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The chronic myeloid leukaemia stem cell: stemming the tide of persistence.

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ABSTRACT

Chronic myeloid leukaemia (CML) is caused by the acquisition of the tyrosine kinase BCR-ABL1 in a haemopoietic stem cell (HSC), transforming it into a leukaemic stem cell (LSC) that self-renews, proliferates and differentiates to give rise to a myeloproliferative disease. While tyrosine kinase inhibitors (TKI) that target the kinase activity of BCR-ABL1 have transformed CML from a once fatal disease to a manageable one for the vast majority of patients, only ~10% of those who present in chronic phase (CP) can discontinue TKI treatment and maintain a therapy-free remission. Strong evidence now shows that CML LSC are resistant to the effects of TKIs and they persist in all patients on long-term therapy, where they may promote acquired TKI resistance, drive relapse or disease progression and inevitably represent a bottleneck to cure. Since their discovery in patients almost two decades ago, CML LSC have become a well-recognised exemplar of the cancer stem cell and have been characterised extensively with the aim of developing new curative therapeutic approaches based on LSC eradication. This review summarises our current understanding of many of the pathways and mechanisms that promote the survival of the CP CML LSC and how they can be a source of new gene coding mutations that impact in the clinic. We also review recent pre-clinical approaches that show promise to eradicate the LSC, and future challenges on the path to cure.
CML: THE CLASSIC STEM CELL DISEASE

Chronic myeloid leukaemia (CML) is a classic example of a stem cell cancer and arises when the t9;22 translocation (the Philadelphia chromosome)\textsuperscript{1-3} occurs in a haemopoietic stem cell (HSC). This event results in the constitutive expression of the fusion tyrosine kinase BCR-ABL1, transforming the HSC into the CML stem cell (referred to here as the leukaemic stem cell or LSC) which then gives rise to a clonal myeloproliferative disease. Early evidence regarding the HSC origins of CML came from observations that transfusion of peripheral blood cells from CML patients into severely neutropenic recipients resulted in temporary homologous BM engraftment and Ph\textsuperscript{+} progeny in the blood\textsuperscript{4}. This was later explained by the presence of high numbers of mobilised LSC in the peripheral blood of chronic phase (CP) CML patients\textsuperscript{5}. A possible haemopoietic progenitor origin of CML was ruled out when BCR-ABL1 expression in murine haemopoietic progenitors failed to confer self-renewal capabilities to BCR-ABL1\textsuperscript{+} cells, and these cells failed to induce leukaemia in mice\textsuperscript{6}. Until very recently, BCR-ABL1 expression was considered sufficient to cause a CML-like disease in mouse models using retrovirus transduction or transgene insertional mutagenesis to express the oncogene in LSC\textsuperscript{7-9}. However, issues with BCR-ABL1 copy number, high oncogene expression and/or secondary mutations arising by retroviral or transgene insertional mutagenesis or genomic instability could theoretically contribute to leukaemogenesis. In a recent knock-in model, a single copy of BCR-ABL1 expressed from the endogenous BCR locus was able to confer enhanced BM engraftment, however this model was unable to induce leukaemia\textsuperscript{10}.

Whilst the cell of origin of CML is generally accepted to be the HSC, several studies implicate an HSC-precursor cell – the multipotent haemangioblast that gives rise to
both haemopoietic and endothelial cells. The BCR-ABL1 fusion can be detected in endothelial cells obtained from BM and peripheral blood of CML patients at varying frequencies\textsuperscript{11,12}. These cells show altered intra-cellular signalling and protein expression that may affect crosstalk between LSC and the bone marrow microenvironment (BMM), alter immune-modulation and LSC exit from quiescence into proliferation\textsuperscript{13,14}. Collectively these data suggest that the acquisition of BCR-ABL1 in the haemangioblast may contribute to both malignant haemopoiesis and endotheliopoiesis.

**THE NATURAL HISTORY OF CML**

CML is a rare stem cell disease with an annual incidence of 1-2 cases per 100,000 individuals peaking in the sixth and seventh decades of life\textsuperscript{15}. Data derived from atomic bomb survivors\textsuperscript{16} suggest that following a latent period of some 7 years, the natural history of CML is for 85-90% of cases to present in CP, but to progress to accelerated phase (AP) and then to either myeloid or lymphoid blast crisis (BC) over a 5 year time frame\textsuperscript{17}. However, the mechanism of disease progression is complex and disease behaviour is highly variable for individual patients, with some progressing within a few months and others remaining in stable CP for up to 20 years. This heterogeneity between patients may relate to the mutations subsequently acquired in the BCR-ABL1 clone\textsuperscript{18}, variations in gene expression patterns between patients\textsuperscript{19}, or the subtype of HSC in which BCR-ABL1 is first expressed - with recent evidence delineating multiple HSC subsets defined by variably fixed lineage potentialities, transcriptional profiles and phenotypes\textsuperscript{20-23}. Furthermore, intriguing work on pre-leukaemia\textsuperscript{24,25} and the detection of BCR-ABL1 in blood cells of normal individuals\textsuperscript{26,27} presents the possibility that heterogeneity could also be driven by mutations acquired before or after BCR-ABL1, or other
factors such as deregulation and skewing of lineage specification, clonal haemopoiesis, DNA damage, activation of inflammatory responses, and epigenetic alterations, all of which occur in haemopoiesis during aging\textsuperscript{28,29}. Some, or all, of these factors may also be required for, or contribute to, disease development in mouse models of CML.

**LSC PERSISTENCE: A BOTTLENECK TO CURE**

The introduction of a potent BCR-ABL1 tyrosine kinase inhibitor (TKI), imatinib, almost two decades ago, followed by subsequent generations of TKI (dasatinib, nilotinib, bosutinib, ponatinib) has transformed the management of CML\textsuperscript{30,31}. What was once a universally fatal disorder, unless treated with an allogeneic transplant, is now well controlled in the outpatient setting and overall survival has improved significantly (\url{http://seer.cancer.gov/statfacts/html/cmyl.html}) with the majority of patients requiring life-long TKI. In keeping with disease heterogeneity, patient responses to TKI are also variable. The majority of cases (50-70\%) achieve major molecular response (MMR) where BCR-ABL1 levels detectable by quantitative PCR (qPCR) in the blood show a 3 log\textsubscript{10} fold reduction, i.e., 0.1\%, compared to a standardised baseline (reviewed elsewhere\textsuperscript{32}). However, patient to patient variation in leukaemic cell blood counts at diagnosis, and variations in BCR-ABL1 expression between early and late stages of cell differentiation can often confound these interpretations. Approximately 10-20\% of all patients develop even deeper molecular responses triggering dose de-escalation and discontinuation/stopping trials (STIM, TWISTER, DADI), where 50\% of patients relapse within 12 months\textsuperscript{33,34}. When CP relapse occurs, the doubling time (\approx 9 days) for increasing disease burden mirrors the CML disease at diagnosis\textsuperscript{35}. A quarter of CP patients fail TKI therapy\textsuperscript{36}, and approximately half of these cases can be explained by BCR-
ABL1 kinase domain mutations\cite{32,37} but the reason for failure in the remaining patients is unclear.

Ironically, the earliest evidence of CML LSC\cite{38} pre-dated the introduction of TKI and this was followed by definitive evidence of a deeply, but reversibly, quiescent subpopulation of leukaemic cells in patients with CML\cite{39}. In the subsequent years, the consensus view has emerged that virtually all CP patients on TKI therapy and in MMR are not cured of CML and show signs of residual disease burden due to the presence of LSC in the BM (termed “LSC persistence”). In a typical cohort of 100 CP CML patients who undertake TKI therapy over a 5 year period, almost two-thirds of them will have this “LSC persistence” phenotype (Figure 1). Researchers have consistently detected BCR-ABL1+ primitive cells in the BM of TKI-treated patients in MMR which are capable of growth in colony forming cell (CFC) and long-term culture initiation cell (LTC-IC) assays, even in patients in deep molecular response with no detectable BCR-ABL1 transcripts by qPCR\cite{40-43}. The most recent of these studies have shown that although LSC are not always detectable in cases of very deep molecular response, most likely due to technical limitations, some patients with no detectable LSC can subsequently relapse after TKI discontinuation\cite{43}. Others have shown that the LSC that persist in patients in MMR express BCR-ABL1 at lower levels than LSC at the point of diagnosis. Furthermore, murine BM cells engineered to express low levels of BCR-ABL1 levels were far less sensitive to imatinib, while those expressing higher levels were prone to de novo mutations\cite{44}. These findings point to LSC persistence as a “low mutator” phenotype, perhaps explaining why the majority of these patients do not develop drug resistance or progress to BC. The eradication of the LSC remains a challenge in the
majority of CML patients, a significant bottleneck to cure, and an area of intensive research.

**GENERAL FEATURES OF THE LSC**

At time of CP diagnosis, BCR-ABL1− cells co-exist with BCR-ABL1+ cells and enriched CD34+ populations require dual-fluorescent in situ hybridisation (D-FISH) to determine the proportion of cells that carry the Ph+ (usually >90% BCR-ABL1+). The more primitive LSC fraction can be purified by fluorescence-activated cell sorting (FACS) in a variety of ways, giving rise to overlapping, primitive, quiescent populations (Figure 2). Phenotypically and functionally, we define the CP CML LSC as those primitive stem/progenitor cells that show a higher capacity to engraft in immunocompromised mice than bulk CD34+ cells, have stem cell properties (self-renewal), are resistant to apoptosis, are prone to genomic instability and have impaired DNA damage responses. Since BCR-ABL1 drives survival and proliferation, it is somewhat of a paradox that CML LSC express BCR-ABL1 but can also be quiescent – a feature which may enable them to become refractory to TKI-induced apoptosis. However, TKI also exert a potent anti-proliferative effect on CML CD34+ cells and LSC to induce quiescence and subsequent evidence has shown that TKI exert additional effects to subvert a number of pathways to promote survival (see below).

**BCR-ABL1 KINASE INDEPENDENT SURVIVAL**

To understand why LSC were refractory to the effects of TKI, we exposed CML CD34+ cells to high concentrations of dasatinib for 12 days, and subjected them, in parallel, to BCR-ABL1 knockdown. These *in vitro* studies were complemented *in vivo* using the inducible transgenic SCL-tTA/BCR-ABL model. BCR-ABL1
expression was induced in mice to lead to the development of CML-like disease, then switched off to determine whether the LSC population required BCR-ABL1 for survival, and then induced for a second time to see whether the LSC were still functional and could again drive the development of CML-like disease. In the in vitro studies, functional BCR-ABL1+ LSC persisted in culture despite evidence for complete kinase inhibition and significant BCR-ABL1 knock-down. In the mouse model, CML-like disease re-occurred following the second induction of BCR-ABL1. This work demonstrated that LSC survival is not dependent on BCR-ABL1 kinase activity\textsuperscript{54} and suggested that BCR-ABL1 may have non-kinase mediated functions that modulate signalling pathways to promote LSC survival. These conclusions were further supported by others who used imatinib to fully inhibit BCR-ABL1 kinase activity in both LSC and quiescent cells\textsuperscript{55}. Taken together, these studies concluded that CML LSC were not “oncogene-addicted” and that targeting of BCR-ABL1 kinase activity alone would not eliminate them. Furthermore, this work has led investigators worldwide to search for LSC selective, BCR-ABL1 kinase independent targets and pathways that might offer potential for improved targeting of LSC in CML. To date, a number of mechanisms, pathways and drug-able targets have been proposed to contribute to the TKI-resistant LSC phenotype (Figures 2-3, Tables 1-2).

**PI3K/AKT/FOXO SIGNALLING**

BCR-ABL1 has been shown to up-regulate PI3K/AKT signalling and AKT-mediated phosphorylation of FOXO transcription factors results in their cytoplasmic localisation where they are inactive (Figure 2). One important consequence of TKI exposure is inhibition of BCR-ABL1 and down-regulation of PI3K/AKT signalling in the LSC (kinase dependent), leading in turn to re-localisation of FOXO1 and
FOXO3a from the cytoplasm to the nucleus, where they modulate expression of CCND1, ATM, CDKN1C, and BCL6 causing a G1 arrest\textsuperscript{56,57} and may fuel an anti-apoptotic phenotype. The transcriptional repressor BCL6, a FOXO3A target, is likely to play an important role in this process by repressing the tumour suppressors p53 and ARF. In this respect, TKI exposure permits FOXO3A-mediated up-regulation of BCL6 resulting in a protective, pro-survival effect. Others have shown that the PI3K signalling axis in LSC is also under the control of TGFβ signalling (kinase independent) and blocking this pathway reversed the effects of FOXO nuclear translocation\textsuperscript{58,59}. However, the precise mechanism of how this occurs is not fully understood and may not to be completely cell-autonomous.

**HEDGEHOG SIGNALLING**

Several studies have implicated the hedgehog pathway in the maintenance (self-renewal) and proliferation of the LSC\textsuperscript{60-62}, where Smoothened (SMO) is a critical mediator. Hedgehog binding to Patched (PTCH) activates SMO which in turn activates the transcription factor GLI1. This leads to reductions of NUMB expression and increased MDM2-mediated degradation of the p53 protein (Figure 2). This would have the effect of suppressing apoptotic responses and/or cell cycle arrest through repression of p53 targets. SMO deletion or pharmacological inhibition in mouse models of CML blocked this pathway and led to loss of LSC\textsuperscript{60,61}. However, TKI treatment alone was unable to block this pathway, suggesting that hedgehog signalling was kinase independent. More recently, similar results were obtained using SMO inhibitors in human CML samples \textit{in vitro} and \textit{in vivo} using xenografts in immunocompromised NOD scid gamma (NSG) mice\textsuperscript{62}.

**CANONICAL AND NON-CANONICAL WNT SIGNALLING**
β-catenin is a central mediator of both canonical and non-Wnt signalling and has a dual role in regulating cell-to-cell contact through tight junctions and acting as a transcriptional regulator when translocated to the nucleus (Figure 2). In the absence of Wnt signalling, cytoplasmic β-catenin is ultimately phosphorylated by GSK3β and targeted for degradation by an axin-mediated multimeric complex. Nuclear β-catenin is required for self-renewal and survival of normal HSC\textsuperscript{63}, and it therefore not surprising that it has also been shown to be a key mediator of LSC survival. Loss of β-catenin in a murine model of CML impaired the development of the disease by inhibiting LSC self-renewal\textsuperscript{64}, and genetic and pharmacological inhibition of β-catenin activity synergised with TKI to target the loss of LSC\textsuperscript{65}. Several alternative Wnt-regulated pathways have been implicated in CML LSC. TKI exposure induced the up-regulation of CD70 ligand-induced CD27 signalling\textsuperscript{66,67}, resulting in β-catenin nuclear translocation and activation of Wnt target genes, including NOTCH, and c-MYC (kinase dependent). TKI exposure also induces a non-canonical Wnt signalling mediated through NFAT signalling which reduces levels of the pro-survival cytokine IL-4\textsuperscript{68} (kinase dependent). Fatty acid metabolism was demonstrated to be important in LSC when arachidonate 5-lipoxygenase, encoded by ALOX5 - was shown to be up-regulated in LSC in a kinase independent manner\textsuperscript{69} where it is thought to regulate β-catenin levels. Inhibition of ALOX5, through genetic deletion or by pharmacological inhibition in mouse models, targeted the loss of LSC, implicating this component as an important mediator of LSC survival.

**JAK/STAT SIGNALLING**

The Janus kinases (JAK) family of intracellular non-receptor kinases play important roles in regulating cytokine-mediated signal transduction via the JAK/STAT pathway.
Activation of the signal transducer and activation of transcription 5 (STAT5) was demonstrated in primary CML and CML cell lines twenty years ago and this involves its phosphorylation and translocation to the nucleus where it regulates transcription. Subsequent evidence has also shown that a single null mutation in the STAT5a isoform can attenuate CML-like disease in mouse models and knockdown can impair Ph+ myeloid colony formation from CML patient samples. Modulating JAK2 activity in human and mouse cell lines reduces BCR-ABL1 and STAT5 signalling, and pharmacological inhibition using ruxolitinib resulted in the loss of LSC both in vitro and in vivo, implicating JAK2 as an upstream mediator of a CML JAK/STAT signalling cascade in LSC. However, BCR-ABL1 has also been implicated in the direct activation of STAT5 (kinase dependent), suggesting that JAK2 may not be necessary for CML disease maintenance. Furthermore, the re-activation of the tumour suppressor and serine-threonine phosphatase PP2A, through either knockdown or pharmacological inhibition of its repressor SET, has been shown to inhibit BCR-ABL1 and STAT5 activation in CML BC. The scenario, however, is different in LSC, where BCR-ABL1 exerts kinase independent roles to recruit JAK2 to modulate JAK/STAT signalling (see also below). Activation of STAT3 has also been implicated in the JAK/STAT cascade, where it exerts a protective effect on CML cells upon exposure to TKI. Inhibition of STAT3 in combination with TKI induced synthetic lethality to target the loss of LSC.

GENOMIC INSTABILITY, DNA DAMAGE AND REPAIR

Whether TKI-induced quiescence contributes to LSC persistence in patients is still an open question. Two possible beneficial consequences of TKI treatment would be to reduce the turnover and expansion of LSC in patients and enhance a "low
mutator” phenotype. However, a more cautionary interpretation of these possible benefits has come from the examination of the mechanisms and pathways that contribute to genomic instability in LSC. BCR-ABL1 kinase activity leads to increased levels of reactive oxygen species (ROS)\textsuperscript{48,49,81}, including H\textsubscript{2}O\textsubscript{2}, and these lead to oxidative DNA damage, including point mutations and double stranded breaks (DSB). In this regard, the RAC2 GTPase has been shown to alter the function of the mitochondrial respiratory chain complex (MRC-cIII) to generate ROS and DNA damage in LSC, as evidenced by the accumulation of chromosomal aberrations and clinically-relevant BRC-ABL1 kinase domain mutations\textsuperscript{49}. This effect was also observed under hypoxia, the conditions that LSC are exposed to in the BM microenvironment, and during exposure to TKI where RAC2 levels were unaffected, thus demonstrating a kinase independent pathway. Inhibition of RAC2 or disruption of the MRC-cIII reduced the level of genomic instability. Similarly, high ROS levels and associated genomic damage were re-capitulated using the transgenic SCL-tTA/BCR-ABL model\textsuperscript{48} where both BCR-ABL1 kinase domain mutations and various base pair additions/deletions in genes linked to progression to BC were identified in LSC in both TKI-naïve and TKI-treated mice. Evidence as to why such DNA damage is tolerated in LSC has also emerged. BCR-ABL1 can inhibit mismatch repair to protect cells from apoptosis\textsuperscript{50}, and can stimulate single-strand annealing, homologous recombination repair (HRR) and non-homologous end-joining, all of which are error-prone in BCR-ABL1 expressing cells\textsuperscript{52,81}. Furthermore, LSC are dependent on the alternative RAD52-RAD51 pathway of HRR to deal with DSB rather than BRCA1/2-RAD51, due to the kinase independent down-regulation of BRCA1\textsuperscript{82}. While we are unable to reconcile these data with a “low-mutator” phenotype\textsuperscript{44}, they point to the LSC as a potent source of clinically
relevant mutations, and also argue that CML is constantly evolving at the molecular level even in CP, countering the clinical view that it is a disease of 3 distinct phases.

THE LSC BONE MARROW MICROENVIRONMENT

While the pathways described above have ostensibly been studied as primarily cell-intrinsic or cell-autonomous, it is likely that some, if not all, of them are regulated through interactions between the CML LSC and the BMM - and a number of these interactions have been identified (Figure 3, Table 2), some of which mediate TKI resistance.

LSC adhesion within the BMM is likely to contribute to homing and lodgement – critical steps in LSC engraftment subsequent to transplantation. CD44, expressed on LSC, are ligands for e-selectins and lack of CD44 reduced homing and engraftment of LSC\(^8\). Similarly, a critical role for selectins and their ligands in engraftment has also been shown\(^8\) and e-selectins can be blocked pharmacologically to reduce the number of LSC. The lectin GAL-3 mediates resistance to TKI through binding \(\beta\)-galactosides on stromal cells and over-expression activated AKT signalling and increased lodgement of LSC in the BM\(^8\). \(\beta1\)-integrins mediate adhesion of LSC to BM stromal cells, a process likely to be regulated by interferon \(\alpha\)\(^8\). TKI-induced up-regulation of N-cadherin in LSC, and adhesion to mesenchymal stem cells led to increased canonical Wnt signalling and protection of the LSC from apoptosis\(^8\). The CXCL12 ligand and its receptor CXCR4 has been linked to intracellular LYN signalling in LSC\(^8\), and the CXCL12/CXCR4 axis is regulated through CXCL12 cleavage by CD26\(^9\). Reduced homing capacity of LSC has also been attributed to alterations of the CXCL12/CXCR4 signalling pathway as a result of increased granulocyte-colony stimulating factor (G-CSF)
levels which conferred a selective growth disadvantage to normal HSC\(^{91}\). LSC also exert other molecular and phenotypic effects on HSC through extrinsic IL-6 signalling in the CML BMM\(^{92,93}\). Indeed, a variety of ligand-receptor mediated signalling pathways regulate CML LSC in the BMM (Figure 3, Table 2).

It is likely that LSC also avoid eradication by modulation of host immune surveillance in the BMM (reviewed in detail elsewhere\(^{94}\)). In this respect, cytotoxic T lymphocytes (CTLs) are unable to elicit an appropriate immune response against CML cells through CTL exhaustion - and this is believed to be mediated by the interaction of the PD-1 receptor expressed on CTLs with its inhibitory ligand PD-L1 expressed on CML cells. PD-L1 is expressed on patient derived CML cells\(^{95}\) and on LSC in mouse models of CML\(^{96}\). Blockade of the PD-1/PD-L1 interaction in combination with T-cell immunotherapy was able to trigger the loss of LSC, and prevent development of CML-like disease\(^{96}\). Our recent work has demonstrated that cytokine-mediated downregulation of MHC-II expression may be an alternative way that LSC evade immune surveillance – and treatment with ruxolitinib or interferon gamma (IFN\(\gamma\)) can reverse this effect \textit{in vitro} and enhance proliferation of responder CD4\(^+\)CD69\(^+\) T cells in mixed lymphocyte reactions\(^{97}\). These examples represent exciting areas of research that could lead to new immune therapy-based therapeutic approaches.

**NEW THERAPIES TO TARGET LSC: RECENT APPROACHES**

The many examples summarised above illustrate the scope of potentially drug-able targets that have been identified in CML to eradicate LSC (Tables 1-2). Disappointingly, drugs against these targets have yet to be implemented in the clinic as standard of care. In the past 3-4 years, additional drug-able targets and
pathway have been identified, while others previously identified have been further elaborated in pre-clinical studies (Figure 4). Our analysis of global proteomics and transcriptomics in drug-naïve primary patient material (bulk CD34+ cells and LSC) pointed towards a dependency of CML cells on a p53 and c-MYC regulated network. This provided a rationale to use a combination of MDM2 and BET inhibitors (MDM2i, BETi respectively) to target the synergistic eradication of LSC through up-regulation of the p53 apoptotic pathway and down-regulation of c-MYC by both drugs (Figure 4A). Given that BETi acts generally as a transcriptional repressor, how its effects lead to up-regulation of apoptosis in CML LSC is not fully understood, although this appears to be a common phenomenon of BETi in pre-clinical cancer studies. We have also used global epigenetic and transcriptomic analysis of drug-naïve primary patient material to reveal that mis-regulation of the PRC2 complex (including kinase independent down-regulation of EZH1 in LSC) results in the functional dependency of LSC on EZH2 and its biochemical readout H3K27me3. Using murine models, others have also reported that CML LSC are dependent on EZH2. Combining an EZH2 inhibitor (EZH2i) with TKI was highly effective at eradicating the LSC population. Our data supports a model whereby apoptosis is induced in CML LSC through the up-regulation of EZH2 targets upstream of p53 (such as ARF), which could lead to increased p53 levels, or through up-regulation of p53 target genes directly which are normally repressed by EZH2 activity (Figure 4A).

Two groups have shown that activators of the peroxisome proliferator-activated receptor gamma (PPARγ) have increased anti-leukaemic activities in combination with TKI. Quiescence of LSCs is regulated by a pathway involving the receptor PPARγ, STAT5, HIF2α, and CITED2 - a master regulator of blood stem cell
quiescence (Figure 4B). Activators of PPARγ result in transcriptional down-regulation of STAT5, whilst TKI block phosphorylation of STAT5 – the combined effects of both drugs significantly down-regulating this pathway and caused LSC to exit quiescence where they were eradicated by TKI\(^{104}\). Recently, EZH2 has been shown to be activated by STAT5 in CML cells\(^{105}\) suggesting possible cross-talk between the effects of PPARγ activators and those of EZH2i.

The TKI-mediated up-regulation of CD70 has been further examined to provide a clear rationale for inhibiting non-canonical Wnt/β-catenin signalling in LSC\(^{67}\). Upon exposure to TKI, the microRNA miR-29 is down-regulated – the consequence of which is up-regulation of CD70 through the opposing roles of miR-29 on SP1 and DNMT1a regulation (Figure 4C). Thus, antibody-based blockade of the interaction between CD70 and CD27 resulted in a potent loss of LSC in the presence of TKI\(^{67}\).

Two other routes for inhibiting β-catenin signalling in LSC have also recently been deduced. In the first, BCR-ABL1 interacts directly with JAK2 in a kinase independent manner to activate a JAK2/β-catenin survival/self-renewal pathway that results in inhibition of PP2A and activation of β-catenin (Figure 4D). Use of PP2A activating drugs (PADs) reversed these effects resulting in GSKβ-dependent degradation of β-catenin and eradication of LSC\(^{77}\). In the second, another enzyme in fatty acid metabolism arachidonate 15-lipoxygenase (15-LO encoded by ALOX15) has been implicated in the kinase independent up-regulation of β-catenin, although the exact mechanism is unclear. However, pharmacological inhibition of 15-LO in combination with nilotinib on human LSCs \textit{in vitro} appeared synergistic\(^{106}\).

In addition, the p-selectin SELP appears to be a key down-stream target of 15-LO, which is normally repressed to promote LSC survival. Further pre-clinical studies
and mechanistic studies are required to provide a clearer rationale for taking 15-LO inhibitors into clinical trials as has been done with zileuton which inhibits 5-LO69.

FUTURE CHALLENGES

We know little about how TKI-resistant LSC clones evolve in patients in MMR and the degree of intra- and inter- patient heterogeneity that is likely to exist – not only at the DNA level, but also with respect to the many pathways that we have identified by studying diagnostic drug-naïve LSC for many years. This is because (i) the TKI-resistant LSC are extremely rare in the BM of these CML patients, and (ii) the LSC cannot be selectively isolated from the normal HSC that reconstitute normal haemopoiesis in the BM subsequent to TKI therapy. Surrogate in vivo analysis has also been problematic since the majority of CML primary samples do not engraft well in commonly used immunodeficient mice strains. These issues most likely underpin the failure of many promising new drugs to deliver results in clinical trials. However, recent advances in tracking individual normal and malignant clones in xenograft models using bar-coding21,107, the development of humanised xenograft models108, an explosion of single cells technologies20,109, and the identification of a number of leukaemia-specific cell surface markers, make the analysis of individual LSC or LSC clones much more accessible. Furthermore, several groups have identified markers that discriminate LSC from HSC (CD2690, IL-1RAP110, CD25111 and CD93112) but how these will perform in samples from patients in MMR has yet to be determined. For those of us that are intent on curing CML, this new era of game-changing technologies provides some tantalizing prospects that will enable us to finally stem the tide on drug-resistant LSC.
REFERENCES


### TABLES

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<td>Wnt/Ca²⁺/NFAT signalling</td>
<td>cyclosporin A</td>
<td>Gregory et al. 2010</td>
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<td>CD25</td>
<td>JAK/STAT signalling</td>
<td>BEZ235</td>
<td>Sadovnik et al. 2015</td>
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<td>CD70/CD27</td>
<td>Wnt/β-catenin signalling</td>
<td>αCD70 mAb</td>
<td>Riether et al. 2015</td>
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<td>c-MYC &amp; TP53</td>
<td>apoptosis</td>
<td>CPI-203; RITA/RG7388</td>
<td>Abraham et al. 2016</td>
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<td>EZH2</td>
<td>histone H3 K27 trimethylation</td>
<td>GSK 343; GSK126; EPZ-6438 (tazemetostat)</td>
<td>Scott et al. 2016; Xie et al. 2016</td>
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<td>farnesyl transferases</td>
<td>RAS signalling; protein farnesylation</td>
<td>BMS-214662</td>
<td>Copland et al. 2008</td>
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<td>FOX03A</td>
<td>TGFβ/AKT/FOXO3a/ BCL6 signalling</td>
<td>n.a.</td>
<td>Pellicano et al. 2013; Naka et al. 2010</td>
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<td>GSK3β</td>
<td>Wnt/β-catenin signalling</td>
<td>SB216763</td>
<td>Reddiconto et al. 2012</td>
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<td>HDACs</td>
<td>histone acetylation</td>
<td>LBH589</td>
<td>Zhang et al. 2010</td>
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<td>JAK2</td>
<td>JAK/STAT signalling</td>
<td>ruxolitinib</td>
<td>Gallipoli et al. 2014</td>
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<td>PML</td>
<td>apoptosis; mTOR repression</td>
<td>arsenic trioxide (As₂O₃)</td>
<td>Ito et al. 2008</td>
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<td>PP2A</td>
<td>JAK/STAT/β-catenin signalling</td>
<td>1,9-dideoxy-forskolin; FTY720</td>
<td>Neviani et al. 2005; Neviani et al. 2013</td>
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<td>PPARγ</td>
<td>STAT5/HIF2α/ CITED2</td>
<td>pioglitazone</td>
<td>Prost et al. 2015; Glodkowska-Mrowka et al. 2016</td>
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<td>RAD52</td>
<td>DNA repair</td>
<td>RAD52 F79 peptide aptamer</td>
<td>Cramer-Morales et al. 2013</td>
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<td>SIRT1</td>
<td>deacetylation of p53</td>
<td>tenovin-6 (TV-6)</td>
<td>Li et al. 2012</td>
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<td>SMO</td>
<td>Hedgehog signalling</td>
<td>cyclopamine; LDE225;</td>
<td>Zhao et al. 2009; Dierks et al. 2008; Irvine et al. 2016</td>
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<td>STAT3</td>
<td>JAK/STAT signalling</td>
<td>BP-5087</td>
<td>Eiring et al. 2015</td>
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**Table 1: Candidate therapeutic targets in CP CML LSCs.** Column one: key survival factors/drug targets intrinsic to the LSC. Column two: the roles of these factors/targets in cellular pathways (if known or as proposed by investigators). Column three: exemplar compounds that have been identified as having potential therapeutic value for each factor/target or pathway (if known). Column four: references where the factors/targets were described. Column five: whether factors/targets or compounds have been evaluated in clinical trials that we know of.

<table>
<thead>
<tr>
<th>TGF-β RI, ALK5</th>
<th>TGFβ/AKT/FOXO3a/BCL6 signalling</th>
<th>Ly364947; EW-7197</th>
<th>Naka et al. 2010[^56]</th>
<th>Naka et al. 2016[^122]</th>
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[^56]: Naka et al. 2010
[^122]: Naka et al. 2016
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<th>TARGET OR SURVIVAL FACTOR</th>
<th>PATHWAY</th>
<th>EXEMPLAR INHIBITORS/ACTIVATORS</th>
<th>REFERENCES</th>
<th>CML CLINICAL TRIAL</th>
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<td>β1-integrins</td>
<td>cell adhesion; proliferation</td>
<td>IFNα</td>
<td>Bhatia et al. 1994&lt;sup&gt;87&lt;/sup&gt;  Bhatia et al. 1996&lt;sup&gt;86&lt;/sup&gt;</td>
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<td>CD26</td>
<td>CXCL12/CXCR4 signalling</td>
<td>glyptins</td>
<td>Herrmann et al. 2014&lt;sup&gt;90&lt;/sup&gt;</td>
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<td>CD44</td>
<td>e-selectin ligands; homing, engraftment</td>
<td>n.a.</td>
<td>Krause et al. 2006&lt;sup&gt;83&lt;/sup&gt;</td>
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<td>CXCR4</td>
<td>CXCL12/CXCR4 axis</td>
<td>AMD3465; AMD3100; plerixafor</td>
<td>Jin et al. 2008&lt;sup&gt;123&lt;/sup&gt;  Weisberg et al. 2012&lt;sup&gt;124&lt;/sup&gt;  Dillmann et al. 2009&lt;sup&gt;125&lt;/sup&gt;  Vianello et al. 2010&lt;sup&gt;126&lt;/sup&gt;</td>
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<td>BMP2/4</td>
<td>BMP signalling</td>
<td>n.a.</td>
<td>Lapерrousaz et al. 2013&lt;sup&gt;127&lt;/sup&gt;</td>
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<td>GAL-3 (Galectin-3)</td>
<td>BM lodgement</td>
<td>n.a.</td>
<td>Yamamoto-Sugitani et al. 2011&lt;sup&gt;85&lt;/sup&gt;</td>
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<td>HIF1α</td>
<td>hypoxia response</td>
<td>n.a.</td>
<td>Zhang et al. 2012&lt;sup&gt;128&lt;/sup&gt;  Ng et al. 2014&lt;sup&gt;129&lt;/sup&gt;</td>
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<td>IFNα</td>
<td>immune surveillance; cytokine-mediated proliferation</td>
<td>interferon alfa-2a</td>
<td>Preudhomme et al. 2010&lt;sup&gt;130&lt;/sup&gt;  Burchert et al. 2010&lt;sup&gt;131&lt;/sup&gt;  Simonsson et al. 2011&lt;sup&gt;132&lt;/sup&gt;  Nicolini et al. 2015&lt;sup&gt;133&lt;/sup&gt;  Hjorth-Hansen et al. 2016&lt;sup&gt;134&lt;/sup&gt;</td>
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<td>IL-1RAP/IL-1</td>
<td>NFKβ/AKT signalling</td>
<td>IL-1RAP mAb (mAb81.2)</td>
<td>Jaras et al. 2010&lt;sup&gt;110&lt;/sup&gt;  Ågerstam et al. 2016&lt;sup&gt;135&lt;/sup&gt;  Zhang et al. 2016&lt;sup&gt;136&lt;/sup&gt;</td>
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<td>IL-6</td>
<td>IL-6Rα signalling from the microenvironment</td>
<td>aIL-6 mAb</td>
<td>Welner et al. 2015&lt;sup&gt;52&lt;/sup&gt;</td>
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<td>LYN</td>
<td>CXCR4/CXCL12 signalling in lipid rafts</td>
<td>methyl-beta-cyclodextrin; PP2</td>
<td>Tabe et al. 2012&lt;sup&gt;89&lt;/sup&gt;</td>
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<td>MHC-II/CIITA</td>
<td>JAK/STAT signalling</td>
<td>ruxolitinib, IFNγ</td>
<td>Tarafdar et al. 1&lt;sup&gt;97&lt;/sup&gt;</td>
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<td>miR-126</td>
<td>exosomal transfer</td>
<td>2-O-Me-miR-126</td>
<td>Taverna et al. 2014&lt;sup&gt;137&lt;/sup&gt;</td>
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<td>N-cadherins</td>
<td>N-cadherin/ Wnt/β-catenin signalling</td>
<td>ICG001</td>
<td>Zhang et al. 2013&lt;sup&gt;88&lt;/sup&gt;</td>
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<td>JAGGED-1</td>
<td>Notch signalling</td>
<td>n.a.</td>
<td>Bowers et al. 2015&lt;sup&gt;138&lt;/sup&gt;</td>
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<td>MPL</td>
<td>JAK/STAT signalling; engraftment</td>
<td>n.a.</td>
<td>Zhang et al. 2016&lt;sup&gt;139&lt;/sup&gt;</td>
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<td>PD-1/PD-L1</td>
<td>immune surveillance</td>
<td>αPD-L1 mAb, αPD-1 mAb</td>
<td>Mumprecht et al. 2009&lt;sup&gt;95&lt;/sup&gt;, Riether et al. 2015&lt;sup&gt;96&lt;/sup&gt;</td>
<td>Yes</td>
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<td>PIGF</td>
<td>VEGF signalling: BM angiogenesis; proliferation; metabolism</td>
<td>5D11D4</td>
<td>Schmidt et al. 2011&lt;sup&gt;140&lt;/sup&gt;</td>
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<tr>
<td>selectins</td>
<td>BM homing; engraftment</td>
<td>GMI-1271</td>
<td>Krause et al. 2014&lt;sup&gt;84&lt;/sup&gt;, Aggoune et al. 2014&lt;sup&gt;141&lt;/sup&gt;</td>
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<td>TGF-β1</td>
<td>osteoblast → LSC TGFβ signalling</td>
<td>parathyroid hormone (PTH)</td>
<td>Krause et al. 2013&lt;sup&gt;142&lt;/sup&gt;</td>
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**Table 2: Candidate therapeutic targets implicated in CP CML LSC/BMM interactions.** Column one: key survival factors that act through LSC/BMM interactions. Column two: the roles of these survival factors in cellular pathways (if known or as proposed by investigators). Column three: exemplar compounds that have been identified as having potential therapeutic value for each target (if known). Column four: references where the targets were described. Column five: whether targets or compounds have been evaluated in clinical trials.
FIGURE LEGENDS

Figure 1: The CP CML patients’ journey through TKI therapy for 5 years. The schematic shows the clinical outcome for a typical 100 CP CML patients with respect to response to imatinib over the course of 5 years, and the decision-tree leading to discontinuing TKI or switching to 2nd or 3rd generation TKI for various reasons. Outcomes were compiled based on data obtained from various sources\textsuperscript{31,143-146}. By the end of year 5: twelve (12) (green segment of the pie chart) of these 100 patients will typically be off TKI and in therapy-free remission (TFR), more than a quarter (26) (red segment of the pie chart) will have failed TKI therapy (even after drug switching or through disease progression to AP or BC), and the majority (62) (amber segment of the pie chart) will remain on long-term TKI therapy but have residual disease due to LSC persistence in their BM.

Figure 2: General features and critical pathways that contribute to CP CML LSC being quiescent, refractory to apoptosis and prone to DNA damage. Typically LSC represent 1-5% of the bulk CML CD34\textsuperscript{+} cells, are enriched by FACS as CD34\textsuperscript{+}CD38\textsuperscript{−}, and show more variable levels of Ph\textsuperscript{+} cells than bulk CML CD34\textsuperscript{+} cells. Some researchers also include Lin\textsuperscript{−}/CD90\textsuperscript{+}/CD45RA\textsuperscript{−} cells as part of the CD34\textsuperscript{+}CD38\textsuperscript{−} LSC definition\textsuperscript{112}. Other FACS approaches can also be used to isolate LSC which employ Hoechst, Pyronin Y and carboxyfluorescein succinimidy ester (CFSE) intracellular staining in combination with CD34 to identify quiescent/undivided cells\textsuperscript{39,46,47,147}. CD34\textsuperscript{+}CD38\textsuperscript{−} CML cells from patients at diagnosis which retain high levels of CFSE (CFSE\textsubscript{max}) or are CD34\textsuperscript{+} and both Hoechst\textsuperscript{lo} and Pyronin Y\textsuperscript{lo}, and survive exposure to TKI, are often considered surrogate \textit{in vitro} models for the TKI-resistant cells that are found in patients with LSC persistence. The schematic diagram of the LSC shows key (but not exhaustive) pathways and components and whether the published evidence points to TKI-dependent (blue) or independent (olive green) mechanisms of regulation. Dotted lines denote translocation of components from the cytoplasm (light red) to the nucleus (white). ROS = reactive oxygen species. TK = tyrosine kinase. Activation and repression are denoted according to convention. Specific details of each pathway are described in the text.

Figure 3: LSC survival signalling in the CP CML bone marrow microenvironment (BMM). The schematic diagram of the BMM shows key (but not...
exhaustive) pathway components that mediate signalling between the LSC (light red) and other BMM cell types. HSC is shown in blue. OB = osteoblast cells (tan); CTL = cytotoxic T-cell (turquoise). Ligands involved in various signalling pathways are shown as small coloured spheres. IL-1/IL-1RAP regulates NFKβ signalling in LSC, and can be blocked using a monoclonal antibody to IL-1RAP. MPL, the thrombopoietin (TPO) receptor, regulates JAK/STAT signalling and CML patients with high MPL expression on their LSC have reduced sensitivity to BCR-ABL1 kinase inhibition with TKI, but a higher sensitivity to JAK inhibitors. Leukemic progenitor expansion is driven by exposure of LSC, overexpressing BMPR1B, to BMP2 and BMP4. The CML BMM is also thought to over-express the NOTCH ligand JAGGED-1 implicating NOTCH signalling in LSC quiescence. LSC stimulate the production of placental growth factor (PIGF) by BM stromal cells which works in a positive feedback loop to increase angiogenesis of the BM and promote CML cell proliferation through FLT1 (VEGFR1) signalling. Stimulation of BM osteoblasts with parathyroid hormone (PTH) resulted in bone remodelling and production of TGFβ1, eradicated LSC by stimulating TGFβ signalling (the opposite effect to other reports of TGFβ signalling in LSC). Similarly, others have shown that expansion of the osteoblast layer of the CML BMM can contribute to creating a hostile environment for HSC – and these effects are mediated by TPO, CCL3 and direct cell-cell interactions that alter TGFβ, NOTCH and pro-inflammatory signalling in the remodelled osteoblasts. Other abbreviations are as described in the text. Other features are as described in Figures 2 and 3.

**Figure 4: Recent therapeutic approaches to target the eradication of CP CML**

**LSC. A.** Dual targeting of c-MYC and TP53 (p53) or combined treatment with TKI and EZH2 inhibitor (EZH2i). Both approaches converge on up-regulating p53-mediated apoptosis through different mechanisms. BET and MDM2 inhibitors (BETi and MDM2i respectively) lead to synergistic repression of c-MYC transcription and up-regulation of p53 target genes. A dependency on EZH2 for LSC survival is accompanied by a TKI-independent down-regulation of EZH1. **B.** Inhibition of STAT5 upstream of the HIF2α-CITED2 pathway that governs LSC quiescence. Combining a PPARγ activator (PPARγa) with TKI inhibits STAT5 transcription and STAT5 phosphorylation respectively and down-regulates HIF2α-CITED2 leading to LSC exit from quiescence. **C.** Inhibition of non-canonical Wnt/β-catenin signalling mediated by
CD70/CD27. TKI up-regulates the Wnt/β-catenin pathway by inhibiting miR-29 expression thus facilitating both increased CD70 expression and CD70/CD27 receptor/ligand interaction. Treatment with a monoclonal antibody that blocks the CD70/CD27 interaction (αCD70) in a TKI background blocks the pathway. D. Activation of PP2A to inhibit a novel CML network driven by JAK2-β-catenin signalling. PP2A activating drugs (PADs) disrupt the PP2A-SET interaction thereby allowing PP2A reactivation which inhibits BCR-ABL1 recruitment of JAK2 (TKI-independent) and impairs β-catenin signalling through GSK-3β activation. E. Inhibition of ALOX15 to inhibit β-catenin and PI3K/AKT signalling. Knockdown of ALOX15 or treatment with a 15-LO inhibitor (15-LOi) which blocks ALOX15 enzymatic activity reduced LSC survival in association with reduced PI3K/AKT and β-catenin levels. This “kill” phenotype was rescued by loss of p-selectin (SELP), which is thought to negatively regulate LSC self-renewal and survival. TK = tyrosine kinase. Activation and repression are denoted according to convention. Drug treatments are shown in yellow. Further details are described in the text.
Figure 1. Holyoake and Vetrie

100 cases of CP CML IM 5 yr

50 on IM

40

10

50 off IM

IM intolerant → switch TKI

IM resistant → switch TKI

disease progression

TKI failure on TKI (LSC persistence) off TKI (TFR)

26

12

62
Figure 3. Holyoake and Vetrie