Chronic myeloid leukemia stem cells are not dependent on Bcr-Abl kinase activity for their survival

Citation for published version:

Digital Object Identifier (DOI):
10.1182/blood-2010-12-326843

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Blood

Publisher Rights Statement:
© 2012 by The American Society of Hematology

General rights
Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and/or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Recent evidence suggests chronic myeloid leukemia (CML) stem cells are insensitive to kinase inhibitors and responsible for minimal residual disease in treated patients. We investigated whether CML stem cells, in a transgenic mouse model of CML-like disease or derived from patients, are dependent on Bcr-Abl. In the transgenic model, after retransplantation, donor-derived CML stem cells in which Bcr-Abl expression had been induced and subsequently shut off were able to persist in vivo and reinitiate leukemia in secondary recipients on Bcr-Abl reexpression. Bcr-Abl knockdown in human CD34+ CML cells cultured for 12 days in physiologic growth factors achieved partial inhibition of Bcr-Abl and downstream targets p-CrkL and p-STAT5, inhibition of proliferation and colony forming cells, but no reduction of input cells. The addition of dasatinib further inhibited p-CrkL and p-STAT5, yet only reduced input cells by 50%. Complete growth factor withdrawal plus dasatinib further reduced input cells to 10%; however, the surviving fraction was enriched for primitive leukemic cells capable of growth in a long-term culture-initiating cell assay and expansion on removal of dasatinib and addition of growth factors. Together, these data suggest that CML stem cell survival is Bcr-Abl kinase independent and suggest curative approaches in CML must focus on kinase-independent mechanisms of resistance. (Blood. 2012; 119(6):1501-1510)

Introduction

In chronic myeloid leukemia (CML), the malignant clone is driven by the oncogene Bcr-Abl. Although there is strong evidence that Bcr-Abl is sufficient to induce CML-like disease in transduction/transplantation and transgenic murine models, it is less clear that Bcr-Abl is always the first hit in CML in humans or that the disease in chronic phase is maintained by Bcr-Abl as the only driver, rather than by additional genetic and/or epigenetic changes, as has been shown recently for Philadelphia-positive and -negative acute lymphoblastic leukemia. Therefore, comparisons between mouse models and primary CML should be interpreted with caution. In primary CML studies, kinase inhibitors, imatinib, dasatinib, or nilotinib induced high rates of apoptosis. Similarly, in transduction/transplantation and transgenic murine models of Ph+ B-cell leukemia or CML, inhibition of Bcr-Abl kinase by kinase inhibitors or by switching off transgene expression, resulted in proliferation arrest, apoptosis, and complete remissions.

Kinase inhibitors have transformed the natural history of CML by inducing cytogenetic and molecular responses in the majority of patients in chronic phase and resulting in transient deep responses in many cases of advanced disease. A proportion of CML cases develops drug resistance, with the incidence increasing in more advanced disease. In most cases, the leukemic cells continue to express Bcr-Abl and often retain their dependence on the oncogene. For example, where the underlying mechanism for drug resistance is expansion of a clone expressing a Bcr-Abl kinase domain mutation resulting in imatinib resistance, the leukemic cells retain sensitivity to second-generation kinase inhibitors.

Those working on CML now believe we are on a “pathway to cure”; however, this belief is tempered by the insensitivity of CML stem cells to kinase inhibitors. We have consistently demonstrated that kinase inhibitors, imatinib, nilotinib, dasatinib, and bosutinib, although exhibiting potent antiproliferative effects, are only weak inducers of apoptosis in CML stem and progenitor cells. In primitive stem/progenitor cells (CD34+38−) harvested from CML patients in durable complete cytogenetic response over 5 years, Bcr-Abl transcripts show no suggestion of a downward trend over time. Furthermore, genomic PCR and PCR on individual long-term culture-initiating cell (LTC-IC) colonies reveals that CML patients on imatinib who achieve complete molecular response remain Bcr-Abl positive. These in vitro and in vivo results, derived from primary human CML stem and progenitor cells, at least hint that CML stem cells may not be “oncogene addicted.” However, in these studies, no conclusive evidence was provided that Bcr-Abl activity had been fully suppressed in the surviving cells and in particular in leukemic stem cells with repopulating potential, with many groups focused on...
issues of drug transport and more potent kinase inhibitors. These data have recently been conducted by a carefully conducted study which concluded that primary CML stem cells are insensitive to imatinib despite inhibition of Bcr-Abl. Similarly, in transgenic mice, multiple rounds of induction and reversion of Bcr-Abl are possible, suggesting long-term persistence of leukaemic stem cells. However, as these studies were performed in primary recipients only, there was no proof that transplantable stem cells were responsible for disease reinitiation. To address these critical points, we used complementary in vivo and in vitro approaches to determine whether Bcr-Abl activity is required for the survival of transplantable murine CML-like and primary human CML stem cells and conclude that this critical population is independent of Bcr-Abl kinase activity for survival.

Methods

Reagents
Dasatinib was obtained from Bristol-Myers Squibb (BMS). Stock solution (10 mg/mL) in DMSO (Sigma-Aldrich) was prepared and stored in aliquots at -20°C.

Primary cell samples
After informed consent, CD34+ cells were enriched from CML chronic-phase samples at diagnosis and non-CML controls and cryopreserved.

In vitro cell culture
HT1080 and 293FT cells were cultured in DMEM, K562, KCL22, and BaF3 cells in RPMI 1640 medium, supplemented with 1% penicillin/streptomycin (10,000 µg/mL/10,000 U/mL), 1% L-glutamine, and 10% FCS (all Invitrogen). IL-3 (10 ng/mL; StemCell Technologies) was added to parental BaF3 cells. Primary CML cells were cultured in serum-free medium, supplemented with a high growth factor cocktail, 100 ng/mL Flt-3 ligand and SCF, and 20 ng/mL each of IL-3, IL-6, IL-7 (StemCell Technologies) and G-CSF (Chugai Pharma Europe Ltd), growth factor cocktail for transduction, 50 ng/mL SCF, 100 ng/mL thrombopoietin (TPO) and Flt-3 ligand, physiologic growth factor cocktail, 0.2 ng/mL SCF, 1502 HAMILTON et al BLOOD, 9 FEBRUARY 2012 VOLUME 119, NUMBER 6
Results

Leukemic stem cells retain their transplantation and leukemogenic potential after complete reversion of Bcr-Abl

We previously described an inducible transgenic mouse model of CML in which p210-Bcr-Abl expression is targeted to stem and progenitor cells of murine BM using the tet-off system.2,8 On tetracycline withdrawal, Bcr-Abl expression is induced and mice demonstrate leukocytosis, splenomegaly, and myeloid hyperplasia. The disease is transplantable using Bcr-Abl+ unfractionated (uf) BM or LSK cells and can be reverted after tetracycline treatment or to a much lesser extent using imatinib.5 In both transgenic and primary transplant recipients, the CML-like disease is fatal after 29-122 days.2,8

Here we used the CD45.1/45.2 system to discriminate donor and host cells in a transplantation setting and were thus able to serially transplant the initially leukemic cells after abrogation of Bcr-Abl expression and reversion of the CML phenotype (Figure 1A). SCLIT/Bcr-Abl double-transgenic (dtg) BM cells from CD45.1 donors were transplanted into 8Gy sublethally irradiated CD45.2 recipients (n = 12). Wild-type (wt) CD45.1 donors were used as controls (n = 12). An alternative approach would have been to use dtg mice as donors and to maintain one cohort on and the other one off tetracycline throughout the experiment. This was considered but decided against in view of concern that tetracycline might lead to undesired effects on either normal or leukemic hemopoiesis. Recipient mice were maintained off tetracycline to induce Bcr-Abl expression as shown previously.2,8 PB analysis on day 21 confirmed that dtg recipient mice had developed disease and this was confirmed in BM and spleen on day 25 when mice were killed. At this time point, donor BM LSK showed a slight but significant 1.2-fold expansion compared with controls (supplemental Figure 1A-G, available on the Blood Web site; see the Supplemental Materials link at the top of the online article).
Tetracycline was then administered to the remaining mice to abrogate Bcr-Abl expression and revert the phenotype (Figure 1Aii). By day 41 on PB sampling the disease had been completely reverted with no difference between dtg and controls (supplemental Figure 1H-ii) and this was confirmed at the time of sacrifice on day 48, with no evidence of leukemia in BM or spleen (supplemental Figure 1I-M). Strikingly, the percentages of mature and immature granulocytic donor cells decreased to control levels in dtg BM and spleen on Bcr-Abl abrogation (compare supplemental Figure 1B-C, I-J), showing that proliferation and survival of mature cells are affected by Bcr-Abl abrogation. Conversely, BM LSK donor cells had continued to increase by equivalent amounts in control and dtg mice (supplemental Figure 1M) suggesting that dtg donor LSK cells showed similar chimerism dynamics as controls. Bcr-Abl was neither detectable in total BM nor spleen cells, nor in FACS-sorted CD45.1+ BM cells from either cohort. Histology of spleen showed no evidence of leukemic infiltration and there was no evidence of splenomegaly. To assess potential residual Bcr-Abl expression in reverted LSK cells, we FACS-sorted these cells from a cohort of primary, transgenic mice that had either been induced for 3 weeks or reverted for an additional 6 weeks. Analysis of BM, spleen, and PB confirmed neutrophilia and splenomegaly restricted to induced, but not reverted dtg or control mice (supplemental Figure 2A-B). RT-PCR using LSK cells showed a > 96% reduction of Bcr-Abl expression in reverted mice back to control levels (supplemental Figure 2C). To assess Bcr-Abl activity, we performed Western blot using lineage-negative BM cells from mice that had either been induced for 4 weeks (supplemental Figure 2E) or mice that had been reverted for 68 days after a 3-week induction period (supplemental Figure 2F). Level of CrkL phosphorylation was increased on induction of Bcr-Abl (supplemental Figure 2E) but was decreased to control level on reversion (supplemental Figure 2F). These results confirmed that by administration of tetracycline the leukemic phenotype had been completely reverted.

As a final step, FACS-sorted, BM-derived, CD45.1+ cells from each cohort were retransplanted, at 1.2 × 10^6 cells/mouse, into 9 Gy sublethally irradiated, secondary recipients (CD45.2/H11003 and LSK of Bcr-Abl–expressing cells but was completely reverted on 100nM dasatinib treatment (Figure 1I). In addition we demonstrated that dasatinib treatment significantly induced apoptosis in leukemic total BM and mature Gr1-positive cells, whereas Bcr-Abl–expressing dtg LSK were comparatively resistant (Figure 1J).

**Partial Bcr-Abl knockdown inhibits proliferation of CML CD34+ cells**

Recently Shah et al argued for evaluation of more than one end point for inhibition of Bcr-Abl to ensure coverage of an adequate dynamic range.14 Measurement of inhibition of phosphorylation of STAT5 and CrkL by flow cytometry and Western blotting was therefore optimized in BaF3 cells expressing Bcr-Abl (supplemental Figure 3A-C). A total of 150nM dasatinib reduced phosphorylation of both CrkL and STAT5 to the baseline seen in parental BaF3 cells, with no effect on Bcr-Abl T315I mutant and no additional effect with increasing concentrations of dasatinib (supplemental Figure 3A-C). Previous data using lentiviral-mediated shRNA delivery confirmed efficient and specific knockdown of Bcr-Abl in Ph+ cell lines expressing B3A25,35 (supplemental Figure 3D-E). Here Bcr-Abl was knocked down in CML CD34+ cells expressing B3A2, cultured in SFM supplemented with physiologic growth factors, and viral transduction of the most primitive CD34+38 population verified (supplemental Figure 4A-B). To measure the extent of Bcr-Abl knockdown, transduced CD34+ CML cells were FACS sorted. Q-PCR confirmed 56% specific down-regulation of Bcr-Abl mRNA (Figure 2A) and Western blot showed clear reduction in Bcr-Abl protein level (48% by densitometry; Figure 2B). We then sought to demonstrate inhibition of Bcr-Abl downstream targets. In primary CML CD34+ cells, 150nM dasatinib was sufficient to reduce p-CrkL27 and p-STAT5 to the same level (described as baseline) as non-CML CD34+ cells (Bcr-Abl-negative control; Figure 2C-D). In Bcr-Abl knockdown, CML cell inhibition of phosphorylation of both CrkL and STAT5 was observed in CD34+ and CD34+38 cells by flow cytometry (gated on GFP+ cells; supplemental Figure 4C), and in CD34+ cells for STAT5 by Western blotting (Figure 2B), although the level of inhibition was not as profound as seen with dasatinib.

While sh-Control expressing cells were able to expand ~ 3-fold over 12 days in the presence of physiologic growth factors, sh-Bcr-Abl cells showed little expansion (Figure 3A), with some induction of apoptosis (Figure 3B). To examine the effect of Bcr-Abl knockdown on survival/proliferation of functional CML stem and progenitor cells, GFP+ cells were plated into CFC and LTC-IC assays (Figure 3C-D). Bcr-Abl knockdown resulted in 47% and 71% reduction in CFC and LTC-IC colonies, respectively. Q-PCR on single colonies for Bcr-Abl and Western blotting for p-CrkL on pooled colonies confirmed Bcr-Abl inhibition in cells expressing sh-Bcr-Abl (Figure 3E-F).

**A proportion of CML CD34+ cells is sensitive to enhanced levels of Bcr-Abl inhibition**

To determine whether CML CD34+ cells would be susceptible to further inhibition of Bcr-Abl activity, GFP+ knockdown and control cells were cultured in physiologic growth factors in the presence and absence of 150nM dasatinib for 12 days, with fresh medium and dasatinib replaced on days 4 and 8. When GFP+ CML CD34+ cells were exposed to 150nM dasatinib (Figure 2E-F), further reductions in phosphorylation of CrkL and STAT5—over those seen with sh-Bcr-Abl, and to previously described baseline levels—were seen, confirming complete inhibition of Bcr-Abl kinase activity. For dasatinib, cell counts on days 4, 8, and 12 illustrated
a sharp reduction in cell numbers evident by day 4, irrespective of
Bcr-Abl knockdown (Figure 3A). Most interestingly, ∼50% of input
cells survived combined partial Bcr-Abl knockdown and dasatinib
treatment for 12 days, indicating that, at least in medium supplemented
with physiologic growth factors, a proportion of CD34
cells can
survive without Bcr-Abl kinase activity. However, with the limited
number of cells available it was not feasible to confirm complete
Bcr-Abl inhibition at all time points, neither was it possible to examine
whether, as expected, surviving cells were enriched for primitive
stem cells.

Complete Bcr-Abl kinase inhibition is achievable at the
stem/progenitor cell level
To investigate in more detail whether CML stem/progenitor cells
would be susceptible to complete inhibition of Bcr-Abl activity,
CML CD34
cells were cultured in SFM, in the presence or
absence of dasatinib, which was replaced with fresh medium on
days 4 and 8. After preliminary experiments (supplemental Figure
5A–D, supplemental Table 1), these cells were cultured without
growth factors to exclude any additional survival signals. In the
remaining viable cells on days 4, 8, and 12 Bcr-Abl activity was
fully inhibited as demonstrated by complete inhibition of p-CrkL
by flow cytometry and by Western blotting (Figure 4A–B). Because
genuine CML stem cells likely represent a small fraction of total
CD34
cells, previous work has focused on those CD34
cells that
remain quiescent in culture by tracking cell division with
CFSE.16,17,28 Here the level of inhibition of p-CrkL by continuous
exposure to dasatinib 150nM was determined on days 4, 8, and 12 for
viable CML CD34
cells that remained undivided in culture or
entered cell divisions 1, 2, or 3. The level of inhibition of p-CrkL
achieved across the entire time course showed no significant
difference for primitive undivided CD34
CML cells compared
with more mature cells able to enter division (Figure 4C, supplemen-
tal Table 2). Inhibition of p-STAT5 by dasatinib gave a very similar
profile as seen for p-CrkL (supplemental Figure 6A). Although no
significant differences were detected in levels of inhibition between
undivided and cells in divisions 1–3, we repeated the experiment
including a higher concentration of dasatinib to ensure that
inhibition of p-CrkL was complete (Figure 4D). Critically, residual
levels of p-CrkL were equivalent for 150 and 1000nM dasatinib.

The most primitive, quiescent CML stem and progenitor cells
are independent of Bcr-Abl kinase for survival
In the mouse, hemopoietic stem cells can be selected to near purity
(1 in 2) based on surface markers. However, this is not yet possible
for normal or leukemic human stem cells. The human stem and
progenitor compartment should therefore be considered as a
continuum and not made up of discrete populations. Here we have
combined accepted surrogate markers for primitive cells based on
phenotype, CFSE retention, absence of Ki-67 staining, expansion
kinetics, CFC, CFC replating, and LTC-IC to demonstrate which
CML cells are growth factor and Bcr-Abl kinase independent. Over
the same 12 days, in the absence of growth factors, untreated CML CD34⁺ cells expanded by ~4-fold, in keeping with their autocrine production of cytokines, whereas with 150nM dasatinib viable cells decreased to 10% of input (Figure 5A). CFSE profiling demonstrated that even in the absence of growth factors and with addition of dasatinib, CML CD34⁺ cells were able to enter divisions 1 and 2, with a few cells progressing as far as division 3 (Figure 5B). The untreated control CML CD34⁺ cells proliferated more actively, with the majority of cells found in divisions 3 and 4. Although CFSE profiling confirmed that most CML CD34⁺ cells had gone through one or more divisions in the presence of dasatinib, by day 12, >85% of residual cells had become quiescent.

Figure 3. Functional analysis of CML CD34⁺ cells following Bcr-Abl knockdown by dasatinib. (A) Proliferation of GFP⁺ sorted cells treated ±150nM dasatinib in physiologic growth factors over 12 days (n = 3). (B) Apoptosis measured in infected cells (gated on GFP) by annexin V and viaprobe (n = 2). (C) CFC (n = 4, P = .025) and (D) LTC-IC assays (n = 1). (E) Bcr-Abl levels were measured by Q-PCR on colonies from CFC assay (n = 7 colonies for sh-scrambled and 8 colonies for sh-Bcr-Abl). (F) The level of total p-CrkL was measured by Western blotting on pooled colonies from CFC assay (n = 1).

Figure 4. Complete Bcr-Abl inhibition is achieved by dasatinib within primitive subpopulations of CML cells. CFSE-stained CD34⁺ CML cells (n = 3) were cultured in SFM ± 150 or 1000nM dasatinib for 12 days. (A) The levels of total p-CrkL were measured by flow cytometry and (B) by Western blotting at day 12. p-CrkL was also measured within each cell division after treatment with either (C) 150 or (D) 1000nM dasatinib.
as demonstrated by low Ki-67 staining (Figure 5Ci-ii). To confirm that the viable residual cells were functional and not irreversibly arrested or senescent, on day 12, both experimental arms were washed and reestablished in culture in SFM with added growth factors. Ki-67 and 7AAD analysis confirmed that the majority of CML CD34$^+$/H11001 cells that had been cultured in the absence of growth factors and in the presence of dasatinib at 150nM for 12 days rapidly entered cell division with only 6% remaining Ki-67 low after a further 7 days of culture (Figure 5Ciii-iv). The pattern of Ki-67 and 7AAD staining was very similar for the untreated control (Figure 5Ciii-iv). Further confirmation that almost all the dasatinib-treated cells entered cell division again by day 7 was provided through CFSE staining (Figure 5Di-ii). Although the residual quiescent fraction was < 10% in both arms, the dasatinib-treated cells expanded more slowly over 7 days with a total 5-fold expansion compared with untreated control, 17-fold expansion (Figure 5E). Both populations showed complete reactivation of Bcr-Abl kinase activity, as measured by p-CrkL staining (data not shown). Enrichment for primitive cells was confirmed by CFSE retention, phenotyping for CD34 and CD133 (Figure 5F) and by residual CFC, CFC replating potential, and LTC-IC activity in the dasatinib-treated cells (Figure 5G-K). Considering that the untreated cells expanded by 4-fold and the dasatinib-treated cells decreased to 10% of input, the final frequency of LTC-IC was 10-fold higher in the dasatinib-treated versus untreated arms. The residual viable cells present at day 12 were also confirmed to express single copy Bcr-Abl by D-FISH (Figure 5L), to express Bcr-Abl by Q-PCR (supplemental Figure 6B) and showed no evidence of mutation by direct sequencing of the kinase domain, thus confirming that these cells were leukemic and that their dasatinib resistance could not be explained by mutation.

Discussion

The discovery that Bcr-Abl exhibited oncogenic properties$^{38}$ and was sufficient to induce CML-like disease in mice$^3$ provided clear evidence that Bcr-Abl was the key initiating event in CML. However, for many years investigators were skeptical that a tumor would remain dependent on the initiating oncogene in view of accumulation of additional genetic and epigenetic events occurring over time. The early studies with imatinib, a relatively selective Bcr-Abl inhibitor, resulted in a genuine paradigm shift with many drug discovery efforts now focused on single oncogenic lesions and the belief that a variety of tumors may depend on the driver oncogene. This concept of oncogene addiction was further strengthened when CML patients with blast crisis, a cancer more akin to solid tumors, also showed dramatic responses to imatinib, providing...
antagonists; (4) inhibit JAK signaling; (5) inhibit kinase inhibitor–induced autophagy; (6) activate PP2A; or (7) exploit differences in epigenetic regulation between normal and CML stem cells. These approaches have been comprehensively reviewed.47

When considering mechanisms of resistance, it is important to consider both the kinase activity of Bcr-Abl and other nonkinase domains of Bcr-Abl that might confer resistance. Using a mouse genetics approach, Chen et al were able to reveal Alox5 as a target whose expression was dependent on Bcr-Abl expression, but not modulated by kinase inhibition.48 To take account of this possibility, we used the combination of Bcr-Abl knockdown with kinase inhibition. However, because we were unable to achieve >50% inhibition of Bcr-Abl using an shRNA specific for the breakpoint and believe that this is not technically possible in primary CML stem cells at this time, we have not formally been able to exclude ongoing signaling via nonkinase Bcr-Abl motifs or proteins within the Bcr-Abl interactome such as Jak2, within the primary cells.49,50

Although complete abrogation of Bcr-Abl expression was achieved in the transgenic mouse model, we accept that relatively short-term transgenic expression may not allow for further genetic or epigenetic changes that likely occur in the primary setting that might affect the degree of oncogene addiction and would again suggest caution comparing mouse models with human disease. Future efforts toward cure in CML patients who are responding well to kinase inhibitors, but continue to show evidence of minimal residual disease, should focus on understanding the mechanisms of proliferation arrest and dormancy on oncogene inactivation in the CML stem cell population and aim to target Bcr-Abl kinase–independent survival pathways that remain active in these cells or are activated on kinase inhibition. In the latter scenario, it is possible that kinase inhibition will be required alongside a second agent to achieve synthetic lethality. Similarly, in solid tumors driven by a single oncogene and sustained by cancer stem cells an enhanced understanding of tumor dormancy will be a critical step to uncovering novel targets in this drug-resistant population.

Acknowledgments

The authors thank all CML patients and UK hematology departments who contributed samples, Dr Alan Hair for sample processing, Dr Sandrine Hayette for performing Q-PCR for Bcr-Abl and Ab1-kinase domain mutation analyses, Linda Kamp for excellent technical assistance, John Dick and Austin Smith for helpful discussions, and Richard and David Rockefeller for supporting this work.

This work was supported by the Medical Research Council United Kingdom (M.S., A.H., and G.V.H.), Cancer Research UK (T.L.H.), Leukaemia & Lymphoma Research United Kingdom (A.H.), the Kay Kendall Leukaemia Fund (G.V.H.), the German Research Foundation (DFG; K021552-2, S.K.), National Institutes of Health grant R01 CA95684 (R.B.), and Leukemia & Lymphoma Society Translational Research Grant (R.B.).

Authorship

Contribution: A.H., G.V.H., and M.S. performed research, analyzed data, and wrote the manuscript; B.Z. and E.K.A. performed research and analyzed the data; S.M. performed research; E.K.A. and C.M.-T. performed research and wrote the manuscript; R.B. contributed vital new reagents, designed research, analyzed data,
The current affiliation for S.K. is Department of Medicine, Oncology, Hematology, and Stem Cell Transplantation, University of Aachen, Aachen, Germany.

Correspondence: Prof Tessa Holyoake, Paul O’Gorman Leukaemia Research Centre, Institute of Cancer Sciences, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, G12 OZD, United Kingdom; e-mail: tessa.holyoake@glasgow.ac.uk.

References


