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Targeting the Chronic Myeloid Leukemia Stem Cell: A Paradigm for the Curative Treatment of Human Malignancies

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1. Introduction

Chronic Myeloid Leukemia (CML) is a clonal myeloproliferative disorder of pluripotent hematopoietic stem/progenitor cells that has been paradigmatic to our understanding of the molecular and cellular basis of human malignancies. It has provided an excellent example of how a specific molecular abnormality can be targeted therapeutically to transform a life-threatening malignancy into a chronic disease. The study of CML has been characterized by a number of 'firsts'. CML was the first malignancy to be: (i) associated with a specific chromosomal abnormality, (ii) associated with a specific molecular alteration (*BCR-ABL*) and (iii) successfully treated with a specifically designed targeted therapeutic agent. As such it seems natural that CML should be the first human malignancy in which a complete medical cure is achieved through the eradication of cancer stem cells. This should be realizable through combining the specific targeting of BCR-ABL and CML stem cells. Once this has been achieved, the challenge will be to successfully transfer the lessons learned from this relatively simple and well-characterized model system to the eradication of cancer stem cells in more complex malignancies.

The treatment of CML has changed dramatically following the introduction into the clinic of the tyrosine kinase inhibitor (TKI) imatinib mesylate and second generation TKIs. These agents directly target the BCR-ABL oncoprotein product of the constitutively active BCR-ABL tyrosine kinase. The specific targeting of BCR-ABL induces durable clinical remission in a high proportion of chronic phase CML (CP-CML) subjects (5 year survival of 89%) (Druker et al, 2006). Although a major molecular response (defined as a 3-log reduction in *BCR-ABL* mutant allele burden) is obtained in many CP-CML patients, only a small number attain PCR negativity as determined by the absence of residual *BCR-ABL* transcripts (Hughes et al, 2003). This is because TKI therapy does not specifically target or eliminate leukemia stem cells (LSCs). Indeed TKI therapy alone is unlikely to ever be curative, as following treatment with TKIs LSCs persist in bone marrow (BM) stem cell niches where they harbor the potential for relapse. The emergence of resistance to TKI monotherapy

through the accumulation of somatic kinase domain mutations that interfere with the binding of TKIs to the BCR-ABL ATP-binding site accounts for around 60-90% of relapses (O'Hare et al, 2006). There is consequently a significant unmet medical need for more effective therapeutic strategies that following effective tumor debulking are able to: (i) inhibit the molecular mechanisms responsible for generating the LSC genomic instability phenotype, (ii) target the essential components of the stem cell niche and the BM microenvironment that generate, protect, and nurture LSCs and (iii) efficiently eradicate LSCs. The micro-evolution of TKI resistance in CML is driven by the intrinsic genomic instability of the LSC, which in the presence of the selective pressure of drug, results in the expansion of a relatively predictable and invariant quasispecies of somatic mutants, which have differing degrees of intrinsic and acquired TKI resistance. The frequency of each of these clones oscillates with time, with a unitary or oligoclonal set eventually dominating the structure of the population and the clinical response to TKI therapy. The spectrum of mutants in any individual may be characterized through mutational analysis and subsequently used to select the most appropriate TKI therapy. The persistence of LSCs, however, allows additional drug-resistant mutants to arise, creating a new repertoire of diversity from which the most resistant and fastest growing clones are selected. As a result the therapeutic effects of first- and second-line TKI therapy are eventually circumvented. Clearly the eradication of the LSCs must form both a necessary and essential component of any therapeutic strategies that aim to achieve a deep and sustained molecular and clinical response, and ultimately a cure. In what follows we outline a number of approaches to the characterization of the CML LSC. The profiling and characterization of the LSC phenotype is expected to contribute to the rational design of LSC-targeted therapy, and as such presents an opportunity to establish a general paradigm for the development of cancer stem cell-directed cures for human malignancies.

2. Clinical and therapeutic challenges in the management of CML

CML is a clonal, multi-step and multi-lineage myeloproliferative disease that typically evolves through three phenotypically and clinically distinct stages (Goldman & Melo, 2003; Jiang X. 2007; Savona & Talpaz, 2008; Sloma et al., 2010). The first of these is an indolent chronic phase (CP) characterized at the time of diagnosis by the presence of a deregulated *BCR-ABL*$^+$ clone. This expands inappropriately and comes to dominate the population of BM progenitor cells, while at the same time continuing to produce phenotypically normal mature blood cells. There is, as a result, an excessive output of myeloid precursors and mature granulocytes into the BM and peripheral blood (PB). The second stage is an accelerated phase (AP) characterized by an incremental increase in the disease burden as demonstrated by an increased frequency of leukemic myeloid progenitor/precursor cells. The third stage is a rapidly fatal acute blast crisis phase (BC) characterized by increased genomic instability, deregulated proliferation and loss of differentiation. BC-CML may be categorized as myeloid or lymphoid (pre-B) by the appearance of increased numbers of differentiation-arrested blast cells that reflect the growth of sub-clones of early myeloid or pre-B cells respectively that have acquired additional somatic mutations (Goldman & Melo, 2003; Jiang X. 2007; Savona & Talpaz, 2008; Sloma et al., 2010). The canonical feature of CML is the presence in hematopoietic stem cell (HSC) derived progeny of a Philadelphia chromosome (Ph$^+$) containing a reciprocal t(9;22)(q34;q11) translocation, which generates a clone-specific *BCR-ABL* fusion oncogene. This encodes a chimeric BCR-ABL oncoprotein

that has significantly enhanced and constitutive tyrosine kinase activity, which drives the pathogenic features of the disease (Druker et al., 1996; Lugo et al., 1990). It produces a range of biochemical changes that impact the growth-factor dependence, turnover, and genomic stability of primitive $CD34^+$ leukemic cells, whilst at the same time having little impact on their ability to differentiate according to predefined molecular programs (Holyoake et al., 2002; Jiang et al., 2007b; Penserga & Skorski, 2007; Sloma et al., 2010; Valent, 2008).

The identification of an invariant molecular genetic alteration (*BCR-ABL*) in the vast majority of cases of CML has facilitated the development of rational targeted therapy focused on the selective inhibition of the dysregulated tyrosine kinase activity of the encoded BCR-ABL oncoprotein (Druker et al., 1996; Shah et al., 2004; Weisberg et al., 2005). Imatinib mesylate (IM, Novartis, Basel, Swizerland) was the first tyrosine inhibitor to be developed as a molecular targeted drug (Druker et al., 1996). It is a competitive inhibitor of the ATP-binding site of the ABL-kinase domain, and prevents a conformational change of the oncoprotein to its active form, resulting in the elimination of most $BCR\text{-}ABL^+$ cells (Druker et al., 1996). This relatively selective agent (it also recognizes the ATP-binding site of the c-Kit and platelet-derived growth factor receptors) has been immensely effective in the treatment of subjects with CP-CML (Druker et al., 2006; Druker et al., 2001; Kantarjian et al., 2002; O'Brien et al., 2003). Nevertheless, early relapse and the emergence of IM resistance are observed in 10-20% of subjects in early CP-CML, and up to 40% of those with advanced phase disease including 1-3% of newly diagnosed CML patients that develop sudden blast crisis (Apperley, 2007; Deininger et al., 2005; Forrest et al., 2008; Kantarjian et al., 2003; O'Hare et al., 2006; Valent, 2008). The inability to successfully discontinue IM therapy following at least five years of therapy, the persistence of a reservoir of clonal leukemic stem cells following the attainment of a complete molecular response (CMR), and the uncertain safety profile of long-term TKI treatment, has led to differing views on the most appropriate choice of therapy in CP-CML (Mahon et al., 2010; Ross et al., 2010a; Ross et al., 2010b; Rousselot et al., 2007; Sobrinho-Simoes et al., 2010). Recently, second-line TKIs including Dasatinib (DA,Bristol-Myers Squibb, New York, NY, USA) and Nilotinib (NL, Novatis) have been licensed for use in this indication and represent alternative therapeutic options either first-line or for resistant or intolerant cases. Both drugs have increased potency against the *BCR-ABL* kinase domain mutants most commonly associated with IM resistance. This along with their differing spectrums of inhibitory activity across the human kinome, has translated into increased clinical efficacy in subjects with IM-resistant disease (Carter et al., 2005; Shah et al., 2004; Weisberg et al., 2005). The major cytogenetic response (MCyR) rate following therapy with DA or NL in subjects with IM-resistant CP-CML is approximately 60%, with a complete cytogenetic response (CCyR) rate of 50% (Hochhaus et al., 2008; Kantarjian et al., 2007). Two recent Phase 3 randomized trials in subjects with treatment naïve early stage CP-CML demonstrated that both drugs are more effective than IM at inducing MCyR and major molecular (MMR) responses (Kantarjian et al., 2010; Saglio et al., 2010). DA and NL were subsequently approved by the U.S. Food and Drug Administration (FDA) as first-line therapies in CP-CML. Clinical experience, however, has shown that some subjects experience inadequate responses to all existing TKI therapies, or have an initial response but then progress rapidly (Kantarjian et al., 2006; Talpaz et al., 2006). As is the case in 60% of subjects with IM-resistant disease, the recalcitrant T315I mutation also routinely dominates the observed resistance with DA and NL (Apperley, 2007; Goldman, 2007). As a result these agents have no benefit over IM in subjects whose resistance is thought to be mediated

principally by T315I somatic mutants. In spite of this, each TKI has a unique spectrum of activity with respect to most of the other commonly encountered mutations that confer resistance to TKI therapy. Subjects with the V299L, T315A, and F317L/V/I/C mutations, for example, are less sensitive to DA (Muller et al., 2009; Soverini et al., 2011; Soverini et al., 2006), whereas the Y253H, E255K/V and F359V/C/I mutations are less sensitive to treatment with NL (Hughes et al., 2009; Soverini et al., 2011). Several third-generation tyrosine kinase inhibitors have been developed, including ponatinib (AP24534, ARIAD, Cambridge, Massachusetts, US) (O'Hare et al., 2009), which is an orally active multi-targeted kinase inhibitor that targets both the wild type and a broad spectrum of mutant forms of BCR-ABL. It was specifically designed to inhibit the autophosphorylation of wild-type and T315I mutant BCR-ABL and is active against most of the commonly encountered IM-resistant mutations including G250E, Y253F and E255K (O'Hare et al., 2009). In a phase 1 study 38 patients with CP-CML, 66% achieved a MCyR and 53% a CCyR. Most significantly, a total of 89% (nine subjects) of the subjects harboring a T315I mutation attained a CCyR (Santos & Quintas-Cardama, 2011). The effectiveness of long-term therapy with ponatinib in IM-resistant patients, however, has yet to be determined. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is currently the only therapeutic option for CML that has curative potential. Its use, however, is restricted to subjects of less than 50 years that have a suitable donor, and even this highly selected group has a high risk of procedure-related morbidity and mortality (Forrest et al., 2008). The salvage rate for subjects with advanced phase disease, especially BC-CML, is poor even with allo-HSCT, with the vast majority dying as a result of their disease within a few years. There is consequently a significant unmet medical need for therapeutic options that prevent the emergence of resistant sub-clones and that can be administered with curative intent as a result of the selective targeting of LSCs.

3. Properties of CML stem/progenitor cells that generate TKI resistance

Primitive quiescent CML stem cells are relatively unresponsive to TKIs (Copland et al., 2006; Graham et al., 2002; Jorgensen et al., 2007) and possess unique features that predispose them to intrinsic and acquired resistance to BCR-ABL targeted therapeutics (Chu et al., 2005; Jiang et al., 2007a; Jiang et al., 2007b; Jiang et al., 2007c; Konig et al., 2008; Sorel et al., 2004). Evidence further suggests that LSCs are responsible for relapse following the discontinuation of IM therapy (Mahon et al., 2010; Ross et al., 2010a; Ross et al., 2010b; Rousselot et al., 2007; Sobrinho-Simoes et al., 2010). The elimination of the LSCs responsible for disease perpetuation and for the intrinsic and acquired TKI resistance observed in CML therefore represents the next logical step in the treatment of CML. An essential component of this enterprise, involves the molecular characterization of the CML stem cell phenotype, BM microenvironment and the stem niche that foster the origin, development, growth and survival of LSCs.

The relative insensitivity of primitive CML cells to treatment with IM was first reported in a quiescent subset of $CD34^+$ CML cells using a carboxy-fluorescein diacetate succinimidyl diester (CFSE) staining cell division tracking assay (Graham et al., 2002; Holyoake et al., 1999). This demonstrated that 3 days of exposure of CML cells *in vitro* to concentrations of IM that were several-fold higher than those achieved in the plasma of subjects treated with 400 mg IM daily, failed to eliminate most of the primitive quiescent CML cells (Graham et

al., 2002). In contrast, cells with replicative competency during the same interval were eliminated. A similar insensitivity of this subset of cells to DA and NL has also been demonstrated (Copland et al., 2006; Jorgensen et al., 2007). Subsequent studies using assays for long-term culture-initiating cells (LTC-ICs) and colony forming assays (CFCs) have

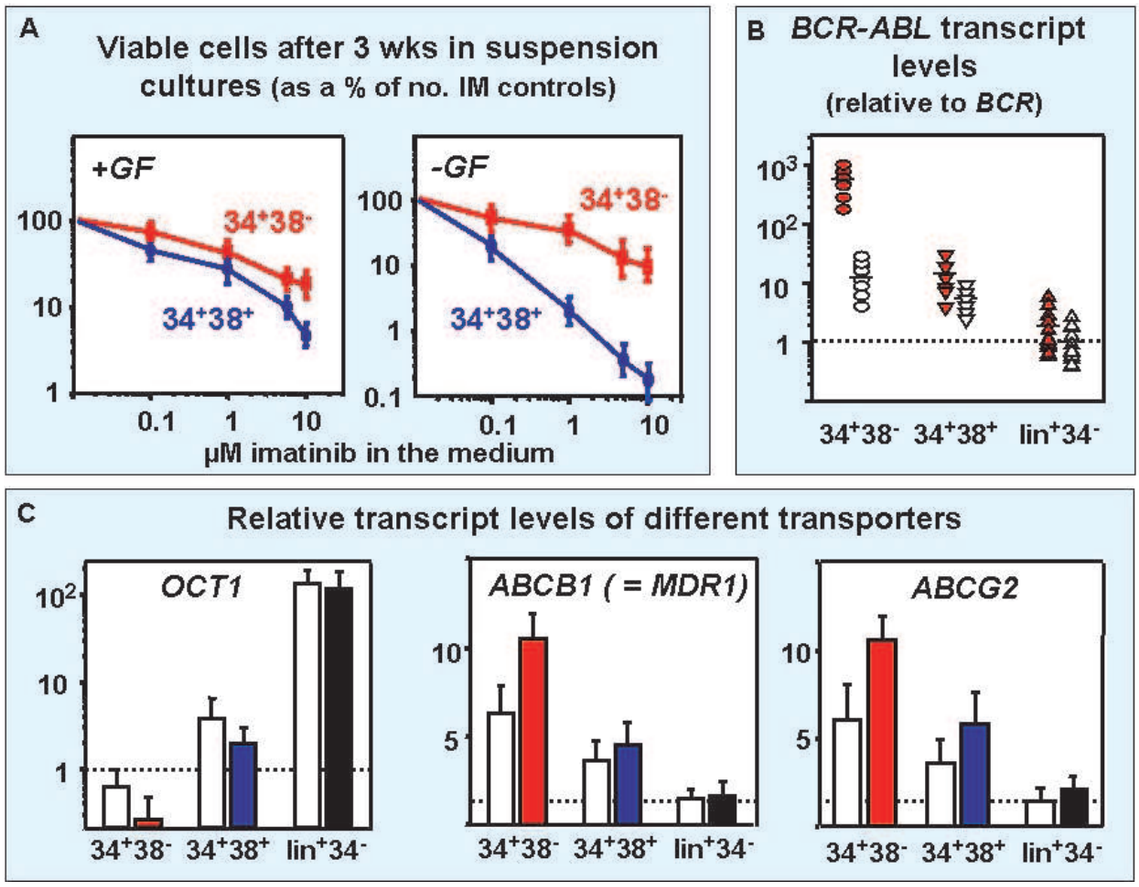


Fig. 1. Unique features of CML stem cells promoting their resistance to BCR-ABL-targeted therapies. (A) Suspension cultures were initiated with FACS-purified lin-CD34+CD38- and lin-CD34+CD38+ CML cells and maintained for 3 weeks in the presence or absence of growth factors and variable concentrations of imatinib (IM). The differential IM sensitivity of lin-CD34+CD38- and lin-CD34+CD38+ CML cells to IM *in vitro* is markedly enhanced under growth factor-deprived conditions. (B) *BCR-ABL* transcript levels relative to *BCR* were measured in RNA isolated from different subsets of cells. Expression of *BCR-ABL* is highly deregulated in lin-CD34+CD38- stem-cell enriched population as compared to their more mature progenitor cells (lin-CD34+CD38+) and differentiated cells (lin+CD34-). (C) *OCT1*, *ABCB1* and *ABCG2* transcript levels relative to *GAPDH* were measured in different subsets of CP-CML and normal BM cells. A reduced level of *OCT1* and elevated levels of *ABCB1* and *ABCG2* were detected in lin-CD34+CD38- stem-cell enriched population as compared to their more mature progenitor cells. The combination of very low expression of *OCT1* (low IM uptake), highly elevated expression of *ABCB1* and *ABCG2* (high efflux of IM and other drugs) and elevated expression of *BCR-ABL* in *CML* stem cells indicates that their general insensitivity to IM and other therapeutics is likely to be explained by multiple abnormal mechanisms.

indicated that although IM is able to inhibit the proliferation of primitive CML cells *in vitro*, it does not induce concurrent apoptosis (Holtz et al., 2005; Holtz et al., 2002). We have shown that the ability of IM to inhibit primitive CML cells depends on their differentiation status (Jiang et al., 2007c). In these experiments, stem cell and progenitor cell enriched CML cell fractions ($lin^-CD34^+CD38^-$ and $lin^-CD34^+CD38^+$ cells, respectively) were isolated and cultured in the presence of varying concentrations of IM for a more prolonged period than in the earlier studies with primary quiescent CML cells (3 weeks vs. 3 to 12 days). The inhibitory effect of IM on the yield of viable cells following a 3 week exposure period was found to be much less (~10- to 20-fold) pronounced in cultures initiated with more primitive $lin^-CD34^+CD38^-$ CML cells as compared with the more differentiated $lin^-CD34^+CD38^+$ cells (Figure 1A). Taken together, these findings suggest that the most primitive CML cells are much less sensitive to IM than the differentiated bulk population. The fact that Ph^+CD34^+ CFCs and LTC-ICs remain detectable in subjects with CML that have achieved hematological remission following treatment with IM, suggests that these *in vitro* findings are likely to translate into the clinic (Bhatia et al., 2003; Chu et al., 2005). They are further supported by a recent report revealing the presence of *BCR-ABL*+ cells in LTC-ICs of CML patients that have achieved prolonged clinical remission following treatment with either interferon-alpha, IM or DA (Chomel et al., 2011). However, whereas most CML cells are oncogene addicted and sensitive to TKI treatment, the growth and survival of CML LSCs do not appear to be BCR-ABL tyrosine kinase activity dependent (Corbin et al., 2011). This suggests that alternative pathways may be active in CML LSCs that drive their proliferation and self-renewal in a BCR-ABL independent manner. Combination therapies aimed at targeting critical components of these pathways are likely to be of key importance in the derivation of a logical CML LSC eradication strategy.

Other studies have shown that CML stem/progenitor cells have multiple unique features that would be expected to contribute to the observed intrinsic and acquired resistance to *BCR-ABL* directed therapeutics (Copland, 2009; Engler et al., 2010; Jiang et al., 2007a; Jiang et al., 2007b; Jiang et al., 2007c). These include: (i) elevated levels of *BCR-ABL* expression and kinase activity in CML stem cells as compared with their more mature progeny (Figure 1B) in a manner that is cell cycle status independent (Barnes et al., 2005; Copland et al., 2006; Jamieson et al., 2004; Jiang et al., 2007b; Jiang et al., 2008; Jiang et al., 2007c), (ii) a corresponding reduced (almost undetectable) level of the transporter gene *OCT1* that is the principal regulator of IM uptake (Figure 1C) (Thomas et al., 2004; White et al., 2006) and whose decreased levels would be expected to reduce the ability of cells to take up IM (Engler et al., 2010; Jiang et al., 2007c) and (iii) elevated levels of expression of the ABC transporter genes *ABCB1(MDR)* and *ABCG2* (Figure 1C) which enhance the cellular efflux of IM and other drugs (Jiang et al., 2007c; Jordanides et al., 2006; Lepper et al., 2005). The combination of an exceptionally low level of *OCT1* expression which impedes cellular IM uptake, a highly elevated expression of *ABCB1* and *ABCG2* which produces a high rate of cellular IM efflux, and elevated expression of the *BCR-ABL* oncogene in CML stem cells, indicates that their insensitivity to IM and other therapeutics is in part explained by a unique portfolio of protective mechanisms (Jiang et al., 2007b; Jiang et al., 2007c). Interestingly studies in a cohort of 30 CML patients have shown that IM-non-responders have lower *OCT1* transcript levels than IM-responders (Crossman et al., 2005), and that the functional activity of the encoded OCT1 protein, as measured by the OCT1-mediated influx of IM into primary CML cells, is predictive of the long-term outcome of CP-CML subjects

treated with IM (White et al., 2010; White et al., 2006). This suggests that measurement of OCT1 expression might provide a useful predictor of the long-term risk of resistance acquisition in subjects with IM-treated CML.

In order to determine whether the unique properties of $CD34^+$ stem/progenitor cells derived from treatment-naïve CML patients correlate with the subsequent clinical response of the patients to IM therapy, we conducted a retrospective analysis of pre-treatment $CD34^+$ PB cells obtained from 25 IM-treated CP-CML subjects with documented clinical outcomes (Jiang et al., 2010). Following the isolation of $CD34^+$ cells from pre-treatment samples, we measured their *in vitro* CFC sensitivity to IM, expression levels of *BCR-ABL*, *OCT1*, *ABCB1/MDR*, and *ABCG2*, and the frequency of *BCR-ABL* tyrosine kinase domain somatic mutations. The data were segregated and analyzed according to whether they were from the 11 clinically defined IM-responders or the 14 IM non-responders. This confirmed the reported features of $CD34^+$ CML cells, and identified two further features that differed significantly between the two groups. These were the responses of the pre-treatment CFCs to IM exposure *in vitro* ($P<0.0001$) and the frequency of mutant *BCR-ABL* transcripts in $CD34^+$ cells ($P=0.0025$) suggesting that these parameters might form the basis of a prospective test for the optimization of CP-CML management (Jiang et al., 2010).

4. Genomic instability and a mutator phenotype as an invariant feature of CML LSCs

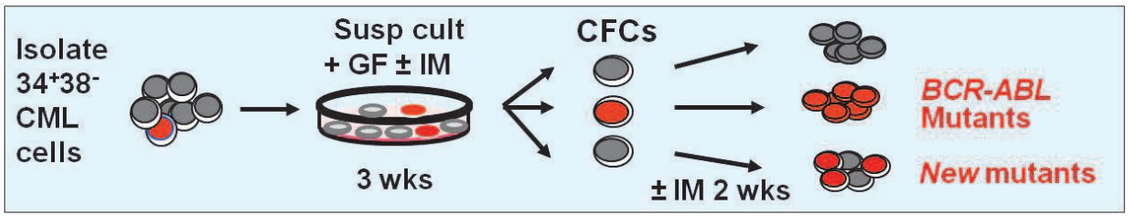
The genomic instability of primitive CML cells induced by the presence of the *BCR-ABL* fusion oncogene has long been thought to be a critical feature of CML. However, it is only in the last decade that data from CML cell lines and transgenic mice have provided definitive evidence that the *BCR-ABL* oncogene is necessary for the induction of genomic instability in hematopoietic cells (Brain et al., 2002; Brain et al., 2003; Canitrot et al., 1999). *BCR-ABL* is able, for example, to induce a mutator phenotype in *BCR-ABL* transfected murine cells. It also produces elevated levels of reactive oxygen species (ROS)-dependent DNA damage as compared with non-transfected controls. This damage to genomic DNA is likely to contribute to the accumulation of the somatic point mutations found in the ATP-binding site of the constitutively expressed BCR-ABL tyrosine kinase, and which are responsible for most cases of TKI resistance (Koptyra et al., 2006; Skorski, 2008). Interestingly, the ROS-dependent mutations in *BCR-ABL* transfected cells were principally detected in a gene encoding the Na^+K^+ATPase *Atp1a1* (Koptyra et al., 2006). Mutant *ATP1A1* transcripts including those containing point mutations, insertions and deletions have been reported at high frequency (15-34%) in $CD34^+$ CML cells rescued from IM nonresponders, a mutational rate similar to that observed in the *BCR-ABL* kinase domain of CML subjects. These mutations are not seen in $CD34^+$ BM cells derived from healthy controls (Jiang et al., 2010). This and other data suggest that *BCR-ABL* induces a mutator phenotype, which results in the genome-wide instability of primitive CML cells.

Investigations into the cellular and molecular causes of IM resistance have shown that the acquisition of somatic mutations in the *BCR-ABL* kinase-encoding domain that reduce the efficiency of IM binding to the ATP binding site represents the most common mechanism of resistance (accounting for 60-80% of relapses) (Deininger et al., 2005; O'Hare et al., 2006; Soverini et al., 2011; Valent, 2008). Copy number amplifications of *BCR-ABL* in contrast are rare (<10% of cases) (Gorre et al., 2001; Hochhaus et al., 2002; Tauchi & Ohyashiki, 2004).

More than 90 different *BCR-ABL* kinase domain point mutations have been reported at varying frequencies in IM-resistant subjects (Apperley, 2007; Hughes et al., 2006; Shah et al., 2002; Soverini et al., 2011). Of these 15 specific amino acid substitutions account for more than 85% of the mutations at the protein sequence level. The mutations responsible for 66% of reported cases, furthermore, have been shown to occur at only six different positions (G250E, Y253F/H, E255K/V, T315I, F359V, H396R/P). The T315I mutation, which confers resistance to IM, DA and NL, is the most frequently detected mutation in IM-resistant patients and is the hardest mutant to treat (Apperley, 2007; Carter et al., 2005; Shah et al., 2004; Soverini et al., 2011; Weisberg et al., 2005). *BCR-ABL* kinase domain mutations are found in IM-naïve patients but not in the germline of healthy controls, indicating that at least some of the mutations are somatically generated prior to the presence of drug selection, and that the increased frequency of mutations observed following TKI therapy occurs as a result of drug-induced selection and associated clonal expansion (Roche-Lestienne et al., 2003; Roche-Lestienne et al., 2002; Willis et al., 2005). The somatic mutations themselves arise as a consequence of underlying genomic instability, which may reflect impaired processes of DNA repair in LSCs. Although some of the mutants are adaptive under the selective pressure of drug exposure, the majority diminish in frequency in the presence of TKI selection. We and others have demonstrated that the *BCR-ABL* fusion gene in $CD34^+$ leukemic progenitor cells (Chu et al., 2005; Jiang et al., 2010) and $CD34^+CD38^-$ stem cell-enriched cells is itself highly unstable (Jiang et al., 2007a) This is reflected in the unusually high frequency of *BCR-ABL* mutations that accumulate in the $CD34^+CD38^-$ compartment in the presence or absence of IM selection. The rapid acquisition of somatic mutations in *BCR-ABL* is also observed in the progeny of CML cells stimulated to proliferate and differentiate *in vitro* (Figure 2) (Jiang et al., 2007a). The rapid and prolific generation of *BCR-ABL* somatic mutants in primary CML cells *in vitro* extends recent findings in *BCR-ABL*-transduced murine BaF3 cells (von Bubnoff et al., 2005), and adult BM cells (Flamant & Turhan, 2005) and indicates that primitive leukemic CML cells have an intrinsically high rate of mutation, and a tendency to fix new somatic point mutations irrespective of the presence or absence of drug. The nature and timing of these apparently stochastic events relative to the size of the primary LSC clone at the time diagnosis and the timing, nature and extent of TKI therapy, may to some extent explain the variable clinical responses observed in different subjects (Jiang et al., 2007a; Roche-Lestienne et al., 2003; Shah et al., 2002; Sorel et al., 2004). A recent study shows that CML subjects defined retrospectively as either IM responders or IM non-responders display significant differences in the frequency of mutant *BCR-ABL* transcripts present in their pre-treatment $CD34^+$ cells ($P=0.0025$), with some of the highly resistant *BCR-ABL* kinase domain somatic mutants such as T315I being amplified from the $CD34^+$ cells of IM non-responsive subjects (Jiang et al., 2010). Overall this suggests that primary CML stem/progenitor cells have a high degree of focal and possibly genome-wide instability, emphasizing the importance of taking the properties of these cells into account when considering new therapeutic approaches. The unique properties of leukemia stem/progenitor cells may, furthermore, help predict individual responses to TKI therapies and in so doing improve clinical management by facilitating personalized treatment decisions.

One approach to targeting the LSC involves defining and inhibiting the generative mechanism causal to the observed genomic instability in LSCs. The ROS that are induced by *BCR-ABL* are known to cause many types of DNA damage including double-strand breaks (DSBs). It has been shown that the error-prone repair of DSBs by non-homologous end-joining (NHEJ) may be responsible for at least some of the somatic point mutations observed

A



B

No. of colonies of each mutant type			
	Wt	Mt	Wt + Mt
Start 34+38- cells	20/26	6/26	-
3 wk - IM	5/18	1/18	12/18
3 wk + IM	3/19	2/19	14/19

C

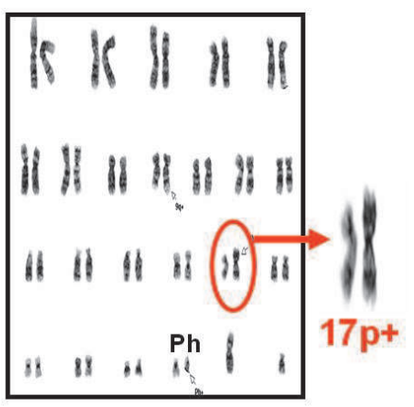


Fig. 2. CML stem cells are highly unstable and generate a high frequency of *BCR-ABL* kinase domain mutations both *in vitro* and *in vivo*. (A) Suspension cultures were initiated with FACS-purified lin-CD34+CD38- stem cell-enriched population and maintained for 3 weeks in the presence or absence of growth factors and IM. The cells harvested from the 3-week cultures were then assayed for CFCs. Mutational analysis was then performed for the detection of *BCR-ABL* kinase domain mutations in freshly isolated lin-CD34+CD38- cells and their CFC progenies. (B) Pre-existing *BCR-ABL* kinase domain mutations could be detected in freshly isolated lin-CD34+CD38- stem cells and new mutations appeared during the growth of primitive CML cells *in vitro*. (C) Chromosomal abnormalities, such as 17 p+, could be observed from single colonies generated from the cells present after 3 weeks in culture with IM.

in early CML and also the large deletions seen in later-stage CML. This is supported by evidence which suggests that key protein components of the major NHEJ pathway, WRN and DNA ligase IIIα that form a molecular complex which is recruited to DSBs, are up-regulated in CML (Sallmyr et al., 2008). There is also evidence that ROS-induced DSBs are repaired, at least to some extent, by single-strand annealing (SSA). This is a rare and very unfaithful repair mechanism whose activity has been shown to be stimulated by *BCR-ABL*. Interestingly the activation of this repair mechanism is attenuated by IM therapy (Cramer et al., 2008). The presence of multiple alternative error-prone mechanisms for repairing DSBs, raises the possibility that the differential recruitment of these alternative repair pathways itself evolves as the disease progresses from CP to BC.

One approach to identifying the generative mechanism that underlies the observed genomic instability in CML, is to generate a compendium of *BCR-ABL* kinase domain somatic mutations to determine if the mutator phenotype observed in LSCs is associated with a distinct mutational signature. In order to establish whether CML is characterized by a

distinct mutational imprint, we analyzed *BCR-ABL* tyrosine kinase domain sequence data from 15 IM-naïve and 316 IM-resistant CP-CML subjects (Grant et al., 2010). This revealed a distinct and non-random distribution of *BCR-ABL* kinase domain mutations with apparent hot spot regions located at codon positions 1 and 2, and several other distinct features that are commensurate with the activity of a distinct mutator. These include a propensity for transitions relative to unselected regions of the human genome across all codon positions, a T-to-C mutational hotspot at codon position 2, a near lack of mutations at codon position 3, and an overall under-representation of C-to-T mutations. These results provide evidence for the activity of a distinct mutator that is active in LSCs, and it is interesting that the frequently observed M244V and D276G mutations arise from T-to-C transitions, both of which are predicted consequences of putative CML LSC mutator activity. The clinically most problematic mutation, T315I, is interestingly generated by a C-to-T transition, indicating the imprint of intense drug-mediated selection. This characteristic mutational signature may provide insights into the mechanism that contributes to the observed genomic instability in LSCs, and which may act in concert with the error-prone repair of DSBs. Candidate mutators include the *MYC* oncogene, which is known to result in aberrant DNA synthesis and which has been shown to be over-expressed in CML subjects at the time of diagnosis. Higher expression levels have, furthermore, been shown to correlate with a poor clinical response to IM. Interestingly *MYC* levels do not directly correlate with *BCR-ABL* levels in subjects treated with IM (Albajar et al., 2011).

5. Strategies for the eradication of leukemic stem cells

The existence of both intrinsic and acquired resistance to TKIs in CML stem cells has prompted considerable interest in identifying multi-targeted therapeutic strategies able to combat the emergence of resistant clones by eliminating the LSCs that generate them. It has been hypothesized that combination strategies able to target both proliferating and primitive quiescent leukemic cells will significantly improve clinical outcomes in CML (Jiang X. 2007; Savona & Talpaz, 2008; Sloma et al., 2010). There is no doubt that the development of stem cell-directed therapies will be critical to the attainment of prolonged remission and ultimately TKI cessation and cure. As expected, the use of molecularly targeted agents such as IM results in the elimination of the majority of the more differentiated leukemic cells, but leaves primitive stem cells largely untouched. So although able to effect a significant initial reduction in leukemic cells of a more mature differentiation stage, LSCs eventually repopulate the malignant cell population following or during ongoing TKI therapy, generating new resistant mutants that leads to disease persistence and clinical recurrence. This situation recapitulates the elimination of rapidly cycling cells and sparing of quiescent leukemic stem cells seen in the deployment of conventional chemotherapeutics. Whereas monotherapy using agents that directly target LSCs may result in only a minimal observable initial response, in the absence of LSCs the leukemia is not expected to be maintained or expanded. Most importantly, the micro-evolutionary process that continuously and dynamically generates a spectrum of mutant clones will be disabled. As a result the disease burden would be expected to decline incrementally, providing significantly improved long-term outcomes. Combination with TKIs or other agents that target proliferating cells of a more mature phenotype will though continue to be essential for debulking. In the situation where resistant clones with *BCR-ABL* kinase domain mutations and/or other critical mutations are already present at high frequency, the simultaneous

targeting of the mutant clonal population with a second generation TKI will be necessary if molecular cure is to be achieved. This process will be facilitated by the development of new and more effective classes of debulking agents. A switch pocket inhibitor DCC-2036, for example, has been developed which targets the hydrophobic pockets distant from the catalytic region that regulate the transition between the active and inactive state of BCR-ABL. This drug appears to successfully inhibit the majority of TKI-resistant mutants, including the critically important T315I gatekeeper mutation (Chan et al., 2011). DCC-2036 inhibits the BCR-ABL oncoprotein in both its active and inactive conformation by inducing and stabilizing the type II inactive conformation. It is highly effective in suppressing the growth of transduced wild-type *BCR-ABL* murine cells, including cells transduced with the T315I mutant both *in vitro* and *in vivo* (Chan et al., 2011). A phase I trial of DCC-2036 in IM resistant subjects carrying either the T315I mutation or two or more alternative TKI mutations is currently underway. However, although useful in the management of TKI-resistant mutations, DCC-2036 alone is unlikely to target LSCs.

Global gene expression analysis and transcriptome profiling, including the identification of deregulated micro-RNAs (miRs) and the genes they target using next-generation sequencing technologies, have been applied in order to facilitate the identification of new molecular targets and biomarkers able to predict TKI responsivity in primary CML cells, IM resistant cells and *BCR-ABL*-transduced cells. Several studies have compared the transcriptome of $CD34^+$ progenitor cells and $CD34^+CD38^-$ stem cell-enriched leukemic cells with their healthy counterparts. These studies have confirmed the functional relevance of the activation of the JAK/STAT, PI3K/AKT, RAS/MAPK and NFκB pathways in LSCs (Janssen et al., 2005; Jongen-Lavrencic et al., 2005; Kronenwett et al., 2005; Nowicki et al., 2003; Radich et al., 2006; Salesse & Verfaillie, 2003; Yong et al., 2006; Zhao et al., 2008). These studies have also identified differentially expressed genes that are involved in the regulation of DNA repair, cell cycle control, cell adhesion, and homing, as well as genes and transcription factors involved in drug metabolism (Diaz-Blanco et al., 2007; Kronenwett et al., 2005; Salesse & Verfaillie, 2003; Yong et al., 2006; Zhao et al., 2008). Several miRs and their target genes, including *miR-203, miR-328* and *miR-17-92* cluster, have been shown to regulate *BCR-ABL* and the expression of other genes known to be critical to the generation and maintenance of the CML phenotype (Bueno et al., 2008; Eiring et al., 2010; Venturini et al., 2007), indicating the potential utility of Micro-RNA profiling in the identification of novel targets for LSC-directed therapies. Of note, several new potential targets have been identified which regulate the maintenance of self-renewal, quiescence and expansion of CML stem and progenitor cells. These include promyelocytic leukemia protein (PML), β-Catenin, RNA-binding proteins (RBPs) and the *BMI1* and *FOXO* transcription factors, suggesting that the specific targeting of these proteins in conjunction with TKIs, may help eliminate residual CML LSCs (Copland, 2009; Eiring et al., 2008; Hu et al., 2009; Ito et al., 2008; Naka et al., 2010; Park et al., 2003; Rizo et al., 2010; Zhao et al., 2007).

The systematic applications of these and other new technologies have resulted in significant advances in our understanding of the molecular properties of CML stem and progenitor cells. They are also helping to define the nature of the BM microenvironment and stem cell niche that support the growth and differentiation of LSCs. The specific targeting of the BM microenvironment and stem cell niche represent alternative and indirect strategies for the elimination of LSCs (Figure 3). The chemokine receptor CXCR4, for example, which is central to stem cell localization and a known chemo-attractant for hematopoietic cells, is induced by IM and causes CML cell migration to the BM and promotes the survival of

quiescent CML progenitors (Jin et al., 2008). This suggests a possible mechanism of IM resistance working through the cross-talk between CML stem/progenitor cells and their BM microenvironment niches, and suggests a rationale for the combination of CXCR4 antagonists with TKIs so as to more effectively eliminate IM-resistant LSCs.

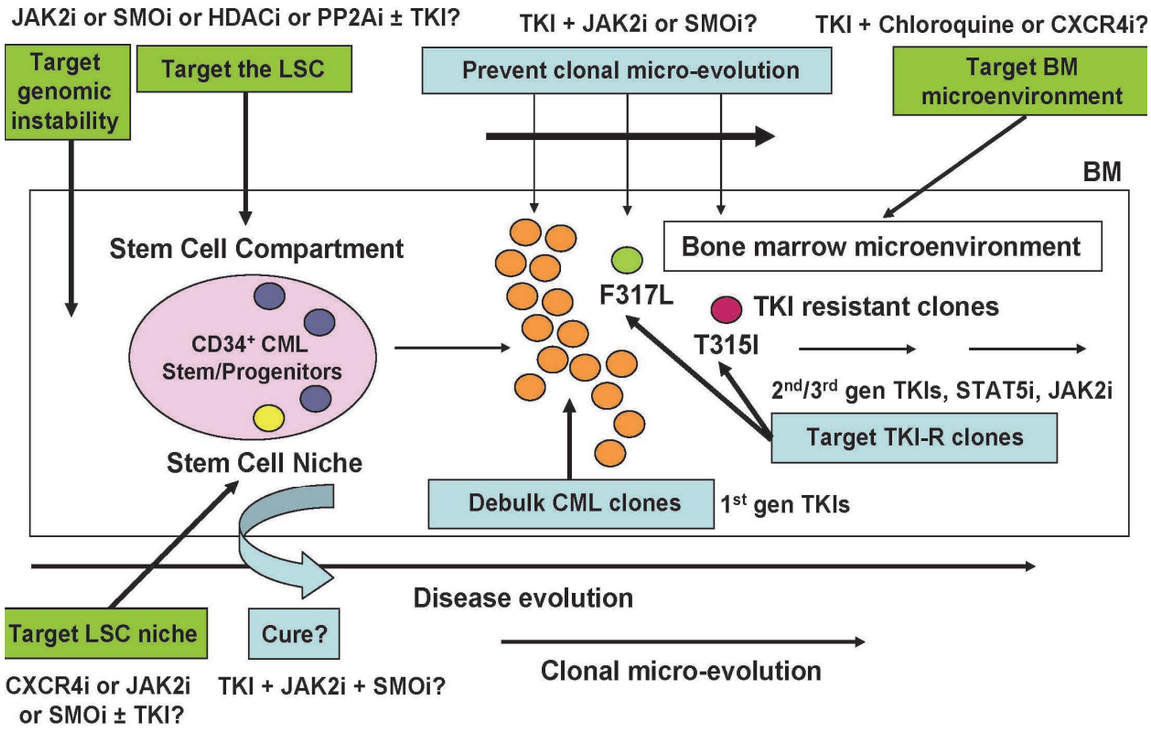


Fig. 3. **Targeting the CML stem cell.** Schematic diagram of the stem cell compartment and stem cell niche, indicating the specific targets of conventional first line TKI drugs, second and third generation TKIs, and the opportunities for targeting the CML stem cells either directly, or through the targeting and manipulation of the stem cell niche or bone marrow microenvironment.

Components of the sonic hedgehog signalling pathway, including the smoothened transmembrane protein (SMO), have similarly been shown to play a critical role in normal and leukemic stem cell development, proliferation and self-renewal, including the regulation of the epithelial-mesenchymal transition (Varjosalo & Taipale, 2008). Knockdown or inhibition of *SMO* impairs HSC self-renewal and abrogates or delays the appearance of CML in several *in vitro* and *in vivo* models (Dierks et al., 2008; Zhao et al., 2009). Conversely, SMO over-activity has been demonstrated in CML cells, with their proliferation being more SMO-dependent than that of healthy HSCs. Clinical trials evaluating the effects of SMO pathway antagonists developed by a number of different pharmaceutical companies and the relevance of this pathway are currently under way in a range of different malignant indications. The combination of NL with the SMO inhibitor LDE225 and DA with PF-04449913 has been reported as having additive effects on the inhibition of primitive CML cells *in vitro* and *in vivo* (Mar et al., 2011). Another study indicates that a combination of histone deacetylase inhibitors (HDACis, e.g. LAQ824) with IM is effective in targeting

quiescent CML stem cells (Figure 3). This study also suggests that an IM/HDAC combination inhibits several genes that regulate hematopoietic stem cell maintenance and survival (Zhang et al., 2010). Another study demonstrates that the targeting of autophagy, a process that allows cells to adapt to environmental stresses, enhances the effects of TKIs in *BCR-ABL*$^+$ cell lines and in primary CML stem and progenitor cells (Bellodi et al., 2009). IM induces autophagy in BC-CML cell lines and in primary CML cells, and is associated with endoplasmic reticulum (ER) stress that is mechanistically non-overlapping with IM-induced apoptosis. Combination treatment with TKIs and inhibitors of autophagy such as chloroquine, is more effective in eliminating CML stem and progenitor cells *in vitro* than TKIs or autophagy inhibitors alone (Bellodi et al., 2009). The effectiveness of this combination treatment in eliminating primary CML stem and progenitor cells *in vivo*, however, remains to be seen. Other promising therapeutic strategies include the induction of protein phosphatase-2A activation by FTY720, which inhibits the survival and self-renewal of CML progenitor cells (Neviani et al., 2007; Neviani et al., 2005). A farnesyltransferase inhibitor (BMS-214662) was also found to target primitive quiescent CML cells, indicating a possible role for this class of inhibitors (Copland et al., 2008).

We have been pursuing a strategy based on the targeting of JAK2 with a view to inhibiting the activity of a biologically important multi-molecular complex that we have identified comprising AHI-1 (a novel signalling molecule encoded by the Abelson helper integration site 1 gene), the BCR-ABL fusion oncoprotein and JAK2 kinase (Zhou et al., 2008). *AHI-1* is upregulated in highly enriched populations of CML stem cells in which the levels of *BCR-ABL* transcripts are also elevated (Jiang et al., 2004; Jiang et al., 2007c). Interestingly, overexpression of *AHI-1* confers a growth advantage *in vitro* and results in leukemia *in vivo*, synergizing with *BCR-ABL* to enhance these outcomes (Zhou et al., 2008). Conversely, the stable suppression of *AHI-1* in CD34$^+$ CML cells using small interfering RNA, reduces their growth autonomy *in vitro*. Importantly, this newly defined AHI-1-BCR-ABL-JAK2 molecular interaction complex appears to mediate leukemic stem cell transformation and plays an important role in the TKI response/resistance of primary CML stem and progenitor cells. JAK2 itself is known to interact with the C-terminus of BCR-ABL, and is one of the most prominent targets of BCR-ABL in *BCR-ABL* transformed CML cells (Miyamoto et al., 2001; Samanta et al., 2006; Xie et al., 2001). BCR-ABL has also been found to interact with the IL-3/GM-CSF receptor, which subsequently contributes to the downstream activation of JAK2 (Wilson-Rawls et al., 1997). Furthermore, in primitive CML cells, *BCR-ABL* expression stimulates the production of IL-3, G-CSF and GM-CSF which, following binding to their cognate receptors, further contributes to the CML progenitor cell resistance to TKIs via the activation of the JAK2/STAT5 pathway (Jiang et al., 1999; Wang et al., 2007). High *STAT5* levels have also been shown to mediate acquired IM-resistance in CML cells and the STAT5 inhibitor pimozide was shown to reduce their survival (Nelson et al., 2011; Warsch et al., 2011). Therefore, targeting the activity of JAK2 could provide an excellent strategy to complement the inhibition of BCR-ABL kinase activity in primary CML stem cells (Figure 4). Indeed, recent studies have demonstrated that JAK2 inhibitors (TG101209, WP1193) and a dual kinase inhibitor of JAK2 and ABL kinases (ON044580) induced apoptosis in IM-sensitive and IM-resistant CML cell lines (Samanta et al., 2011; Samanta et al., 2010) and that treatment with TKIs in combination with TG101209 results in greater inhibition of CML stem and progenitor cells as compared to when the same cells are treated with either TKIs or TG101209 alone or a combination of TKIs (DeGeer et al., 2010; DeGeer et al., 2009). Several

JAK2 inhibitors with varying degrees of intra-JAK family and intra-kinome selectivity are currently in various stages of clinical development. However, the myelosuppressive effects of these inhibitors on normal hematopoietic stem/progenitor cells remain a concern. The development of highly selective and less toxic (fewer hits across the kinome) JAK2 inhibitors in combination with first or second generation TKIs provides an attractive option for the specific targeting of LSCs (Figure 3 and 4). The permanent eradication of the leukemia stem cell in conjunction with tumor debulking, is expected to result in a functional cure, and in so doing to provide the study of CML with its ultimate crowning achievement. Importantly it should also provide molecular medicine with a general paradigm for the medical cure of cancer through stem cell eradication that with appropriate modifications should be applicable to multiple tumor types.

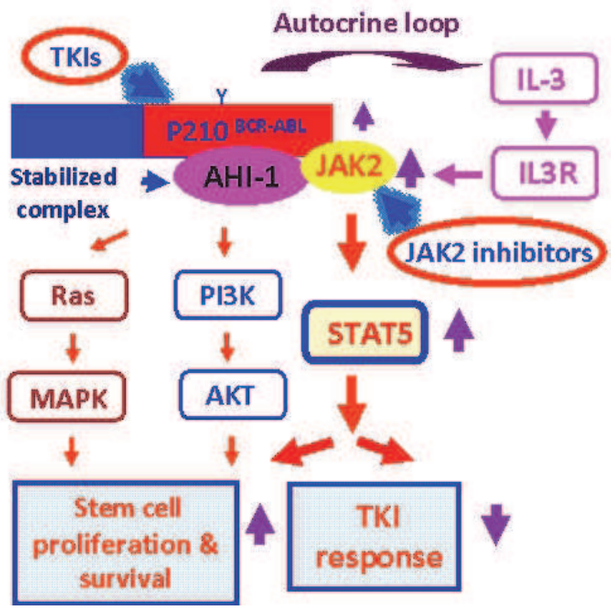


Fig. 4. Model of the targeting of the AHI-1-BCR-ABL-JAK2 complex in CML stem cells by combination treatment of TKI and JAK2 inhibitors. Schematic diagram of the AHI-1-BCR-ABL-JAK2 interaction complex that regulates constitutive activation of BCR-ABL and JAK2/STAT5 and results in increased proliferation and a reduced TKI response in CML stem and progenitor cells. Targeting both BCR-ABL and JAK2 activities to destabilize this protein interaction complex may represent a potential therapeutic option for CML.

6. Conclusion

The discovery of tyrosine kinase inhibitors marked a major advance in CML therapy and other cancers. Although highly successful, selective tyrosine kinase inhibition has not resulted in a functional cure. As CML is driven by genetically unstable pluripotent leukemic stem cells, therapeutic approaches that target these cells will be required for definitive curative therapies. The systematic characterization of the unique biological properties of CML LSCs promises to deliver new insights into the process of malignant transformation and disease progression. As it comprises a relatively simple and well-understood model system, it is envisaged that the elimination of LSCs in CML will provide a general paradigm

for cancer stem cell eradication and the consequent provision for the basis of medical cures across a broad range of different malignancies.

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8. Conflict-of-interest disclosure

A. Woolfson is an employee of Bristol-Myers Squibb, some of whose products are discussed in this chapter.

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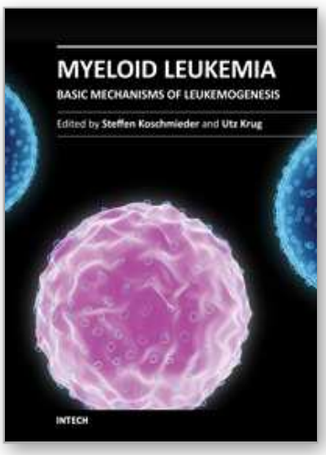

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The current book comprises a series of chapters from experts in the field of myeloid cell biology and myeloid leukemia pathogenesis. It is meant to provide reviews about current knowledge in the area of basic science of acute (AML) and chronic myeloid leukemia (CML) as well as original publications covering specific aspects of these important diseases. Covering the specifics of leukemia biology and pathogenesis by authors from different parts of the World, including America, Europe, Africa, and Asia, this book provides a colorful view on research activities in this field around the globe.

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