition may carry the risk of augmented toxicity, raising the possibility that these drugs may have more long-term side-effects [176]. It still needs to be determined if this holds true for BMS-354825.

Another challenging problem that remains is the T315I kinase mutation against which neither AMN107 nor BMS-354825 showed significant activity. To date, this mutation accounts for approximately 20% of imatinib-resistant cases, a relatively significant proportion of patients for which no adequate therapy is currently available. ON012380 may overcome this problem, but no clinical data for this drug are currently available. The percentage of cases bearing mutations in the T315-proximal region is likely to further increase if patients are repeatedly treated with AMN107 or BMS-354825, both of which are incapable of inhibiting these ABL kinase mutations and therefore act as “selectors”. It has been suggested that T315 in ABL may hold a “gatekeeper function” for ATP-competitive small-molecule kinase inhibitors through direct contact with ABL [82]. This mutant may therefore be difficult to inhibit with small molecule drugs that target the ATP binding site and additional strategies are required to overcome resistance caused by this mutation. One of these forward approaches includes combination therapy, for example small molecule kinase inhibitors combined with compounds that target downstream proteins of the BCR–ABL oncoprotein such as Ras or mTOR.

To date, one widespread opinion is that ABL inhibition results not in an ultimate cure, but attains a plateau of response where low levels of BCR–ABL positive hematopoietic progenitors are still present [38,177]. The majority of patients treated with imatinib achieve a 3-log reduction in leukemic burden. Unfortunately, only few achieve *BCR–ABL* transcript levels that cannot be detected by sensitive PCR methods. A magic bullet would have the ability to eradicate all neoplastic clones that harbor the *BCR–ABL* transcript, however, it may still be sufficient to develop drugs that at least inhibit the expansion of BCR–ABL positive stem cells. The presence of AMN107 and BMS-354825 resistant mutants suggests that even though great progress has been made, this goal will not be easily achieved with these drugs and continues to foster the demand to find novel therapies. There is potential that the introduction of combined therapies including tyrosine kinase inhibitors and vaccination strategies or immune modulation may lead to a solution in patients with CML and other disorders with transforming tyrosine kinase activities.

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