The level of BCR-ABL1 kinase activity before treatment does not identify chronic myeloid leukemia patients who fail to achieve a complete cytogenetic response on imatinib

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ABSTRACT

Imatinib is currently the first line therapy for newly diagnosed patients with chronic myeloid leukemia. However, 20-25% of patients do not achieve durable complete cytogenetic responses. The mechanism underlying this primary resistance is unknown, but variations in BCR-ABL1 kinase activity may play a role and can be investigated by measuring the autophosphorylation levels of BCR-ABL1 or of a surrogate target such as Crkl. In this study we used flow cytometry to investigate the *in vitro* inhibition of Crkl phosphorylation by imatinib in CD34$^+$ cells in diagnostic samples from two groups of patients distinguished by their cytogenetic response. No difference in inhibition of Crkl phosphorylation was observed in the two groups. The observation that increasing the dose of imatinib *in vivo* did not increase the level of cytogenetic response in some non-responders suggests that in at least a proportion of patients imatinib resistance may be due to activation of BCR-ABL1-independent pathway.

Key words: CD34, CML, p-Crkl, imatinib.

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder resulting from the malignant transformation of hematopoietic stem cells by the BCR-ABL1 oncoprotein.[1] Imatinib mesylate (IM), the specific inhibitor of BCR-ABL1 oncoprotein, is a well established first line therapy for newly diagnosed CML patients.[2] IM induces complete cytogenetic response (CCyR) in approximately 80% of newly diagnosed chronic phase CML patients. However, 20% of patients do not achieve CCyR (primary resistance) and up to 20% of patients who achieve CCyR may subsequently lose it (secondary resistance).[3] Mutations in the BCR-ABL1 kinase domain are the best described mechanism associated with secondary resistance but play only a limited, if any, role in primary resistance.[4]

As CML is a stem cell disorder, CD34$^+$ stem cells collected at time of diagnosis have been used in various studies to predict a patient's response to IM.[5-7] Insufficient suppression of the Philadelphia-positive CD34$^+$ stem cell population by therapeutic levels of IM at diagnosis can be investigated by measuring the reduction in phosphorylation of BCR-ABL1 and its downstream targets.[8,9] However, because the BCR-ABL1 oncoprotein is degraded upon lysis of primary CML cells, the activity of BCR-ABL1 in primary cells is often evaluated by measuring a surrogate target for phosphorylation, such as p-Crkl.[10-13]

As Crkl is phosphorylated by BCR-ABL1 kinase in primary CML CD34$^+$ cells[10,14,15] and not detectable in BCR-ABL1 negative cells[16] it represents a leukemia-specific target to measure BCR-ABL1 activity.

A study by White *et al.*[17] measured *in vivo* p-Crkl in mononuclear cells (MNC) after two or three weeks of IM treatment and showed a direct correlation between reduction of p-Crkl and achievement of CCyR. They also showed that the *in vitro* sensitivity to IM-induced inhibition of ABL1 kinase activity in MNC predicted major molecular response at 12 months after starting IM.[18] Hamilton *et al.*[7] developed a flow cytometry based method of measuring p-Crkl inhibition in CML CD34$^+$ stem cells to evaluate the response to IM. Their study was limited to observations made in 8 patients, and although the methodology was feasible and reproducible, a

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larger cohort of patients would be valuable to corroborate their preliminary findings.

We identified 36 chronic phase CML patients from whom CD34+ stem cells were available from time of diagnosis and who received IM as first line therapy. The methodology described by Hamilton *et al*. was used to investigate the reproducibility of p-Crkl as a prognostic marker for IM response in CML patients.

Design and Methods

Patients

Leukapheresis samples from 36 newly diagnosed CML patients and prior to the start of IM therapy were frozen and stored in the Cellular Therapy Centre at Hammersmith Hospital, London, UK. Ethical approval from the local Committee and patients' consent were obtained prior to sample collection. Twenty-four of these patients achieved CCyR on standard dose of IM (400mg per day). Of the remaining 12 patients, 8 failed to even achieve a partial cytogenetic response (PCyR, ≤35% Ph-positive metaphases). Among these 12 patients the dose of IM was increased to 600 (in 3 patients) and to 800 mg (in one patient) with no further improvement of their cytogenetic response. Patients' response was monitored using cytogenetic analysis, FISH, molecular monitoring of the BCR-ABL1 transcript and mutation screening as previously described.[4,19]

CD34+ cell separation

Polymorphonuclear (PMN) cells and mononuclear cells (MNC) were separated using Ficoll-HyPaque™ Plus (GE Healthcare, Uppsala, Swden) centrifugation. CD34+ stem cells were separated from the MNC fraction using a CD34 positive selection kit (StemCell Technologies, Inc). Purity of the separated CD34+ cells was checked by flow cytometry using human CD34 antibody in addition to a mouse IgG1 isotype control antibody (CALTAG Laboratories).

Culture of different cell sub-populations and P-Crkl analysis

Cell culture and p-Crkl analysis by flow cytometry were done as described by Hamilton *et al*.[7] 5 μM IM was used for culturing the CD34+ cells from the 36 patients. K562 and HL60 (Ph positive and negative cell lines, respectively), were used as positive and negative controls. The inhibition of p-Crkl was expressed as the percentage of the geometric mean fluorescence intensity (GMFI) of p-Crkl in IM treated cells compared to untreated cells.[7]

Western blot

Western blot was performed using standard techniques with the same specific anti-p-Crkl primary antibody (1:1000) (New England Biolabs) as used in flow cytometry and an anti-rabbit IgG, HRP-linked secondary antibody (5 in 10000) (New England Biolabs). Anti-Crkl antibody (4:1000) (Santa Cruz BioTech) was used for loading control.

Results and Discussion

IM causes a dose dependent inhibition of p-Crkl in K562 and CML CD34+ cells

The effect of IM treatment on p-Crkl levels was first examined in the K562 cell line. Cells were treated with increasing concentrations of IM from 0.5 to 10 μM. Maximum inhibition of p-Crkl in K562 cells was achieved using 5 and 10 μM of IM. K562 were lysed and p-Crkl was measured by Western blot. Flow cytometry and Western blot showed good correlation of inhibition with increasing IM concentrations. As flow cytometry also required a lower number of cells than Western blotting, flow cytometry was selected as the method of choice in our study.

In order to assess the optimal cell population from primary samples for investigation, three different sub-populations (PMN/granulocytes, MNC and CD34+ cells) prepared from 5 fresh primary CML samples were analyzed. CD34+ cells showed the highest detectable p-Crkl level. Maximum p-Crkl inhibition was observed at an IM dose of 5 and 10 μM (Figure1A) and at 2 h incubation with no further changes at 16 h (Figure 1B).

CD34+ cells from 36 newly diagnosed CML patients were then isolated after thawing the frozen samples as described earlier. FISH analysis confirmed that at least 90% of the separated CD34+ cells were Ph positive. In 2

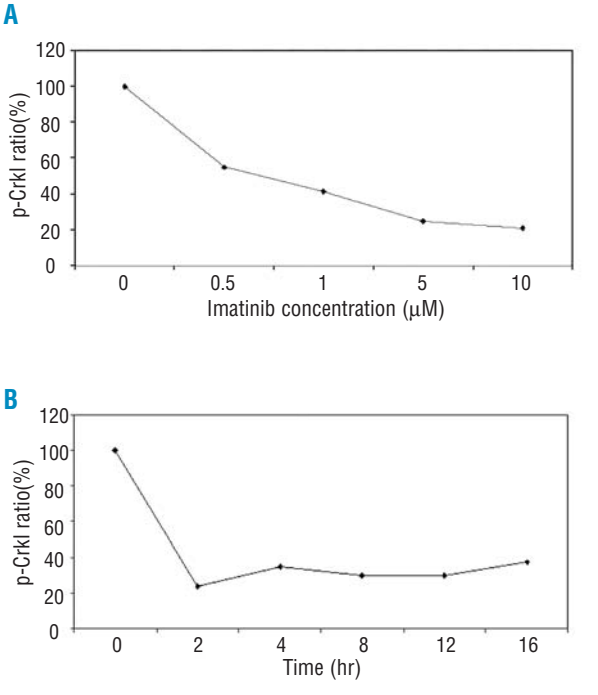

Figure 1. Reduction of p-Crkl at different time points by imatinib mesylate in CD34+ cells from 5 *de novo* chronic myeloid leukemia. (A) P-Crkl is reduced in chronic myeloid leukemia CD34+ cells as the dose of imatinib mesylate increases. The time of exposure to drug in this experiment was 2 h. The X axis represents the dose of imatinib mesylate. (B) P-Crkl decreases significantly after 2 h of drug exposure (at 5mM) and remains relatively stable for the next 14 h. The X axis represents the time of exposure to imatinib mesylate. The Y axis represents the percentage of p-Crkl ratio for both 1A and B.

cases, CD34 separation was performed in duplicate at different time points from the same leukapheresis, with less than 10% difference in the paired results.

P-Crkl phosphorylation tests in responding and non-responding patients

The p-Crkl ratio in IM (at 5 μM concentration) treated CD34+ cells was analyzed in patients grouped according to either cytogenetic or molecular response. The p-Crkl ratio obtained in 24 patients who achieved CCyR ranged from 28 to 64% (median 50%) (Figure 2A) compared to 25 to 68% (median 40%) in 12 patients who failed to achieve CCyR. In the latter group, p-Crkl ratio ranged between 25 and 68% (median 41%) in the 8 patients who failed to achieve even a PCyR.

When patients were classified according to molecular responses, patients ranged from those who achieved a 4 log reduction in BCR-ABL1/ABL1 ratio (group e; Figure 2B) to those who failed to achieve any detectable molecular response (group a; Figure 2B). There was no significant difference in p-Crkl activity among any of these groups (Figure 2A and B).

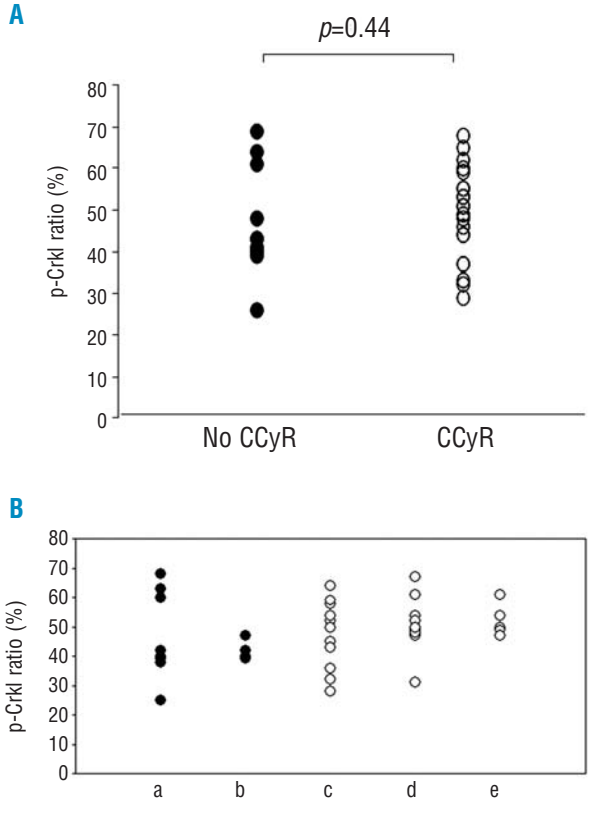

Figure 2. Inhibition of p-Crkl in different sub-groups of patients based on their best clinical response to imatinib mesylate. (A) P-Crkl ratio was not statistically different between the patients who achieved CCyR on imatinib mesylate therapy and those who did not (p=0.44). (B) P-Crkl ratio in the different imatinib response sub-groups. a) hematologic response without achieving PCyR; b) PCyR; c) CCyR but no major molecular response (MMR is defined as 3 or more log reduction in BCR-ABL1 transcripts from standardized control for untreated patients); d) MMR with less than 4 log reduction in BCR-ABL1 transcripts; e) MMR with 4 log or higher reduction in BCR-ABL1 transcripts.

No BCR-ABL kinase domain mutations were detected in any of the patients in this study tested at 12 months after initiation of treatment.

About 20-25% of CML patients treated with IM as first line therapy fail to achieve CCyR. Resistance in the presence of effective inhibition of the BCR-ABL1 kinase could indicate that the leukemic cells are independent of BCR-ABL1 kinase activity and rely on other pathways for survival and proliferation. This is commonly described in the more advanced phases of the disease.[20]

Identifying those patients who will fail to achieve an optimal response to treatment prior to initiation of therapy is of clinical importance, particularly now that more potent agents are available. As CML is a stem cell disorder, we considered that the *in vitro* response to IM in CD34+ cells (as measured by inhibition of Crkl phosphorylation) might predict therapeutic response. We applied the method of Hamilton *et al.*[7] to measure *in vitro* inhibition of p-Crkl by IM in CD34+ cells and found that the CD34+ fraction of the MNC had the highest levels of p-Crkl and also showed the greatest degree of inhibition following treatment with IM, thus supporting the use of CD34+ cells.

In all patients the reduction in p-Crkl level was observed. However, contrary to previous reports, we found no difference in the level of p-Crkl inhibition in CD34+ cells obtained at diagnosis between patients who later achieved, and patients who failed to achieve CCyR. Our data suggest that the *in vitro* inhibition of BCR-ABL1 in CD34+ cells does not predict for future clinical response to IM. The IM dose of 5 μM is higher than the concentration achieved by a 400mg daily dose *in vivo*. However, in 4 patients who failed to achieve CCyR on 400 mg of IM, the dose of the drug was increased to 600 (in 3 patients) and 800 mg (in one patient) with no effect on cytogenetic response, thereby excluding any confounding effect of the use of a higher *in vitro* dose of IM in our study.

As the IM responders and non-responders showed a similar level of BCR-ABL1 inhibition *in vitro*, the presence of *in vivo* factors may be proposed which either prevent the inhibition of BCR-ABL1 kinase by IM or permit the leukemia cells to proliferate and survive regardless of BCR-ABL1 inhibition.

IM may also have a different effect on different cell populations. I*n vitro* inhibition of p-Crkl was used to measure the $IC50^{imatinib}$ in MNC from newly diagnosed CML patients by White *et al.*[18] They showed that a higher dose of IM for inhibition of p-Crkl in MNC was associated with suboptimal response. The predictive value of *in vitro* inhibition of p-Crkl in their study and its failure in our study may reflect differences in IM activity on different cell populations (MNC versus CD34+ stem cells). This may then translate into a lower prediction of response to IM in CD34+ cells compared to more committed CML cells.

The smaller cohort of patients (7 responders and one non-responder) reported by Hamilton *et al.* compared to the substantially larger cohort in our study (24 responders and 12 non-responders, of which 4 failed even to achieve a PCyR) may explain the discrepant results. Indeed, in their conclusions they suggested that their

preliminary data needed to be confirmed in a larger series.

An alternative explanation for the lack of a measurable difference between the two groups of patients in our study could be the activation of *BCR-ABL1* independent signaling pathways, some of which have been studied by others. For example Wang *et al*. showed that autosecretion of GM-CSF mediated *BCR-ABL1*-independent IM resistance via activation of the antiapoptotic JAK2/STAT5 pathway.[21] Jilani *et al*.[22] showed that patients with resistance to IM therapy had significantly lower levels of BCR-ABL1, Crkl and AKT phosphorylation than previously untreated patients. Their observation emphasizes that IM resistance is not necessarily dependent on *BCR-ABL1* kinase activity only, but is likely to be due to the activation of other pathways such as *STAT5*. To investigate the role of other pathways which might predict response, measuring the activity of other markers such as total phosphotyrosine might be useful, as suggested by Schultheis *et al*.[5]

Insufficient *in vivo* inhibition of *BCR-ABL1* by IM, or activation of alternative pathways that maintain the cells in a leukemic phenotype despite *BCR-ABL1* kinase inhibition, may be responsible for the lack of optimal response to IM therapy in a number of patients. The mechanism of Crkl phosphorylation in CML may be more complex and further investigation is required to clarify the underlying mechanisms.

Authorship and Disclosures

Conception and design: JSK, SW, LF, JVM, JMG, JFA; provision of study materials or patients: LG, DMa, DMi, HP, Rezvani K, Loaiza S, Davis JG, Apperley JF; collection and assembly of data: JSK, LG, DM, AGR, HP, SW, GG; data analysis and interpretation: JSK, SW, LG, DM, AGR, LF; manuscript writing: JSK, LG, AGR, DM, JMG, JFA, LF.

The authors reported no potential conflicts of interest.

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