Characterization of cancer stem cells in chronic myeloid leukaemia

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Abstract
CML (chronic myeloid leukaemia) is a myeloproliferative disease that originates in an HSC (haemopoietic stem cell) as a result of the t(9;22) translocation, giving rise to the Ph (Philadelphia chromosome) and bcr-abl oncoprotein. The disease starts in CP (chronic phase), but as a result of genomic instability, it progresses over time to accelerated phase and then to BC (blast crisis), becoming increasingly resistant to therapy. bcr-abl is a constitutively active tyrosine kinase that has been targeted by TKIs (tyrosine kinase inhibitors), including IM (imatinib mesylate), nilotinib and dasatinib. We have developed various flow cytometry techniques to enable us to isolate candidate CML stem cells from CP patients at diagnosis that efflux Hoechst dye, express CD34, lack CD38 and are cytokine-non-responsive in culture over periods of up to 12 days in growth factors. These stem cells have been shown to regenerate bcr-abl-positive haemopoiesis in immunocompromised mice upon transplantation. We previously demonstrated that IM was antiproliferative for CML stem cells but did not induce apoptosis. Clinical experience now confirms that IM may not target CML stem cells in vivo with few patients achieving complete molecular remission and relapse occurring rapidly upon drug withdrawal. Our recent efforts have focused on understanding why CML stem cells are resistant to IM and on trying to find novel ways to induce apoptosis of this population. We have shown that CML stem cells express very high levels of functional wild-type bcr-abl; no kinase domain mutations have been detected in the stem cell population. Dasatinib, a more potent multitargeted TKI than IM, inhibits bcr-abl activity more efficiently than IM but still does not induce apoptosis of the stem cell population. Most recently, we have tested a number of novel drug combinations and found that FTIs (farnesyl transferase inhibitors) have activity against CML. BMS-214662 is the most effective of these and induces apoptosis of phenotypically and functionally defined CML stem cells in vitro, as a single agent and in combination with IM or dasatinib. The effect against CML stem cells is selective with little effect on normal stem cells. The drug is also effective against BC CML stem cells and equally effective against wild-type and mutant bcr-abl, including the most resistant mutant T315I. In association with apoptosis, there is activation of caspase 8 and caspase 3, inhibition of the MAPK pathway, IAP-1 (inhibitor of apoptosis protein-1), NF-κB (nuclear factor κB) and iNOS (inducible nitric oxide synthase). Furthermore, BMS-214662 synergizes with MEK1/2 [MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated kinase) kinase 1/2] inhibitors, suggesting a second mechanism other than RAS inhibition for induction of apoptosis. Our intentions are now to explore the activity of BMS-214662 in other cancer stem cell disorders and to move this preclinical work to a clinical trial combining dasatinib with BMS-214662 in CML.

CML (chronic myeloid leukaemia), a paradigm for stem cell-derived malignancy
CML was one of the first malignancies to which a defined genetic abnormality was ascribed and is a paradigm for stem cell-derived cancer. Following the post-war atomic testing in the Pacific, increased detection of myeloid leukaemias such as CML was noted among witnesses to those tests. However, little is actually known about the initiating oncogenic event that precipitates CML, other than that the aberration must arise in an immature HSC (haemopoietic stem cell), which has the potential to produce many daughter cells as well as identical copies of itself (self-renew). In 1960, Nowell and Hungerford [1] described a shortened chromosome 22 [the so-called Ph (Philadelphia chromosome)] attributable to reciprocal transfer of genetic material between the long arms of chromosomes 9 and 22. Simultaneous breaks occur in the bcr gene on chromosome 22 and the abl gene on chromosome 9, such that the novel fusion oncogene created on chromosome 22 is called BCR-ABL [2]. From this gene is transcribed and translated a constitutively active tyrosine kinase of the same name (BCR-ABL), which is causative in CML and the target of therapeutic intervention [3].

CML is a triphasic myeloproliferative disorder normally presenting in a relatively benign CP (chronic phase) in which

Key words: BCR-ABL, farnesyl transferase, myeloid leukaemia, quiescence, stem cell, tyrosine kinase.

Abbreviations used: BC, blast crisis; CFSE, carboxyfluorescein succinimidyl ester; CML, chronic myeloid leukaemia; CP, chronic phase; FISH, fluorescence in situ hybridization; FTI, farnesyl transferase inhibitor; HSC, haemopoietic stem cell; IM, imatinib mesylate; LTC-IC, long-term culture-initiating cell; Ph, Philadelphia chromosome; TKI, tyrosine kinase inhibitor; QPCR, quantitative PCR.

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Ph is the only genetic abnormality. After an average of 5–7 years, CP transforms into AP (accelerated phase), characterized by an increased number of blast cells present in the bone marrow and peripheral blood, which in a relatively short time (6–9 months) terminates in BC (blast crisis; 3–6 months duration) where numerous additional genetic aberrations may be noted in Ph+ HSCs, e.g. trisomy 8 or 17 or i(17q) [4]. The therapeutic options for newly diagnosed patients with CML have expanded recently with the introduction of targeted, orally active small-molecule TKIs (tyrosine kinase inhibitors) [5,6]. Nevertheless, leucapheresis, a method of obtaining supplies of leucocytes, is performed at diagnosis to provide cells to bank, for example, for possible future use in an autologous stem cell transplant. From this source of material, which is very much in excess from leukaemia patients with high leucocyte counts (>10×10⁶ cells/litre), the leukaemic HSCs can be harvested for research purposes. This is achieved by labelling and isolating cells based on their CD34 expression, a cell-surface marker indicative of primitive haemopoietic cells.

Quiescent stem cell isolation and tracking

Within the total CD34+ stem/progenitor cell population, <1% will be true stem cells, which are quiescent (out of cycle), phenotypically (CD34high/CD38low) and functionally (capable of giving rise to Ph+ progeny in secondary recipients) primitive. In order to be able to study HSC behaviour in vitro, FACS technology can be utilized to not only isolate but track these rare cells in a number of ways.

Vital staining of CD34-enriched HSCs with CFSE (carboxyfluorescein succinimidyl ester) enables tracking of cell division history in vitro. Thus cells maximally labelled with CFSE that do not divide will remain CFSEmax in culture and can be enumerated separately from cells that have undergone cell division such that daughter cells fluoresce at half the intensity of the parent [7]. Alternatively, the quiescent nature of HSCs can be exploited using the DNA and RNA dyes, Hoechst 33342 and pyronin Y respectively such that nature of HSCs can be exploited using the DNA and RNA intensity of the parent [7]. Alternatively, the quiescent cell division such that daughter cells fluoresce at half the can be sorted from cells progressing through the cell cycle (G2, S, G2 and M), which accumulate tetraploid DNA before cell division with increased RNA expression. Finally, CD34high/CD38low/nil cell-surface expression can be used as an approximation of the quiescent stem cell pool (approx. 5% of total CD34+ cells).

CML HSCs identified by the various FACS methods described above have been confirmed to be leukaemic [Ph+ by FISH (fluorescence in situ hybridization) and BCR-ABL+ by QPCR (quantitative PCR)] [8,9]. Their quiescent status has been ascertained by gene expression (cdc25−, Ki67−, p21+) that has been corroborated by FACS analysis for cyclins D1–D3 and Ki67. These cells are small with low forward scatter by FACS, and are non-responsive to cytokines in culture. Quiescent CML cells expressing a primitive phenotype (CD34+ CD38−) are functionally primitive in that they are transplantable into murine recipients from which human CD34+ and CD34− leukaemic cells can be harvested.

CML stem cells are insensitive to TKIs, e.g. imatinib

Using our FACS-based technology, we have identified that quiescent CML HSCs are insensitive to TKI designed to specifically knock out the kinase activity of BCR-ABL. IM (imatinib mesylate; Gleevec®, Novartis Pharma) was the first inhibitor to reach the market, and did so in record time owing to its remarkable specificity, safety and efficacy in CP CML [5]. Compared with the previous standard pharmacotherapy, IFN-α (interferon-α), IM induced significant haematological (normalization of leucocyte count) and cytogenetic (absence of Ph+ cells in bone marrow spreads) responses in treated patients [6]. Nonetheless, most of these patients have minimal residual disease detectable by sensitive QPCR. From our in vitro work notably carried out in primary HSCs and not in cell line models, we have shown that CML HSCs escape the effects of IM through drug-induced, reversible cell cycle arrest [7,10]. This ‘antiproliferative’ effect results in HSC accumulation, the antithesis to stem cell eradication.

Evidence in support of the hypothesis that these persistent IM-insensitive HSCs identifiable in vitro exist in vivo include: molecular relapse on stopping IM sometimes directly to BC; the low incidence of molecular remission among treated patients in the first place [6]; and the persistence of up to 20% BCR-ABL+ CD34+ cells as well as LTC-ICs (long-term culture-initiating cells) in all tested patients who have achieved a complete cytogenetic response [11].

Why do CML HSCs escape IM?

IM-insensitivity among CML HSCs can be explained in one of two ways, that is, by BCR-ABL-dependent or -independent mechanisms. In the former scenario whereby there may be BCR-ABL amplification or overexpression, gene mutation or simply inadequate intracellular drug levels, the HSCs remain dependent on BCR-ABL for growth and survival. This means that BCR-ABL is still a relevant target in these cells. On the other hand, resistance that is BCR-ABL-independent implies that cells are no longer oncogene ‘addicted’ and potentially have acquired additional genetic mutations that drive alternative survival mechanisms even when BCR-ABL activity is optimally inhibited by TKIs.

We have analysed cells surviving IM treatment in vitro to ascertain which mechanism underlies their TKI-insensitivity. Gene copy number was assessed by standard FISH, mRNA expression level by QPCR, and a screen for kinase domain mutations done by BCR-ABL sequencing. BCR-ABL kinase activity was determined by flow cytometric assessment of intracellular CrkL phosphorylation status (downstream of BCR-ABL) as well as by total pTyr (phosphotyrosine)/BCR-ABL phosphorylation by Western blotting [12].

No gene amplification or mutation was detected in a consecutive series of primary CP CML samples [13]. However, it was notable that CD34+38 HSCs exhibited a significant (10-fold) increase in message level compared with total mature mononuclear cells that was reflected in an increase in BCR-ABL activity (pCrkL and pTyr). We next
Overcoming resistance by TKI-targeted drug combinations

There are multiple possible pathways downstream of BCR-ABL signalling to which drugs may be targeted to be used in combination to effect greater cell kill than TKI alone (Figure 1). For example, an HSP90 (heat-shock protein 90) antagonist such as 17-AAG (17-allylamino-17-demethoxygeldanamycin) would be a logical choice as it would interfere with BCR-ABL chaperoning such that the mature protein would be unable to fold correctly into its quaternary structure, leaving it susceptible to proteasome degradation. The Ras/Raf pathway is intimately linked to BCR-ABL through the adaptor molecules Grb2 (growth-factor-receptor-bound protein 2) and CrkL, inhibition of which with an FTI (farnesyl transferase inhibitor), such as lonafarnib, would theoretically reduce nuclear transcription

sought rational combinations of drugs that would synergize with TKI to eradicate HSCs.

<table>
<thead>
<tr>
<th>Pathway / molecular target</th>
<th>Example of Inhibitor</th>
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<tbody>
<tr>
<td>1  Bcr-Abl point mutations</td>
<td>Nilotinib, Dasatinib</td>
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<tr>
<td>2  mRNA</td>
<td>siRNA vs anti-apoptotic genes</td>
</tr>
<tr>
<td>3  HSP-90 antagonists</td>
<td>17-AAG</td>
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<tr>
<td>4  Tyrosine kinase inhibitors</td>
<td>Adaphostin</td>
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<tr>
<td>5a Farnesyl transferase inhibitors</td>
<td>Lonafarnib, R115777,</td>
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<tr>
<td></td>
<td>BMS-214662, BMS-225975</td>
</tr>
<tr>
<td>5b Farnesyl / geranylgeranyl transferase</td>
<td>Zoledronate</td>
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<tr>
<td>6  Mek (MAPK or ERK kinase) inhibitors</td>
<td>PD098059, PD184352, U0126</td>
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<tr>
<td>7  Phosphatidyl inositol 3-kinase inhibitors</td>
<td>LY294002</td>
</tr>
<tr>
<td>8  mTor inhibitor</td>
<td>Rapamycin</td>
</tr>
<tr>
<td>9  Active transporters</td>
<td>Range of agents</td>
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</tbody>
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Figure 1 | Possible signalling pathways that may be drug targeted to complement TKI activity in CML stem cells

4E-BP1, eukaryotic initiation factor 4E-binding protein 1; mTOR, mammalian target of rapamycin; siRNA, small interfering RNA; S6K, S6 kinase; SoS, Son-of-Sevenless.
CML stem cells are insensitive to most drug combinations

The number of undivided CD34\(^+\) cells remaining in vitro after 72 h in the growth factor (GF)-only control was set to 1 in each experiment for each individual CML patient’s cells tested (\(n = 8\)), and the recovery of undivided CD34\(^+\) cells (HSCs) for each condition was then normalized to this value. None of the five agents tested at their respective IC\(_{50}\) reduced the number of recovered HSCs, alone or in combination with IM with the exception of the FTI, lonafarnib, which in combination with 5 \(\mu\)M IM caused a slight reduction in the number of recovered HSCs (indicated by arrows).

required for cell proliferation and growth. In addition, the PI3K (phosphoinositide 3-kinase) axis with Akt that impinges on anti-apoptotic pathways could be interrupted by a specific inhibitor such as LY294002.

None of these combinations, however, with the possible exception of lonafarnib (although this did not reach statistical significance), could reduce the number of HSCs recovered after in vitro treatment compared with TKI alone [10] (Figure 2). Moreover, some of the combinations resulted in further accumulation of quiescent cells.

**Novel agents**

**Dasatinib, a dual Src/ABL kinase inhibitor**

Following on from the successful introduction of IM into the clinic, a novel dual Src/ABL TKI, dasatinib (formerly BMS-354825), was developed by Bristol Myers Squibb. This novel orally active agent was reportedly 50–100-fold more potent than IM at inhibiting BCR-ABL\(^+\) cell proliferation with an IC\(_{50}\) in vitro of 10 nM although more than 200 nM is achievable in vivo [14]. Dasatinib has activity against most of the IM-resistant kinase mutations with the exception of T315I and inhibits BCR-ABL in CD34\(^+\)CD38\(^-\) CML stem cells, although not completely, more effectively than nilotinib or IM [13].

**BMS 214662, a putative FTI**

A cytotoxic as opposed to a cytostatic FTI (such as lonafarnib), BMS-214662 reportedly selectively inhibits farnesyl transferase over geranylgeranyl transferase and demonstrates preferential cytotoxicity against non-proliferating (quiescent) solid tumour cells [15]. We therefore wished to determine this compound’s activity against CML HSCs and its mechanism of action.

BMS-214662 significantly reduced the number of non-proliferating (CFSE\(_{\text{max}}\) CD34\(^+\)) cells remaining after 6 days in culture with respect to no drug control, IM (5 \(\mu\)M) or dasatinib (150 nM). Moreover, the combination of BMS-214662 with each of these TKIs resulted in further reductions in quiescent cell numbers compared with either agent alone [16].

We next determined the effect of BMS-214662 on LTC-IC, a primitive colony-forming cell. Briefly, cells were cultured with drugs in growth factor-supplemented media for 72 h, washed, then added to irradiated cytokine-releasing feeder layers for 5 weeks with replacement of half the medium supernatant on a weekly basis. Surviving cells were harvested and plated in semi-solid culture media to allow outgrowth of colonies after 14–16 days. The readout is indicative of the number of primitive LTC-IC that survived initial drug treatment. IM and dasatinib protected CML stem cells with an increased colony formation over no drug control. Significantly, all arms containing BMS-214662 virtually eliminated colony formation with no significant difference between BMS-214662-containing arms, indicating that this was a BMS-214662-driven effect. Most of the cells in residual colonies were BCR-ABL\(^-\) by FISH with BMS-214662 less toxic to normal CD34\(^+\) cells than CML (reduced colony formation in the former to approx. 40% of the no drug control; \(P = \text{NS}, n = 3\)).

BMS-214662 is effecting cell kill through apoptosis as shown by increased caspase 3 activity in treated CML compared with normal CD34\(^+\)CD38\(^-\) cells. However, this is not via inhibition of Ras as originally hypothesized as
another FTI with equivalent Ras inhibition, BMS-225975, neither reduced the number of CFSE<sup>max</sup>CD34<sup>+</sup> cells or Ki67<sup>neg/7AAD<sup>lo</sup> cells nor increased caspase 3 activity in these cells compared with no drug control (M. Copland, F. Pellicano, L. Richmond, E.K. Allan, A. Hamilton, F.Y. Lee, R. Weinmann and T.L. Holyoake, unpublished work). Irrespective of its enigmatic activity, BMS-214662 is the first drug reported to be highly effective against primitive, quiescent CML stem cells (Figure 3). We are currently investigating the true mechanism of action of BMS-214662 in CML stem cells with a view to further drug development and use in other leukaemias and cancer stem cell disorders.

**Conclusion**

In summary, CML is a paradigm for stem cell disease and molecularly targeted therapy. While rationally designed TKIs have proven effective in disease management, they do not offer a cure owing to the persistence of insensitive HSCs. One possible explanation for this molecular persistence is oncogene overexpression at the message, protein and kinase activity levels that is not overcome by possible subtherapeutic levels of TKI within HSCs. Nevertheless, introduction of novel agents such as the putative FTI BMS-214662 will make significant advances towards the goal of elimination of the diseased stem cell population, preferentially targeting quiescent leukaemic cells over normal. Further elucidation of its true mechanism of action will potentially inform drug development for use in other leukaemias and stem cell-derived cancers.

**References**


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