c-Myc inhibition decreases CIP2A and reduces BCR-ABL1 tyrosine kinase activity in chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a malignant disease of the primitive hematologic cell which is driven by BCR-ABL1 tyrosine kinase activity.[1] Although in recent years CML treatment has been drastically improved by the tyrosine kinase inhibitor (TKI) imatinib, at least one-third of patients will eventually fail imatinib treatment[2,3] and a significant proportion of these will progress to blast crisis (BC). c-Myc is a transcription factor which regulates genes involved in proliferation, cell growth, differentiation, and apoptosis. The C-terminal domain of c-Myc has a basic helix-loop-helix leucine zipper domain (bHLHZip), necessary for the dimerization with MAX and for DNA binding. c-Myc exerts its oncogenic activity via the hetero-dimerization with MAX. Inhibition of c-Myc/MAX interaction has been shown to inhibit c-Myc induced cellular transformation. c-Myc is crucial for BCR-ABL1 mediated cellular transformation[4] and is over-expressed at transformation to blast crisis.[5] Furthermore, in CML, elevated levels of c-Myc may promote aneuploidy, contributing to disease progression.[6,7] Many malignancies, including CML, are associated with inhibition of protein phosphatase 2A (PP2A).[8,9] A novel protein inhibitor of PP2A, cancerous inhibitor of PP2A (CIP2A, *KIAA1525*) is associated with poor outcome in many malignancies. In CML, CIP2A protein level at chronic phase diagnosis is a prospective biomarker of disease progression in imatinib-treated CML patients. Moreover, high

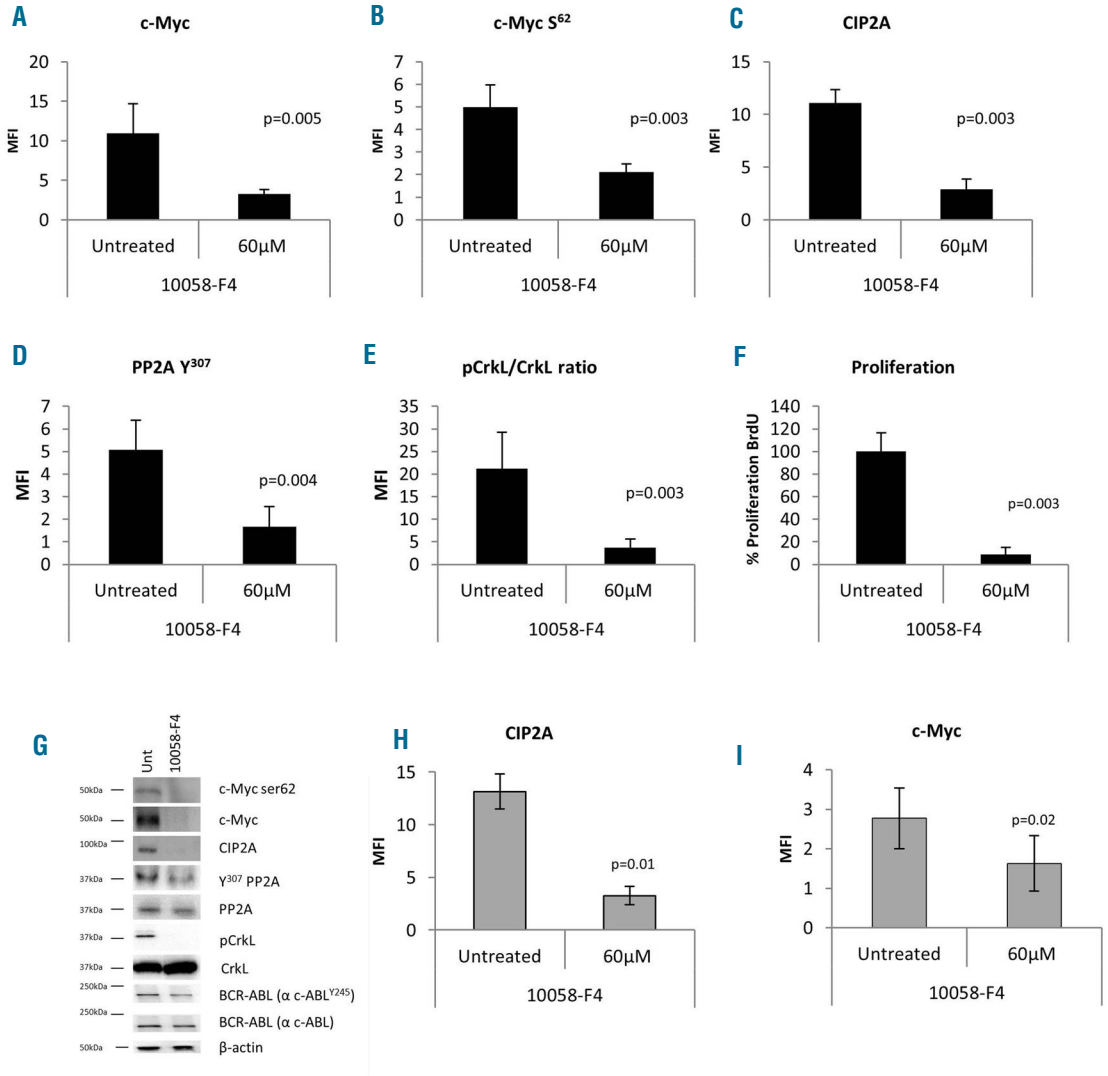

Figure 1. 10058-F4 inhibits c-Myc and reduces CIP2A and BCR-ABL1 tyrosine kinase activity. K562 cells were treated with the c-Myc inhibitor 10058-F4 for 48 h and the CIP2A pathway was assessed by flow cytometry and western blot (n=5). (A) c-Myc. (B) c-Myc S62. (C) CIP2A. (D) PP2A Y307. (E) pCrkL/CrkL ratio. (F) BrdU Proliferation assay and (G) western blot analysis. (H-I) c-Myc inhibition leads to a decrease in CIP2A in AGS cells. (AGS cells are CIP2A positive but BCR-ABL1 negative). FACS analysis of the CIP2A and c-Myc following 48 h of 10058-F4 treatment (n=4).

CIP2A levels are associated with high c-Myc and high BCR-ABL1 tyrosine kinase activity.[9] CIP2A acts by impairing PP2A activity leading to the stabilization of c-Myc,[10] and this stabilization is accompanied by phosphorylation at serine residue 62 (S^{62}). CIP2A is an attractive therapeutic target since high levels are only found in malignant cells. The structure of CIP2A is unknown, thus specific small molecule inhibitors targeting CIP2A have not been developed. The aim of this study was to inhibit the c-Myc using the small molecule inhibitor 10058-F4 which inhibits c-Myc/MAX interaction in order to disrupt the CIP2A/c-Myc interaction, and thus attempt to indirectly suppress CIP2A.

K562 and AGS cell lines and newly diagnosed chronic phase patients' cells were cultured with 60 μM 10058-F4 (Sigma-Aldrich, UK) for 48 h and changes to the CIP2A/C-Myc pathway were assessed by flow cytometry and western blot methodology, as previously described,[9,11] and were used for the detection of PP2A, PP2A Y307, CIP2A, c-Myc and c-Myc S62. The following antibodies were used: Anti-PP2A (Merck Millipore, UK), PP2A Y307 (Epitomics, USA), CIP2A (Santa Cruz Biotechnology, USA), c-Myc (New England Biolabs, UK), c-Myc S62 (Abcam, UK), anti-mouse and anti-rabbit Alex fluor 488 (Invitrogen, UK). Levels of pCrKL and CrKL were used as an assay of BCR-ABL1 activity, measured by flow cytometry, as previously described.[11] c-Myc siRNA (Thermo Scientific. MA, USA) was transfected into K562 and $CD34^+$ cells for 72 h prior to analysis. Cellular proliferation was assessed by bromodeoxyuridine (BrdU) incorporation (Roche Diagnostics, UK).

To investigate whether modulating c-Myc could affect CIP2A levels, K562 cells were initially treated for 48 h with 60 μM 10058-F4. 10058-F4 significantly decreased both c-Myc (P=0.005) (Figure 1A) and c-Myc S62 (P=0·003) (Figure 1B). Interestingly, c-Myc inhibition led to a decrease in

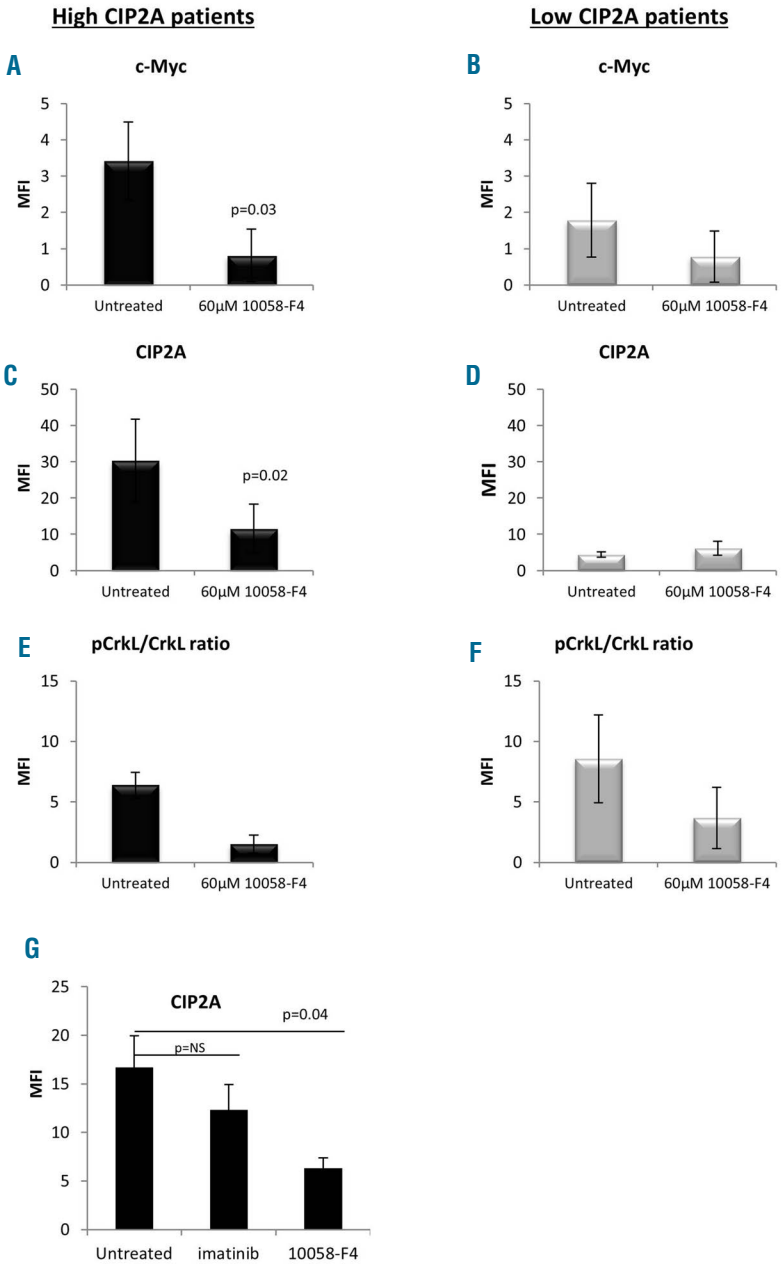

Figure 2. c-Myc inhibition decreases CIP2A in CML patients diagnostic MNC from high and low CIP2A patients treated with 10058-F4 for 48 h (n=10). (A and B) shown c-Myc. (C and D) show CIP2A. (E and F) pCrKL/CrKL ratio for high and low CIP2A patients, respectively. G 10058-F4 significantly reduces CIP2A protein levels to a greater degree than observed with imatinib in high CIP2A patients.

CIP2A (P=0.003) (Figure 1C), and this was associated with increased PP2A activity (i.e. decreased PP2A Y307) (Figure 1D) and decreased BCR-ABL1 tyrosine kinase activity, as assessed by decreased pCrKL/CrKL ratio (P=0.003) (Figure 1E). 10058-F4 also significantly reduced the rate of cellular proliferation (P=0.003), (Figure 1F). Results were also confirmed by western blot (Figure 1G). 10058-F4 treatment decreased both c-Myc and BCR-ABL1 mRNA expression (P=0.002 and 0.004, respectively) (*Online Supplementary Figure S1*). No effect on CIP2A mRNA expression was observed (*data not shown*). To investigate whether the decrease in CIP2A protein was a direct result of c-Myc reduction or an indirect effect via BCR-ABL1, AGS cells (a gastric carcinoma line which has high CIP2A levels but is BCR-ABL1-negative) were treated with 60μM 10058-F4 for 48 h. Again, c-Myc inhibition resulted in a decrease in CIP2A (P=0.001) (Figure 1H and I). These data in a BCR-ABL1 negative cell line are in line with the view that the effect of 10058-F4 on c-Myc and CIP2A was independent of BCR-ABL1.

The effects of c-Myc inhibition using 10058-F4 were extended to primary CML cells. In patients with a high diagnostic CIP2A level, 10058-F4 significantly reduced c-Myc (P=0.03) (Figure 2A) and CIP2A protein levels (P=0·02) (Figure 2C). In those patients with low diagnostic CIP2A level, a reduction in c-Myc and c-Myc S62 was also observed (Figure 2B), though no effect was seen on the already low CIP2A protein level (Figure 2D). Furthermore, as in the K562 cell line, c-Myc inhibition decreased the BCR-ABL1 tyrosine kinase activity in both high and low CIP2A patients (Figure 2E and F). In High CIP2A patients treatment with 10058-F4 significantly reduces CIP2A protein levels to a greater degree than observed with imatinib (Figure 2G). Taken together, these data in CML cell lines and primary cells suggest that it is possible to target c-Myc inhibition as a surrogate for CIP2A inhibition.

We then investigated the role of c-Myc in the CIP2A pathway, using c-Myc siRNA on K562 cells and CML CD34$^+$ cells (Figure 3) with similar findings to those seen with c-Myc inhibition by 10058-F4. Specifically, Figure 3A and B show a significant reduction in c-Myc and c-Myc S62. Inhibition of c-Myc again resulted in an 85% decrease in CIP2A protein (P=0.01) (Figure 3C), reactivation of PP2A activity and a 60% decrease in BCR-ABL1 tyrosine kinase activity (P=0.01) (Figure 3D and E).

Coupled with our previous publication demonstrating that CIP2A siRNA causes a decrease in c-Myc,[9] these data support the notion that CIP2A and c-Myc act to stabilize each other at the protein level by mutual protection from proteolytic degradation. Similarly, inhibition of c-Myc by siRNA in CML CD34$^+$ cells resulted in an 80% reduction in CIP2A protein levels (P=0.04) (Figure 3F).

Our previous work and that of others has shown that CIP2A acts to stabilize c-Myc. c-Myc plays a critical role in

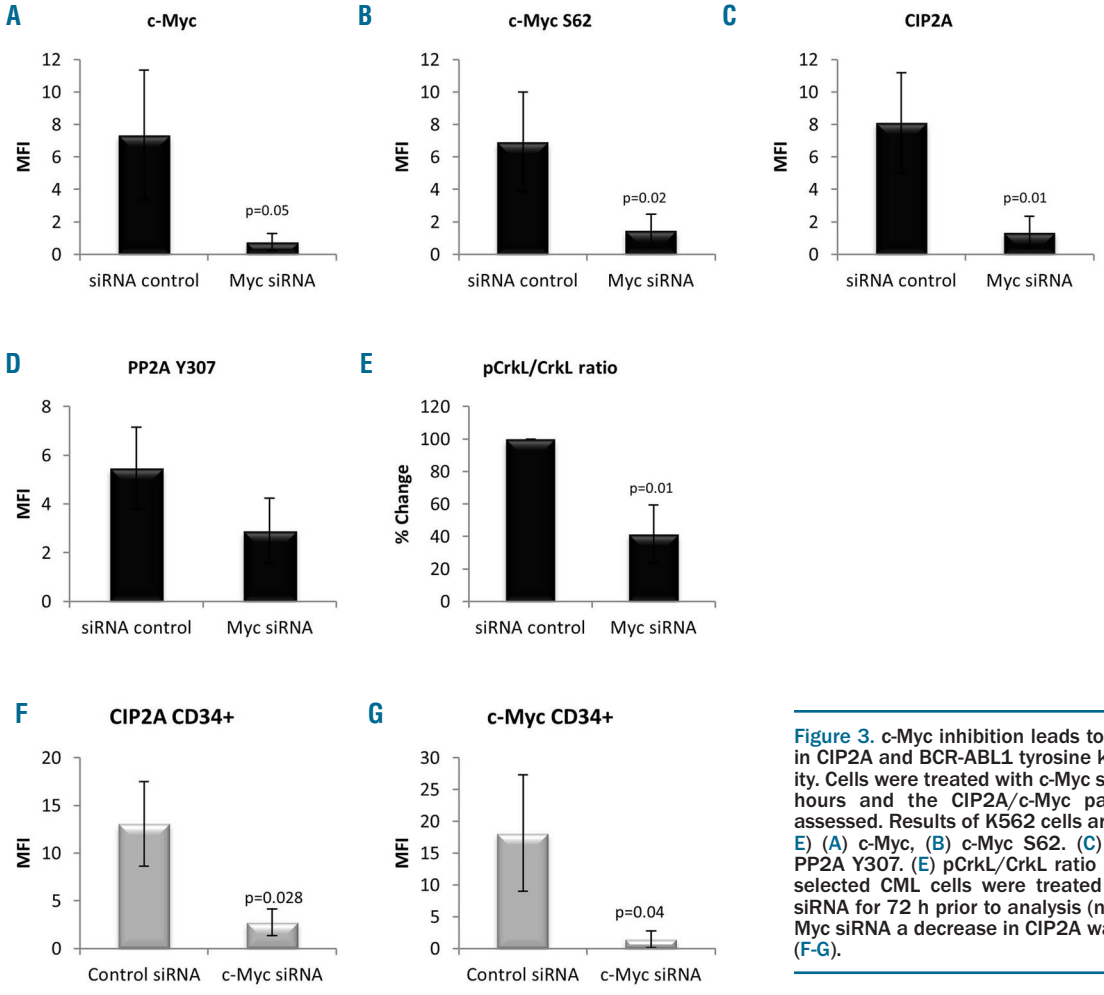

Figure 3. c-Myc inhibition leads to a decrease in CIP2A and BCR-ABL1 tyrosine kinase activity. Cells were treated with c-Myc siRNA for 72 hours and the CIP2A/c-Myc pathway was assessed. Results of K562 cells are shown (A-E) (A) c-Myc, (B) c-Myc S62. (C) CIP2A. (D) PP2A Y307. (E) pCrkL/CrkL ratio (=5). CD34$^+$ selected CML cells were treated with c-Myc siRNA for 72 h prior to analysis (n=4). With c-Myc siRNA a decrease in CIP2A was observed (F-G).

proliferation and cell cycle, and levels are elevated in those patients with a high diagnostic CIP2A level.[9] In AML cell lines and primary cells, 10058-F4 has been shown to inhibit growth, induce cell cycle arrest and cause differentiation.[12] Here, we have demonstrated that c-Myc inhibition by using the small molecule inhibitor 10058-F4 or siRNA leads to a decrease in CIP2A, reactivation of PP2A, a decrease in BCR-ABL1 tyrosine kinase activity (as assessed by pCrKL), and reduces cellular proliferation. In those patients with a high diagnostic CIP2A level, inhibition of c-Myc reduced CIP2A. However, 10058-F4 also reduced BCR-ABL tyrosine kinase activity irrespective of the patient's diagnostic CIP2A level. These data suggest that c-Myc inhibition would be beneficial for all CML patients. Recently, it has been shown that there are putative binding sites for c-Myc and MAX within the BCR promoter and that c-Myc/MAX hetero-dimers up-regulate BCR-ABL1 expression,[13] this may explain why 10058-F4 treatment reduces BCR-ABL1 tyrosine kinase activity in high- and low-CIP2A patients. Our study has demonstrated that c-Myc inhibition via either 10058-F4 or siRNA results in a decrease in CIP2A in K562, mono-nuclear cells and CD34$^+$ cells, and suggest that c-Myc inhibition may merit further study, especially as it is a surrogate target for CIP2A.

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