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Stem Cells in Chronic Myeloid Leukaemia

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Over the last decade, the stem cell field has been one of the most fascinating areas of medical research. In several types of malignancy, both haematological and solid tumours, tissue stem cells are now implicated as cancer initiating cells. Chronic myeloid leukaemia (CML) is a paradigm cancer in this field. It is a clonal disorder of the haemopoietic stem cell (HSC) that affects 1 to 2 individuals per 100,000 every year and is associated with an increased production of mature and immature myeloid cells as a consequence of the formation of the Philadelphia chromosome (Ph) and *Bcr-Abl* oncogene. Extensive knowledge regarding the central role of Bcr-Abl in disease pathogenesis, acquired over many years, has facilitated development of new drugs that selectively target Ph$^+$ malignant cells. In this article, we review the main characteristics of cancer stem cells and discuss novel therapeutic approaches that aim to eradicate CML stem cells.

Stem cells are characterised by the capacity to divide and produce identical copies of themselves (self-renewal) and to differentiate into many other types of cell. They are present in several regions of the human body at every stage of development from embryo to adult. Because of their versatility, stem cells have been studied extensively with the purpose of repairing and/or replacing damaged human tissue in a variety of diseases and injuries, including Parkinson's disease, stroke and diabetes.

Stem cells are usually divided into two different subgroups, pluripotent stem cells, which differentiate during embryonic development into cells of the

endoderm, ectoderm, and mesoderm (i.e. embryonic stem cells) and multipotent stem cells, which are lineage specific and include e.g. HSC, neuronal stem cells and hepatic stem cells (Reviewed in [47]).

The HSC is one of the best-characterised stem cell systems [88]. Progeny derived from HSCs consist of oligopotent progenitors for lymphoid and myeloid cells. As with all stem cells, HSCs have the ability to self-renew indefinitely and to generate the cells of our blood and immune system, such as white and red blood cells and platelets. In adults, HSCs are produced in the bone marrow and, under the right conditions, are induced to migrate out into the blood stream (mobilisation). The signals that trigger a stem cell to undergo differentiation, self-renewal or to mobilise *in vivo* are still unclear, however it has been shown *in vitro* that HSC undergo differentiation upon treatment with SCF, Flt3L, Tpo, and IL-3 [14, 27, 30, 32, 61, 70, 98], whereas Wnts, Notch, and Sonic hedgehog (Shh) promote HSC proliferation [2, 7, 53, 93].

Cancer stem cells, which have been identified in many forms of leukaemia, are thought to be generated, either by mutation in a normal stem cell which already has the capacity for self-renewal, or in a progenitor cell [23, 44, 55] which then acquires self-renewal potential – thus abnormal self-renewal is considered one of the most distinguishing hallmarks of cancer cells. Self-renewing cancer stem cells give rise to all the malignant progeny in a tumour, and if not completely eradicated by chemotherapy, they establish a reservoir of drug-resistant cells responsible for relapse after a chemotherapy-induced remission.

Biomarkers in CML

CML is an excellent example of a leukaemia in which the initiating event for early chronic phase (CP) occurs within the normal HSC compartment, yet for progression to blast crisis (BC) further mutation within the progenitor compartment with acquisition of self-renewal potential has been proposed (16, 19).

CML arises as a consequence of a rare mutational event within a single pluripotent HSC, resulting in a reciprocal translocation between the long arms of chromosomes 9 and 22, leading to the creation of the Ph [83]. Disease progression can be divided into 3 phases: (1) CP, characterised by an excessive production of differentiated but functionally immature granulocytes; (2) accelerated phase (AP) characterised by an increased number of primitive precursors in the bone marrow (BM) and/or peripheral blood (PB); (3) and BC, where there is a block to differentiation leading to accumulation of primitive blast cells and acquisition of additional chromosomal abnormalities [15].

The creation of the Ph results in the expression of a constitutively active tyrosine kinase, (p210)-Bcr-Abl [26], an oncoprotein that shows greater tyrosine kinase activity than its wild-type counterpart, c-abl [60]. Forced over-expression of Bcr–Abl causes cytokine independent survival and cell growth and protects HSC from apoptosis in response to either growth factor withdrawal or DNA damage [29, 67, 73]. Bcr-Abl activity is necessary to cause stem cells to transform both *in vitro* and *in vivo,* leading to the development of leukaemia [84]. As a tyrosine kinase, Bcr–Abl acts constitutively to activate multiple signal-transduction cascades involved in cell growth and differentiation. In particular, Bcr-Abl expression triggers a number of signalling pathways that positively affect stem cell turnover, alter cellular adhesion and block apoptosis. Bcr-Abl substrates include CRKL [72, 74], RAS [62], RAF [75], phosphatidylinositol-3 kinase (PI3K) [90], JUN kinase [79], MYC [85], and STAT [89].

Conventionally treatment response in the clinic is determined using standard laboratory techniques including full blood count, cytogenetics for Ph in BM and fluorescence in situ hybridisation and Q-RT-PCR for Bcr-Abl levels in PB.

In the setting of clinical trials this has been greatly extended to assess the ability of new drugs to block proliferation or induce cell death and to inhibit Bcr-Abl and its down-stream effectors. These biomarkers include, for example, markers of proliferation (Ki67, BrdU) and apoptosis (active caspase-3, annexin V, TMRE, TUNEL), Bcr-Abl phosphorylation, STAT 1, 3 and 5 signalling and AKT phosphorylation, aiming to correlate individual or

combinations of biomarkers with plasma drug levels obtained at similar time-points. More exploratory pharmacogenetic and pharmacogenomic assessments examine whether individual genetic variation relating to the target pathway for the drug of interest, or relating to overall drug metabolism, impact upon treatment response or predisposition to serious side effects. In certain instances more global tumour-related genomics, proteomics and metabonomics have been conducted, aiming to identify additional pathways altered by the drug of interest or associated with either response to therapy or disease progression despite therapy. One example of successful biomarker development utilised Western blotting for P-CRKL in total leucocytes derived from PB to determine imatinib mesylate (IM) sensitivity *in vivo* and showed a positive correlation between IM sensitivity and clinical response [97].

Our group has identified a subpopulation of deeply quiescent stem cells in all patients with CP CML. We have demonstrated that this quiescent subpopulation has a primitive phenotype (CD34$^+$, CD38$^-$, HLA DR$^-$, CD45RA$^-$, CD71$^-$) and can induce Ph$^+$/Bcr-abl$^+$ reconstitution following transplantation in immunocompromised mice, suggesting that *in vivo* the quiescent state is only temporary and reversible under specific circumstances [40]. The presence of quiescent CML stem cells with self-renewal and transplantation capability may explain the historical failure of intensive cell cycle-dependent chemotherapy to eradicate CML, with subsequent relapse in all patients. Distinct from their normal counterparts, CML HSC are characterised by autocrine production of IL-3 and G-CSF and are therefore able to survive and proliferate in the absence of exogenous growth factors [45, 46]. Interestingly, autocrine IL-3 production is switched on as quiescent cells move into cell cycle, suggesting a possible future target for therapy [41]. In terms of CML stem cell related biomarkers, the rarity of the target population makes all such studies challenging. However the activity of Bcr-Abl, before and after drug treatment *in vitro* or *in vivo*, can now be tracked in small cell samples using flow cytometry detection of p-CRKL [36].

Targeted Therapeutics and New Research in CML

Recent advances in our understanding of stem cell molecular and cellular biology has enabled scientists to begin to explore their use in cell-based therapies and as tools in drug discovery. Over all tumour areas work with cancer stem cells is less advanced, however in the leukaemias stem cells are detectable in both BM and PB which are easily accessible. An added advantage for CML is that the underlying molecular and cellular biology are reasonably well understood. In order to eradicate CML stem cells we need to identify relevant selective targets, which in turn, requires a comprehensive understanding of how and why cancer/CML stem cells differ from normal stem cells, including the factors that are important in triggering malignant transformation and disease progression. Our research is now focusing on understanding what controls maintenance of CML stem cell quiescence and the molecular effects induced by Bcr-Abl expression at the stem cell level. This review will discuss three different approaches recently used to selectively target CML stem cells: (1) targeting Bcr-Abl activity using _T_yrosine _K_inase _I_nhibitors (TKI); (2) combining growth factor stimulation to induce CML cell cycle re-entry with TKI; (3) targeting pathways downstream of Bcr-Abl, such as RAS (table 1).

Targeting Bcr-Abl Activity Using TKIs

The current first line therapy for patients with CP CML is IM (Gleevec or Glivec, Novartis Pharmaceuticals, Basel, Switzerland) [4], a compound belonging to the 2 phenylaminopyrimidine family. IM is a rationally designed TKI of Abl, platelet-derived growth-factor receptor (PDGFR) α and β, and KIT [76]. Since the (p210)-Bcr-Abl tyrosine kinase originates from Abl, IM shows an impressive efficacy against proliferating Ph[+]/Bcr-Abl^{+} leukaemic cells [65]. The mechanism of action of IM was originally thought to be by acting as a competitive inhibitor of the ATP-binding pocket. Recently it has been shown that IM occupies only part of the ATP-binding pocket of the enzyme, and that it acts by binding to and stabilising the inactive, non-ATP-binding form of Bcr-Abl [86].

IM induces a rapid haematological and cytogenetic response in the majority of CP CML patients (complete cytogenetic response 82% at 60 months) [25]. However, IM does not eliminate Bcr-Abl transcripts in the majority of patients, suggesting the persistence of minimal but detectable residual disease [8, 43]. A reservoir of resistant leukaemic stem cells is likely to explain this residual disease, as well as the rapid kinetics of relapse observed in most patients who discontinue IM, even a long time after achieving an apparent molecular remission [68] [66, 82]. The mechanism(s) through which these cells are inherently insensitive to IM, or become IM-resistant, remains unclear. One explanation may be the presence or generation of kinase domain mutations in the leukaemic stem cell compartment [34], however our group has been unable to detect such mutations following *in vitro* drug exposure and the rapid relapses following IM withdrawal in patients have shown wild type and not mutant Bcr-Abl. Our group and others have clearly demonstrated that CD34-enriched CML stem cells are not targeted by IM, even at high concentrations, with the most primitive and quiescent cells most resistant to the drug [35, 40]. In addition, IM exerts a reversible anti-proliferative effect on these primitive quiescent CML cells, preventing their entry into cell cycle, thus making their targeting and eradication even more difficult [35, 39].

New TKIs with higher potency against wild type Bcr-Abl and with activity against the majority of IM-resistant mutants of Bcr-Abl have recently been developed and brought to the clinic. Two of these are nilotinib (formerly AMN107, Novartis) and dasatinib (formerly BMS-354825, Bristol-Myers Squibb).

Nilotinib is a novel selective inhibitor of Bcr-Abl designed by modification to the molecular framework of IM, with activity based on the same mechanism of action: it binds to the inactive conformation of Abl, but with a better topographical fit. Pre-clinical data showed that *in vitro* nilotinib has significant activity against a number of IM-resistant Bcr-Abl mutants [33, 63]. *In vivo,* nilotinib promotes survival in IM-resistant CML mouse models and in mice that have been injected with Bcr-Abl-transformed cell lines or primary leukaemia cells [96]. A phase I clinical trial has recently shown that nilotinib induces

cytogenetic responses in 53% of patients with IM-resistant CML in CP [52]. Although nilotinib is more potent than IM, it is not effective against the T315I mutant [96].

Dasatinib is an orally active multi-targeted Src- and Abl-kinase inhibitor that, differently from IM and nilotinib, binds to both the active and inactive conformation of the Abl kinase [58, 87]. Dasatinib is more effective than IM or nilotinib in the majority of cell lines expressing IM-resistant Bcr-Abl mutations, but not against those populations expressing the T315I mutation [96]. Despite the enhanced Bcr-Abl inhibitory activity, recent data from our lab showed that nilotinib and dasatinib, even at high concentrations, are ineffective against the quiescent CML stem cell population [21, 51]. Combination of the three Bcr-Abl TKIs may be more effective against IM-resistant subclones, as synergism of IM with nilotinib has been shown in cell lines and a murine model of CML [96]. As a consequence of disease persistence and resistance, TKIs cannot be considered a cure for the majority of patients with CML. If used in combination these drugs may be more effective against resistance mutations, however there is no evidence that this will increase their efficacy against the stem cell population.

In the laboratory setting, mutation screens are commonly used to predict the profile of the mutations that confer TKI resistance in order to design a rational approach for combination strategies [3, 11, 19, 94, 95]. More than twenty-five amino acid substitutions at 21 positions have been identified so far to confer IM resistance in CML patients undergoing treatment [71]. Many of these mutations sterically preclude the TKI from binding to Bcr-Abl or cause a conformational change in the conserved phosphate binding (P) loop, diminishing drug binding [5, 12, 34, 37, 38].

Recently, mathematical models capable of describing the development and progression of tumours and their response to treatments have been proposed. These models provide quantitative understanding of the dynamics of tumourigenesis with respect to mutation, selection, genetic instability and

tissue architecture. The effects of IM on the autophosphorylation of Bcr-Abl and its downstream protein CRKL have been examined in CML cells using a mathematical model that predicted a minimal concentration for drug effectiveness [17]. More recently, a mathematical model has been used to analyse the *in vivo* kinetics of IM in CML patients [69]. The model indicates that IM is a potent inhibitor of the production of differentiated CML cells, but it does not totally eradicate CML. In addition, the model allows one to predict the probability of developing IM resistance mutations. Finally, a third mathematical model has proposed a selective functional effect of IM on proliferative leukaemia stem cells to explain Bcr-Abl transcript dynamics over time [80].

Combining Growth Factor Stimulation with TKIs

In vitro studies of AML and CML cells have previously shown that the effectiveness of cell cycle–specific chemotherapeutic agents can be enhanced by prior exposure to growth factors [6, 16, 92]. Furthermore, a clinical trial in AML confirmed improved responses for patients with standard risk disease who received growth factor priming [59]. Therefore, since TKIs are primarily targeting proliferating cells and IM is known to have anti-proliferative activity, an approach to eradicate primitive quiescent CML cells would be to induce them to re-enter the cell cycle before and after treatment with TKIs. Our group has recently shown that *in vitro* pulsing with growth factors, such as granulocyte-colony stimulating factor (G-CSF), promotes cell cycle re-entry prior to and following treatment with IM and significantly improves stem cell kill in comparison to IM alone [49]. G-CSF has already been used in order to overcome IM–induced neutropenia in patients with CML, leading to improved cytogenetic responses. Although assumed to be a consequence of increased exposure to IM, these improved responses could also represent a direct effect of G-CSF exposure on the progenitor compartment [50] [65, 78, 91]. The combination of IM and G-CSF has now been translated by our group into an NCRI clinical trial protocol named G-CSF and imatinib mesylate intermittently (GIMI). Results from this trial are expected 2008.

Targeting Pathways Downstream of Bcr-Abl

Recent advances in understanding the mechanisms of defective molecular pathways in cancer cells have enabled the identification of novel targets and therapeutic compounds. A new class of anti-cancer drugs that has recently been investigated is the farnesyl transferase inhibitors (FTIs). The FTIs competitively inhibit the enzyme farnesyl transferase (FT), irreversibly interrupting essential cell-signalling pathways. FTs act by coupling a 15-carbon isoprenyl group to the target protein, allowing correct intracellular localisation and signal transduction [31, 54].

FTIs were initially designed to inhibit prenylation of RAS. The RAS-MAP family is composed of protein-serine/threonine kinases that are highly conserved in evolution. These proteins are activated in response to a variety of growth factors and are known to regulate cell growth, death and differentiation [18]. Since RAS activation is implicated in leukaemogenic transformation by Bcr-Abl, inhibition of FT could be of therapeutic value in CML. Furthermore, mutations in the RAS family genes (H-RAS, K-RAS, and N-RAS) frequently occur in many types of cancer [10] and in haematological malignancies, suggesting wider application. However the observed anti-tumour effects of FTIs are not solely due to RAS inhibition [7, 81], but may involve inhibition of farnesylation of other proteins, including RhoB, RAC, centromere binding proteins and lamins [56] [1]. In fact malignancies containing non-mutated RAS may also be sensitive to FTIs, suggesting activity against an alternative pathway. For these reasons, the exact mechanism of action of FTIs in individual cancers is still unknown.

FTIs tested in clinical trials include BMS-214662 (Bristol-Myers Squibb, Princeton, NJ,), L778123 (Merck & Co., Inc., Whitehouse Station, NJ,), tipifarnib (R115777; Zarnestra; Ortho Biotech Products, L.P., Bridgewater, NJ,), lonafarnib (SCH66336; Sarasar; Schering-Plough Corporation, Kenilworth, NJ,), FTI-277 (Calbiochem, EMD Biosciences, San Diego), and L744832 (Biomol International L.P., Plymouth Meeting, PA,) [13, 24].

BMS-214662 is a non-peptidomimetic cytotoxic FTI [64] that produces potent tumour regression and curative responses in human tumour xenografts and transgenic tumour models [28, 57]. BMS-214662 differs from other cytostatic FTIs, including lonafarnib and tipifarnib, which have non-curative activity against these tumours [28, 57]. BMS-214662 has been shown to preferentially kill non-proliferating cells (*Proceedings of the AACR* 42, 260S, 2001) and has anti-leukaemic activity in AML [22]. Our group has demonstrated that single agent BMS-214662 significantly reduces the quiescent CML HSC population *in vitro*, and is able to overcome the anti-proliferative accumulation that is seen with IM and dasatinib, if used in combination with these agents [20]. Although BMS-214662 exerts a strong FTI activity *in vitro* [81], its overall mechanism of action in killing the stem cell fraction is still not totally clear.

Lonafarnib is a non-peptidomimetic FTI that showed activity against Bcr-Abl-induced leukaemia in a murine model and against primary cells from patients with CML [42, 77]. *In vitro*, lonafarnib enhanced the toxicity of IM against K562 and against the quiescent CD34$^+$ cells [48], whereas a phase I clinical trial showed that, in CML patients in CP or AP resistant or intolerant to IM, lonafarnib has modest activity [9].

Conclusions

CML is the first malignancy recognised to originate from a single clonal mutation. This abnormality leads to the formation of a population of CML stem cells known to be primitive, quiescent and cytokine independent. Allogeneic bone marrow transplantation is considered to be the only curative treatment for patients with CML. However, the restricted number of donors, advanced age of patients and toxicity of this approach limits its application.

Although major advances in the treatment of CML have occurred in recent years with the development of IM, dasatinib and nilotinib, quiescent CML stem cells remain insensitive to these compounds, at least *in vitro*. Therefore, treatment strategies involving combination of TKIs or inhibitors of the Bcr–Abl

signal-transduction pathway are under intense investigation. Researchers are now focusing on drugs, such as FTIs, that specifically target quiescent stem cells. Remarkably, these drugs, alone or in combination with TKIs, have provided promising preliminary results *in vitro* (Figure 1). In particular BMS-214662, by inducing selective apoptosis of quiescent leukaemic stem cells in CP CML, represents a useful tool for developing novel approaches to target cancer stem cells and for future drug discovery.

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