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

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Letter to Haematologica

Chronic myeloid leukaemia (CML) is a malignant disease of the primitive haematological 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 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 stabilisation of c-Myc,[10] and this stabilisation 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 work 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 suppress CIP2A indirectly.

K562 and AGS cell lines and newly diagnosed chronic phase patients' cells were cultured with 60µM 10058-F4 (Sigma-Aldrich, UK) for 48 hours and changes to the CIP2A/C-Myc pathway were assessed by flow cytometry and western blotting methodology as previously described[9, 11] and were used for the detection of PP2A, PP2A Y^{307}, CIP2A, c-Myc and c-Myc S^{62}. The following antibodies were used: Anti-PP2A (Merck Millipore, UK), PP2A Y^{307} (Epitomics, USA), CIP2A (Santa Cruz Biotechnology, USA), c-Myc (New England Biolabs, UK), c-Myc S^{62} (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 hours prior to analysis. Cellular proliferation was assessed by a bromodeoxyuridine (BrdU) incorporation (Roche Diagnostics, UK).

To investigate whether modulating c-Myc could affect CIP2A levels, K562 cells were initially treated for 48 hours with 60µM 10058-F4. 10058-F4 significantly decreased both c-Myc (p=0·005, Figure 1A) and c-Myc S^{62} (p=0·003, Figure 1B). Interestingly, c-Myc inhibition led to a decrease in CIP2A (p=0·003, Figure 1C), and this was associated with increased PP2A activity (i.e. decreased PP2A Y^{307}) (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 p=0.004 respectively, Supplementary Figure 1). 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 is has high CIP2A levels but is BCR-ABL1 negative) were treated with 60µM 10058-F4 for 48 hours. Again, c-Myc inhibition resulted in a decrease in CIP2A (p=0.001, Figure 1H-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 S^{62} 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-F). 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 significant reduction in c-Myc and c-Myc S^{62}. 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-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 stabilise 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 stabilise c-Myc. c-Myc plays a critical role in 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] Herein 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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Authorship

CML performed experiments, designed the study and wrote the manuscript.

AG performed experiments.

RJH and REC designed the study and wrote the manuscript

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Disclosures

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Figure legends

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 hours and the CIP2A pathway was assessed by flow cytometry and Western blot (n=5). Panel A. c-Myc, Panel B. c-Myc S^{62}. Panel C. CIP2A. Panel D PP2A Y^{307}. Panel E pCrkL/CrkL ratio. Panel F BrdU Proliferation assay and Panel G western blot analysis. Panel 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 48h of 10058-f4 treatment (n=4).

Figure 1.

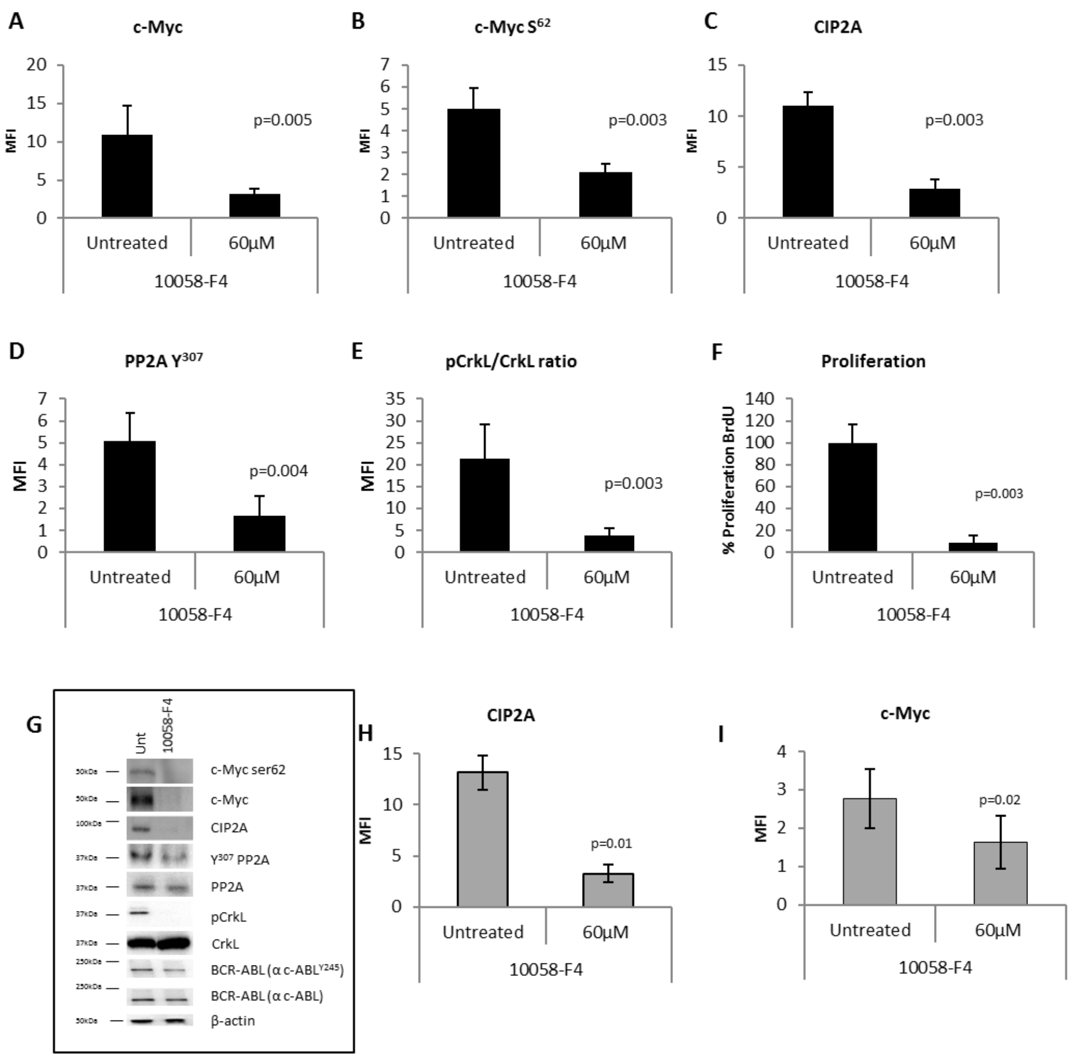


Figure 2. c-Myc inhibition decreases CIP2A in CML patients

Diagnostic MNC from high and low CIP2A patients treated with 10058-F4 for 48 hours (n=10). Panel A and B shown c-Myc. Panels C and D show CIP2A. Panels 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

Figure 2.

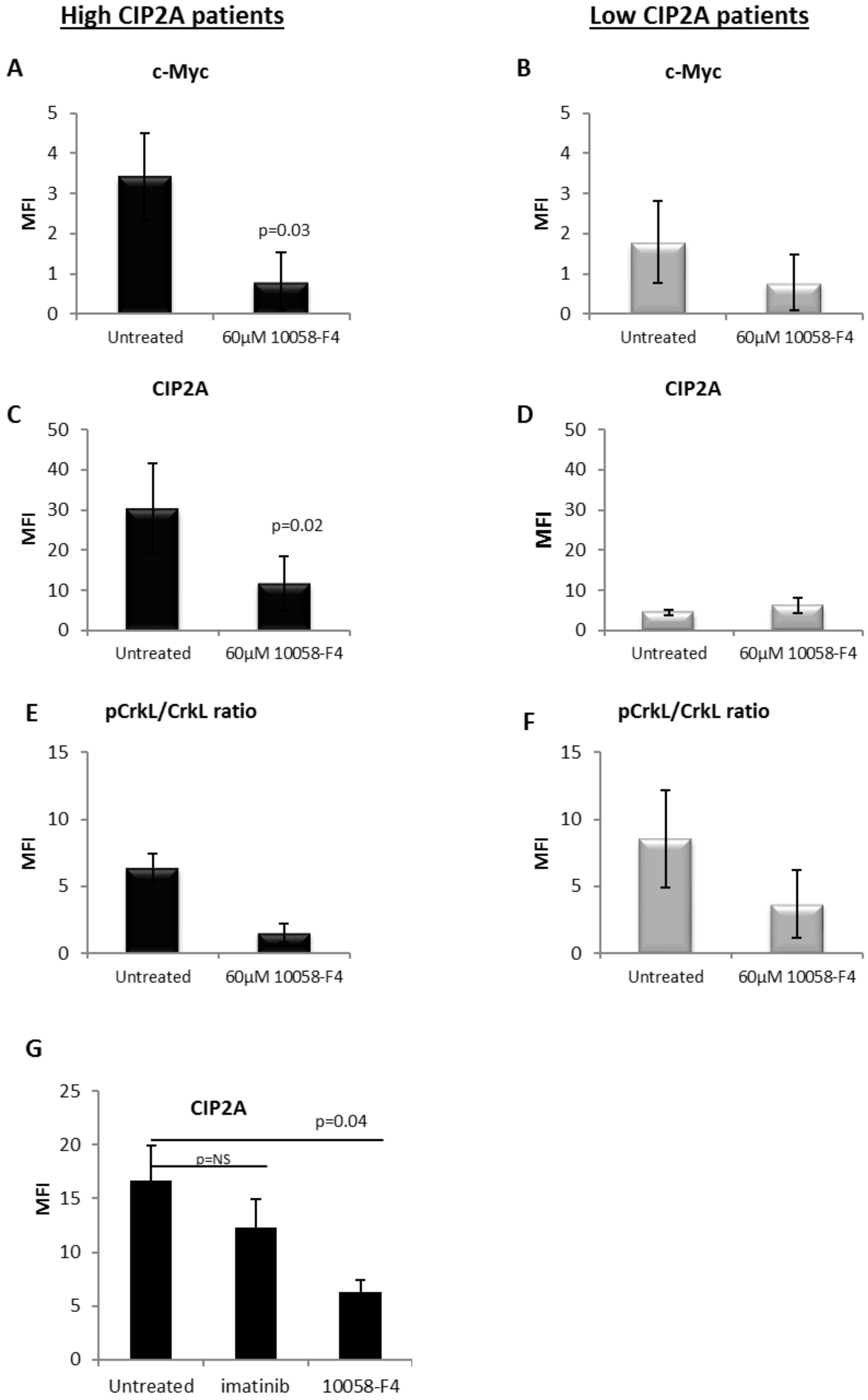
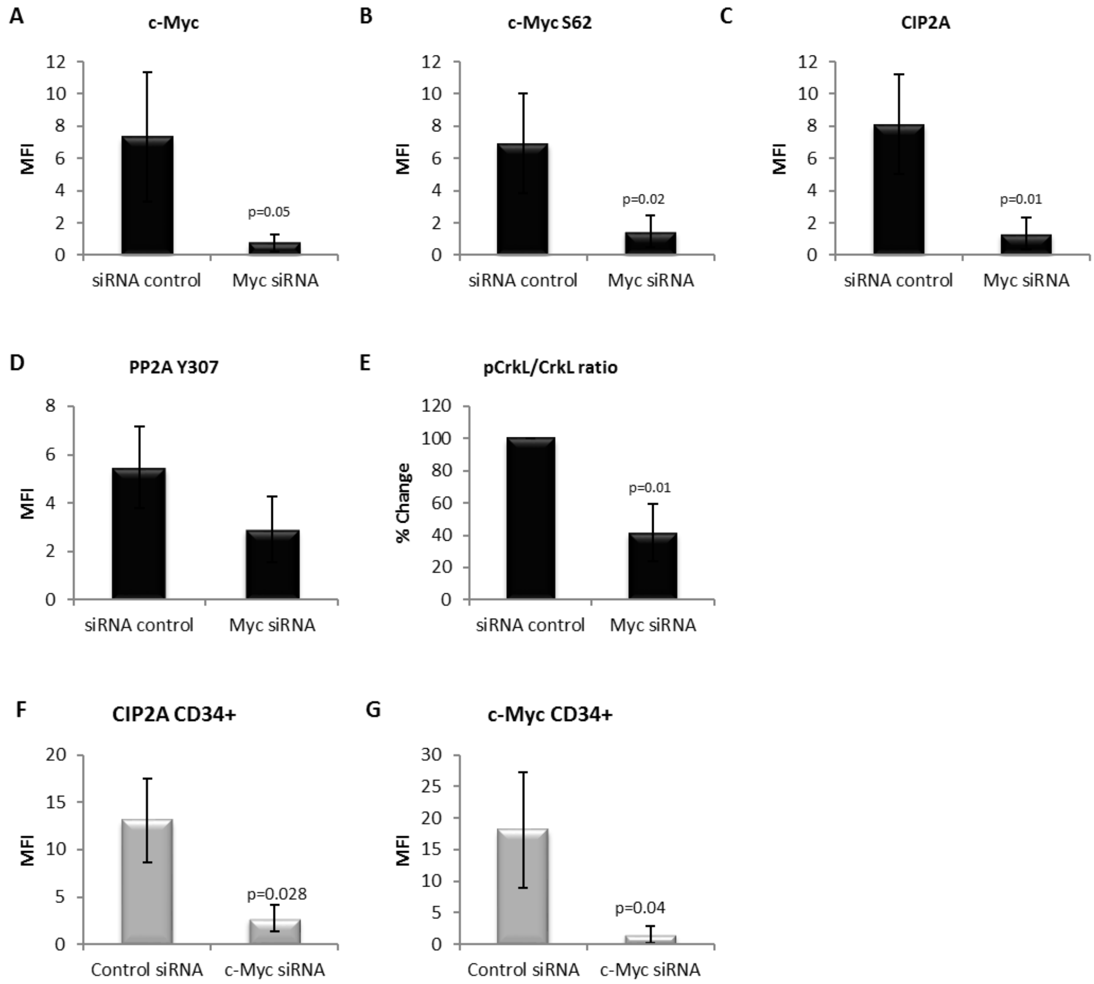

High CIP2A patients
Low CIP2A patients
A
c-Myc
MFI
p=0.03
Untreated
60μM 10058-F4
B
c-Myc
C
CIP2A
p=0.02
D
CIP2A
E
pCrkL/CrkL ratio
F
pCrkL/CrkL ratio
G
CIP2A
p=0.04
p=NS
imatinib
10058-F4

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. Panel A. c-Myc, Panel B. c-Myc S^{62}. Panel C. CIP2A. Panel D PP2A Y^{307}. Panel E pCrkL/CrkL ratio (=5). CD34+ selected CML cells were treated with c-Myc siRNA for 72 hours prior to analysis (n=4). With c-Myc siRNA a decrease in CIP2A was observed (panels F-G).

Figure 3.



Supplementary Figure 1. c-Myc inhibition leads to a decrease in BCR-ABL1 mRNA. Panel A, c-Myc mRNA expression and Panel B BCR-ABL1/ABL ratio mRNA expression.