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ORIGINAL ARTICLE

BCR-ABL activity and its response to drugs can be determined in CD34$^+$ CML stem cells by CrkL phosphorylation status using flow cytometry

A Hamilton[1], L Elrick[1], S Myssina[1], M Copland[1], H Jørgensen[1], JV Melo[2] and T Holyoake[1]

[1]Section of Experimental Haematology, Division of Cancer Sciences & Molecular Pathology, University of Glasgow, Glasgow, UK and [2]Department of Haematology, Imperial College, Hammersmith Hospital, London, UK

In chronic myeloid leukaemia, CD34$^+$ stem/progenitor cells appear resistant to imatinib mesylate (IM) *in vitro* and *in vivo*. To investigate the underlying mechanism(s) of IM resistance, it is essential to quantify Bcr-Abl kinase status at the stem cell level. We developed a flow cytometry method to measure CrkL phosphorylation (P-CrkL) in samples with $<10^4$ cells. The method was first validated in wild-type (K562) and mutant (BAF3) *BCR-ABL*$^+$ as well as *BCR-ABL*$^-$ (HL60) cell lines. In response to increasing IM concentration, there was a linear reduction in P-CrkL, which was Bcr-Abl specific and correlated with known resistance. The results were comparable to those from Western blotting. The method also proved to be reproducible with small samples of normal and Ph$^+$ CD34$^+$ cells and was able to discriminate between Ph$^-$, sensitive and resistant Ph$^+$ cells. This assay should now enable investigators to unravel the mechanism(s) of IM resistance in stem cells.

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Introduction

Chronic myeloid leukaemia (CML) is a clonal myeloproliferative disorder of haemopoietic stem cells, characterised by the reciprocal translocation between chromosomes 9 and 22.[1] This translocation generates the oncogenic *bcr-abl* fusion gene, which encodes a protein that demonstrates higher and constitutively active tyrosine kinase activity compared with the normal Abl counterpart.[2] Although Bcr-Abl is known to play a central role in the pathogenesis of CML, the specific mechanisms by which it mediates the disease are still unclear. However, there is evidence that it induces tyrosine phosphorylation of downstream signalling substrates, including CrkL. CrkL is the major tyrosyl-phosphoprotein detected in the peripheral blood cells of patients with CML, suggesting that its association with Bcr-Abl plays an important role in the pathogenesis of the disease.[3–5] The specificity of CrkL phosphorylation to Bcr-Abl signalling, partnered with the stability of the phosphoprotein complex, has led to its acceptance as an excellent method to assess Bcr-Abl status.[6,7] CrkL phosphorylation has previously been determined by Western blot in Bcr-Abl-expressing cell lines and primary CML cells; however, this method is time consuming and requires large numbers of cells, which are difficult to obtain from primary cell samples. As part of our efforts to characterise CML stem cells,[8–11] we have now developed a method to detect phosphorylated CrkL by flow cytometry, using an anti-phospho-CrkL (P-CrkL) antibody, requiring minimal cell numbers from patient samples.

Correspondence: Professor T Holyoake, Section of Experimental Haematology, Division of Cancer Sciences & Molecular Pathology, University of Glasgow, Level 3 Queen Elizabeth Building, Royal Infirmary, 10 Alexandra Parade, Glasgow G31 2ER, UK.
E-mail: tlh1g@clinmed.gla.ac.uk
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Materials and methods

Reagents

Imatinib mesylate (IM) was a gift from Novartis Pharma (Basle, Switzerland).

Cell lines

The *BCR-ABL*-positive K562 and *BCR-ABL*-negative HL60 cell lines were grown in suspension in RPMI 1640 (Sigma-Aldrich Company Ltd, Dorset, UK) with 10% fetal calf serum (Invitrogen, Paisley, UK), 1% glutamine (100 mM) and 1% penicillin–streptomycin (100 mM) (Invitrogen) (RPMI$^+$), in a humidified incubator at 37°C in an atmosphere of 5% (v/v) CO_2. Every 3–4 days, cells were counted using a haemocytometer and set up at 1×10^6 cells per 75 cm^2 flask with fresh RPMI$^+$. BA/F3 cells containing wild-type or Bcr-Abl with kinase domain mutations were maintained in RPMI$^+$ supplemented with 10% conditioned medium from WEHI-3B cells.

Primary cell samples of fresh leukapheresis products from patients with chronic-phase CML, before any exposure to IM *in vivo,* or from patients undergoing autologous stem cell transplantation, were enriched for CD34$^+$ cells using a clinical scale method of magnetic-activated cell sorting – CLINIMACS (Miltenyi Biotec Ltd, Bisley, UK). The selected cells were then cryopreserved in 10% (v/v) dimethyl sulfoxide in ALBA (4% (w/v) Human Albumin Solution, Scottish National Blood Transfusion Service, Edinburgh, UK) and stored in the vapour phase of liquid nitrogen until required. All human cell samples were obtained with informed consent. Cells were cultured in Iscove's modified Dulbecco's medium (Sigma) supplemented with a serum substitute (BIT; StemCell Technologies, Vancouver, Canada), 0.1 μM 2-mercaptoethanol (Sigma), 1% glutamine (100 mM) and 1% penicillin–streptomycin (100 mM) (serum-free medium, SFM). Serum-free medium was further supplemented with a five growth factor cocktail comprising 100 ng/ml Flt3-ligand and 100 ng/ml stem cell factor and 20 ng/ml each of interleukin (IL)-3, IL-6 (all from StemCell Technologies) and granulocyte colony stimulating factor (Chugai Pharma Europe Ltd, London, UK).

CrkL phosphorylation assay

Cell lines were incubated with increasing concentrations of IM for 16 or 48 h, with drug replenished after 24 h. CD34$^+$ cells

were incubated with 5 μM IM for 16 or 72 h, with drug replenished within 24 h of the end of assay. The later time point differed for K562 and CD34$^+$ cells, as K562 are more sensitive to IM and undergo apoptosis by 48 h, whereas CD34$^+$ cells require 72 h. HL60 cells were used as *BCR-ABL*-negative controls for K562 and BA/F3 cells and Ph$^-$ CD34$^+$ cells were used as *BCR-ABL*-negative controls for CD34$^+$ CML cells. For each data point, $\geqslant 1 \times 10^4$ cells were re-suspended in 100 μl Fixing Reagent ('Fix and Perm' kit, CALTAG Laboratories, Silverstone, UK), incubated at room temperature (RT) for

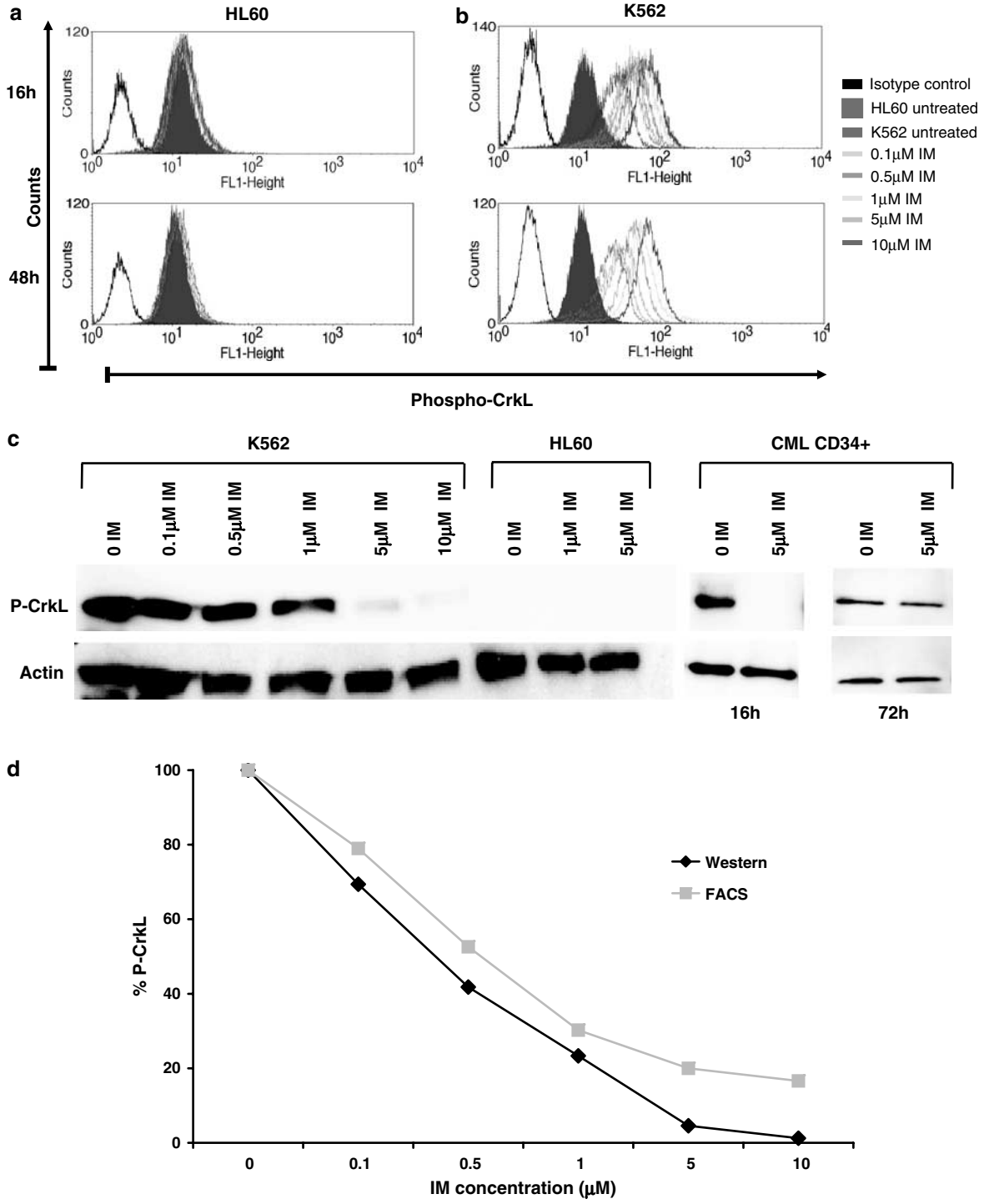

Figure 1 Effect of increasing concentrations of imatinib mesylate (IM) on K562 and HL60 cell lines and chronic myeloid leukaemia (CML) CD34$^+$ cells. K562 and HL60 cells were cultured ± increasing concentrations of IM (0.1, 0.5, 1, 5 and 10 μM) for 48 h. Intracellular flow cytometry was used to measure P-CrkL status at 16 and 48 h. Panel a shows that IM had no effect on P-CrkL status in the Bcr-Abl-negative cell line, HL60. Panel b demonstrates the dose-dependent decrease in P-CrkL in IM-treated K562 cells. Panel c shows Western blot for cells treated with increasing concentrations of IM for 16 h (K562, HL60 and CML CD34$^+$) and 72 h (CD34$^+$ only). The blot was reprobed with anti-pan-actin antibody to confirm equal sample loading. Panel d demonstrates the equivalence between flow cytometry and Western blot methods, in P-CrkL reduction in K562 cells after 48 h treatment with IM.

15 min, washed with 3 ml phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA)/0.1% sodium azide and centrifuged at 1000 rpm for 5 min. Twenty-five microlitres of Permeablising Reagent (CALTAG Laboratories) were added to the cell pellet with 2.5 μl of P-CrkL-specific antibody (New England Biolabs (UK) Ltd, Hitchin, UK) and the cells were vortexed and incubated at RT for 40 min. The wash step was repeated twice, the cells were re-suspended in 100 μl PBS/BSA/sodium azide and the secondary antibody (anti-rabbit IgG FITC-conjugate (Sigma)) and incubated at RT for 30 min in the dark. The wash step was repeated twice and the samples were analysed by flow cytometry. Isotype controls were included for cytometer set-up. The assay was also run on fresh compared to cryopreserved aliquots of the same samples to confirm that P-CrkL baseline levels were unchanged by cryopreservation (data not shown).

The amount of P-CrkL in the sample was determined as the geometric mean fluorescence intensity (MFI) of the P-CrkL-labelled untreated sample minus the MFI of the *BCR-ABL*-negative control (baseline P-CrkL). The P-CrkL status of the IM-treated samples was expressed as a percentage of the untreated control (100%).

K562 *Western blot* and HL60 were cultured in the presence or absence of IM for 48 h. CD34$^+$ CML cells were cultured in the presence or absence of IM for 16 and 72 h. 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, horseradish peroxidase-linked secondary antibody (1:3000) (New England Biolabs). The membranes were then stripped in 1 × 'Re-Blot Plus Strong Antibody Stripping Solution' (Chemicon, Hampshire, UK) for 15 min at RT and re-probed with anti-pan-actin antibody (1:1000) (New England Biolabs) in 5% BSA/phosphate-buffered saline with BSA, to confirm equal sample loading. Developed films were scanned with an Epson scanner and the integrated density of protein bands was determined with ImageQuant software (Molecular Dynamics). Densitometry data were normalised so that the untreated control had 100% P-CrkL and the result from each IM-treated sample was calculated based on this.

Results and discussion

Effect of IM on CrkL phosphorylation in K562, HL60 and BAF3-BCR-ABL cells

In order to determine Bcr-Abl kinase status in response to IM treatment in rare stem cell populations, we first validated an intracellular flow cytometry assay measuring P-CrkL in the *BCR-ABL*-positive cell line K562 and the *BCR-ABL*-negative cell line HL60. IM treatment of HL60 cells had no effect on baseline P-CrkL status at either 16 or 48 h (Figure 1a). Figure 1b and its corresponding MFI data presented in Table 1 show that IM-treated K562 cells demonstrated a dose-dependent decrease of P-CrkL, with only 16.6% phosphorylation after 48 h treatment with 10 μM IM, as compared to no drug control (100%). To prove the specificity of the P-CrkL antibody in the flow cytometry-based detection method, the effect of IM on P-CrkL was also assessed by Western blot on K562, HL60 and CML CD34$^+$ cells. Figures 1c and d confirm the results from the flow cytometry assay, showing that increasing concentrations of IM reduced expression of P-CrkL in a dose-dependent manner in K562 cells. Further, following 16 h treatment with the highest clinically achievable dose of IM,[12] P-CrkL was inhibited in CML CD34$^+$ cells. However, after 72 h treatment, the surviving stem cells (42.7% in the case shown in Figure 1c and mean 31.3% ($n = 8$)) showed increased expression of P-CrkL. The overall

Table 1 MFI values and corresponding % P-CrkL for K562 cells after 48 h treatment with increasing doses of IM

IM (μM)	*MFI – Ph$^-$*	*% P-CrkL*
0	55.26	100
0.1	43.65	79
0.5	29.08	52.6
1	16.71	30.24
5	11.07	20
10	9.18	16.6

Abbreviations: IM, imatinib mesylate; MFI, mean fluorescence intensity.

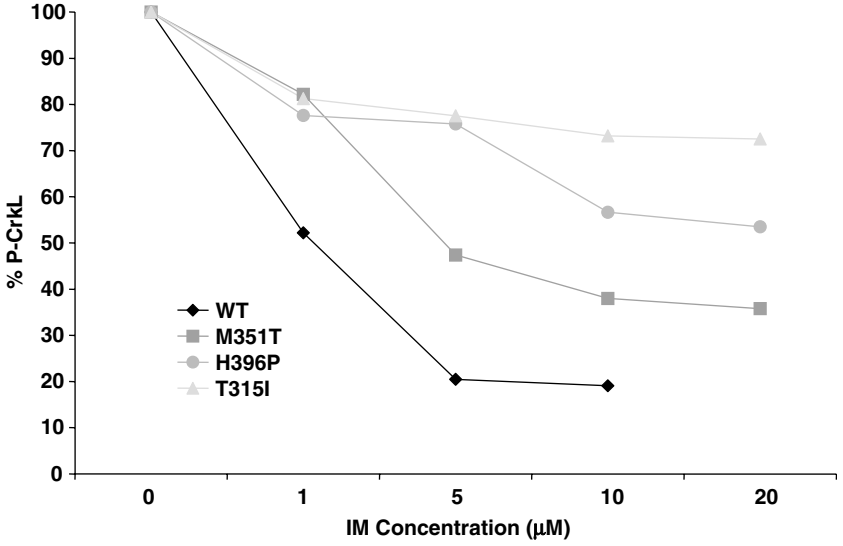

Figure 2 Effect of increasing concentrations of imatinib mesylate (IM) on BAF3 cell lines with defined Bcr-Abl kinase mutations. BAF3-*BCR-ABL* wild type, M351T (low-level IM resistance), H396P (intermediate IM resistance) and T315I (high IM resistance) were cultured ± increasing concentrations of IM (1, 5, 10 and 20 μM) for 16 h. P-CrkL levels were then determined by intracellular flow cytometry. A variable P-CrkL response, which correlated with each BAF3-*BCR-ABL* mutant's degree of IM resistance is demonstrated.

$CD34^+$ protein level (actin loading control) at 72 h was lower owing to the decrease in overall cell number. P-CrkL expression was not evident in HL60 cells.

We next investigated how cell lines with verified resistant phenotypes would perform in this assay. BAF3-*BCR-ABL* wild type, M351T (low-level IM resistance), H396P (intermediate IM resistance) and T315I (high IM resistance) were compared for P-CrkL inhibition at 16 h after IM exposure. As shown in Figure 2, the individual cell lines could be identified by their differing degrees of resistance as was expected from their known mutations.[13]

Effect of IM treatment on primary $CD34^+$ CML cells

Previous investigators have demonstrated the differences in total phosphotyrosine inhibition between $CD34^+$ cells from cytogenetic responders and non-responders to IM treatment.[14] Therefore, to more directly assess Bcr-Abl kinase activity in response to IM treatment, we cultured $CD34^+$ cells from normal donors and CML patients ($n=8$) in the presence and absence of 5 μM IM for 72 h. P-CrkL was measured at 16 h to determine the immediate effects on P-CrkL, and at 72 h, in order to examine the P-CrkL status in those cells that survived drug treatment. Figure 3a demonstrates the P-CrkL profile of the only CML

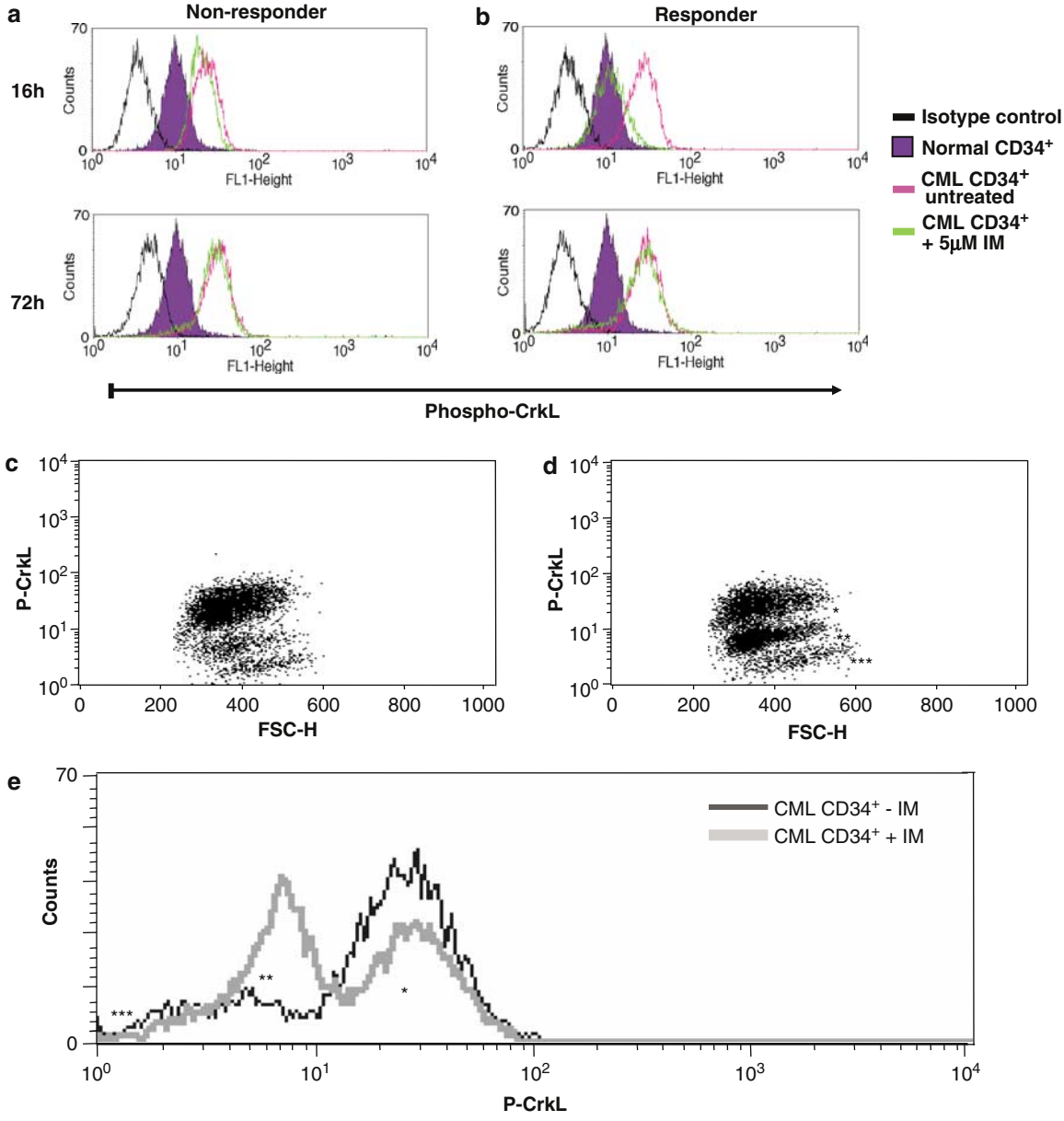

Figure 3 Effect of imatinib mesylate (IM) treatment on $CD34^+$ chronic myeloid leukaemia (CML) cells. $CD34^+$ CML cells ($n=8$) were cultured ± 5 μM IM for 72 h. P-CrkL status was measured in each of the samples at 16 and 72 h. Panel a demonstrates the P-CrkL profile from the only 'non-responder' to IM in this series. At both 16 and 72 h, IM-treated $CD34^+$ CML cells showed maximal phosphorylation of CrkL, as compared to no drug control (100%). Panel b shows a representative profile from a 'responder' to IM. After 16 h, there was only a 7% expression of P-CrkL in IM-treated $CD34^+$ CML cells, whereas, at 72 h, there was no decrease in the phosphorylation of CrkL in these cells, as compared to no drug control (100%). Panels c and d demonstrate dot plots of $CD34^+$ CML cells, control and treated with 5 μM IM, respectively, for 72 h. The three cell populations are denoted as follows: *drug resistant; **drug sensitive; ***$BCR\text{-}ABL$ negative. Panel e shows the overlay of the IM-treated cells from Panel d (light grey) with an untreated control (dark grey). The three cell populations (*, ** and ***) are as above.

patient in this series of eight who did not respond optimally to IM treatment (i.e. did not achieve cytogenetic response by 6 months). After both 16 and 72 h, IM-treated $CD34^+$ CML cells showed no reduction in P-CrkL, consistent with the presence of IM-resistant cells. We were unable to demonstrate the presence of a known IM resistance mutation for this case at diagnosis using direct sequencing, consistent with the literature,[15,16] and by Double-fluorescent *in situ* hybridisation, there was no evidence of *BCR-ABL* amplification, either in the starting population of $CD34^+$ cells or after drug exposure. Figure 3b shows a representative P-CrkL profile of a CML patient who responded well to IM treatment (i.e. complete haematological remission by 4 weeks and complete cytogenetic remission by 6 months). This was the pattern seen for seven of the eight cases. After 16 h, there was only 7% phosphorylation of CrkL in IM-treated $CD34^+$ CML cells, relative to no drug control (100%), indicating that most of the cells were sensitive to IM. However, after the 72 h time point, the cells that had survived IM treatment demonstrated a return to baseline of P-CrkL. This suggests that following the death of IM-susceptible $CD34^+$ CML cells, there was enrichment for an IM-resistant cell population. Figures 3c–e demonstrate the heterogeneity of response to IM treatment in $CD34^+$ CML cells. Rather than producing the expected single P-CrkL peak, in the absence of IM, three different peaks were evident in this profile, suggesting the presence of three cell populations within the CML sample (Figure 3c). This patient was diagnosed early with total WBC of only 18×10^9/l. Baseline cytogenetics confirmed a significant proportion (40%) of Ph^- metaphases. Following IM treatment, a clear population shift was evident (Figure 3d). The population with medium intensity antibody staining (**) after exposure to IM was IM responsive, with significant reduction in P-CrkLs from baseline, relative to the untreated control (Figure 3c). It is probable that the population denoted (***) therefore represented normal Ph^- $CD34^+$ cells, whereas the cells with highest intensity staining (*) proved to be IM resistant.

Overall, these results validate a rapid method to detect P-CrkL protein at the single cell level by flow cytometry. The minimum number of cells may be reduced to $<10^4$ for test and control, enabling an accurate assessment of Bcr-Abl kinase activity in rare stem cell populations in response to novel tyrosine kinase inhibitors.

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