JAK2/STAT5 inhibition by nilotinib with ruxolitinib contributes to the elimination of chronic phase CML CD34+ cells in vitro and in vivo

Paolo Gallipoli1†, Amy Cook2, Susan Rhodes1, Lisa Hopcroft1, Helen Wheadon1, Anthony D. Whetton3, Heather G. Jørgensen1, Ravi Bhatia2, Tessa L. Holyoake1

1Paul O’Gorman Leukaemia Research Centre, College of Medical, Veterinary & Life Sciences, Institute of Cancer Sciences, University of Glasgow, Scotland, UK
2Division of Hematopoietic Stem Cell and Leukemia Research, Beckman Research Institute, City of Hope Cancer Center, Duarte, CA, US
3Stem Cell and Leukemia Proteomics Laboratory, Manchester Academic Health Science Centre, University of Manchester, Manchester, UK
†Note Paolo Gallipoli current affiliation is Cambridge Institute for Medical Research, University of Cambridge, UK

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Address correspondence to:

Tessa L. Holyoake
Paul O’Gorman Leukaemia Research Centre, College of Medical, Veterinary & Life Sciences
Institute of Cancer Sciences, University of Glasgow, UK
21 Shelley Road
Gartnavel General Hospital
G12 0ZD
e-mail: tessa.holyoake@glasgow.ac.uk
Phone: 0044(0)1413017881/0
Fax: 0044(0)1413017898

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Key points

- The JAK2/STAT5 pathway is a relevant therapeutic target in chronic myeloid leukaemia (CML) stem/progenitor cells (SPCs)

- Targeting the JAK2/STAT5 pathway by nilotinib and ruxolitinib in combination leads to enhanced eradication of primitive CML stem cells

Abstract

Chronic myeloid leukaemia (CML) stem cell survival is not dependent on BCR-ABL protein kinase and treatment with ABL tyrosine kinase inhibitors (TKIs) cures only a minority of CML patients, thus highlighting the need for novel therapeutic targets. The JAK2/STAT5 pathway has recently been explored for providing putative survival signals to CML stem/progenitor cells (SPCs) with contradictory results. We investigated the role of this pathway using the JAK2 inhibitor, ruxolitinib. We demonstrated that the combination of ruxolitinib, at clinically achievable concentrations, with the specific and potent TKI nilotinib, reduced the activity of the JAK2/STAT5 pathway in vitro relative to either single agent alone. These effects correlated with increased apoptosis of CML SPCs in vitro and a reduction in primitive quiescent CML stem cells, including NOD.Cg-Prkdc<sup>scid</sup> IL2rg<sup>+/−</sup> WJl/SzJ mice (NSG) repopulating cells, induced by combination treatment. A degree of toxicity towards normal SPCs was observed with the combination treatment, although this related to mature B cell engraftment in NSG mice with minimal effects on primitive CD34<sup>+</sup> cells. These results support the JAK2/STAT5 pathway as a relevant therapeutic target in CML SPCs and endorse the current use of nilotinib in combination with ruxolitinib in clinical trials to eradicate persistent disease in CML patients.
Introduction

Chronic myeloid leukaemia (CML) arises in a haemopoietic stem cell (HSC) as a result of the reciprocal translocation between chromosomes 9 and 22 (t9;22), leading to the formation of the fusion oncogene BCR-ABL. The BCR-ABL protein has constitutive tyrosine kinase (TK) activity which drives myeloid progenitor cell expansion and is both necessary and sufficient for the transformed phenotype\(^1\). The introduction of ABL kinase inhibitors (TKI) has dramatically changed the management of newly diagnosed chronic phase (CP) CML patients, with the vast majority now achieving deep molecular responses, while enjoying good quality of life\(^2\). However 15-20% of patients show variable degrees of resistance to currently available TKI\(^3\) and even in patients achieving deep responses, including those with undetectable BCR-ABL transcript levels, there is evidence of persistence of BCR-ABL\(^+\) cells at the stem cell level\(^4-5\) and of positivity for BCR-ABL genomic DNA by PCR\(^6-7\). Furthermore, over 50% of patients achieving sustained undetectable BCR-ABL transcript levels showed evidence of molecular relapse upon TKI discontinuation\(^8\). Leukaemic stem cell (LSC) persistence determines the need for lifelong TKI treatment in the ever growing CML patient population, with associated implications in terms of compliance, adverse events and costs. Recent evidence has demonstrated that CML LSC persistence is secondary to their insensitivity to TKI despite effective BCR-ABL kinase inhibition, suggesting that other pathways contribute to their survival\(^9-10\). Identifying such pathways and trying to exploit them therapeutically is paramount to achieve CML LSC eradication and disease cure.

During normal haemopoiesis the intracellular TK JAK2 is activated following binding of haemopoietic growth factors (GF) to their receptors. JAK2 subsequently phosphorylates the transcription factor STAT5, leading to its nuclear relocation. Nuclear STAT5 exerts its activity by regulating the transcription of genes involved in normal haemopoiesis\(^11\). The central role of the JAK2/STAT5 axis is clearly demonstrated by the profound effects on haemopoiesis, resulting in embryonic lethality, of JAK2 knockout (KO) mice\(^12-14\). Both JAK2 and STAT5 are constitutively active in BCR-ABL\(^+\) cells\(^15-16\) with evidence supporting a role for each in CML leukaemogenesis. BCR-ABL\(^+\) cell clones transfected with kinase inactive JAK2 mutant displayed reduced clonogenic potential and tumourogenic activity\(^17\). Recently the existence of a JAK2/BCR-ABL protein complex which helps to stabilise BCR-ABL kinase activity has been demonstrated\(^18-19\). Disrupting this complex using either JAK2 chemical inhibitors or RNA interference was shown to increase eradication of BCR-ABL\(^+\)
cells, including primary CML CD34+ cells\textsuperscript{18,20}. Similarly, STAT5 deleted murine bone marrow (BM) cells transduced with BCR-ABL retrovirus were unable to generate and maintain BCR-ABL\textsuperscript{+} leukaemias \textit{in vivo}\textsuperscript{21-23}. Furthermore, high levels of STAT5 were protective for BCR-ABL\textsuperscript{+} cells treated with TKI\textsuperscript{24} and specific targeting of STAT5 activity increased eradication of BCR-ABL\textsuperscript{+} cells, including primary CML CD34\textsuperscript{+} cells and CML cells resistant to TKI\textsuperscript{25}. Pharmacological interference with the JAK2/STAT5 pathway therefore represents an attractive therapeutic strategy in CML, however STAT5 is a difficult drug target as it lacks an enzymatic domain and a simpler approach to interfere with STAT5 function is to inhibit its predominant activating kinase, JAK2.

In contrast to normal haemopoiesis, the role of JAK2 in activating STAT5 in BCR-ABL\textsuperscript{+} leukaemias remains controversial. Investigations using both a dominant negative JAK2 mutant\textsuperscript{26} and BCR-ABL transduced \textit{JAK2 KO} murine BM cells\textsuperscript{27} suggested that BCR-ABL is able to directly phosphorylate STAT5, rendering the role of JAK2 dispensable. It has also been suggested that the reported effects of most JAK2 inhibitors on BCR-ABL\textsuperscript{+} cells were secondary to their off-target inhibition of BCR-ABL kinase\textsuperscript{27-28}. These data have questioned the role of JAK2 as a \textit{bona fide} therapeutic target in CML.

The relevance of understanding the role of the JAK/STAT pathway in CML has increased with the clinical development of numerous JAK2 inhibitors. Amongst these, ruxolitinib (RUX) has emerged as a potent and orally bioavailable JAK1/2 inhibitor\textsuperscript{29} which is now licensed for the treatment of primary myelofibrosis following results from phase 3 clinical trials\textsuperscript{30-31}. As a result, a therapeutic strategy employing RUX in CML could now easily be pursued and early phase clinical studies aiming to assess the ability of RUX and TKI in combination to eradicate CML stem/progenitor cells (SPCs) are already underway (ClinicalTrials.gov identifiers: NCT01702064 and NCT01751425). Here we aimed to further characterise the role of JAK2 in human primary CML CD34\textsuperscript{+} cells and complementary mouse models. The effects of RUX alone and in combination with nilotinib (NL), on JAK2 and STAT5 activity, were assessed aiming to clarify whether JAK2 modulated STAT5 activity in CML cells, especially in the context of a fully inhibited BCR-ABL kinase. Moreover the effects of RUX, with or without NL, on the survival and proliferation of primary human CD34\textsuperscript{+} CML and normal cells \textit{in vitro} and on leukaemia engraftment \textit{in vivo} were tested to assess their efficacy in CML and potential toxicity to normal cells.
Methods

Reagents

RUX and NL were supplied by Novartis Pharmaceuticals and stored at 10mM in dimethyl sulfoxide (DMSO) at -20°C.

Primary cell samples and in vitro culture

Primary cells were obtained following consent, according to the Declaration of Helsinki, from peripheral blood leukapheresis samples of newly diagnosed CP CML patients (supplemental Table 1) and lymphoma patients without BM involvement or cord blood (CB) as normal controls. Enrichment for CD34⁺ expression, fluorescence activated cell sorting (FACS) of CML CD34⁺ CD38⁻, detection of BCR-ABL fusion in CML SPC/LSC cells by fluorescence in situ hybridisation (FISH) and in vitro culture in physiological (CML/CB) or high (adult normal CD34⁺) GF-supplemented serum-free medium were conducted as previously reported⁹, 32. Total cell counts were performed using the trypan blue dye exclusion method or CountBright Absolute counting beads for flow cytometry (Life Technologies).

Colony-forming cell (CFC) assays, apoptosis assessment and cell division tracking

CFC assays, apoptosis measurement by Annexin-V staining and cell division tracking using carboxyfluorescein diacetate succinimidyl diester (CFSE; Life Technologies) of CML and normal CD34⁺ cells were performed according to standard protocols as previously described⁹.

Engraftment of human cells in immunodeficient mice

CML CD34⁺ cells (1x10⁶ cells/mouse) or CB CD34⁺ cells (1x10⁵ cells/mouse) were cultured for 72 hours in the absence of drug (control), or with addition of NL (1μM) alone, RUX (500nM) alone, or their combination. Cells were then harvested, washed and transplanted via tail vein injection into sublethally irradiated (300 cGy) 8 weeks old NOD.Cg-Prkdc<sup>scid</sup> IL2rg<sup>tm1Wjl</sup> /SzJ mice (NSG mice, The Jackson Laboratory). Mice were euthanised after 16 (CB) and18 (CML) weeks and marrow contents of femurs, spleen cells and blood cells were obtained at necropsy. To assess human cell engraftment, cells were labeled with anti-human CD45-APC antibody (#17-9459-42, eBioscience) and analysed by flow cytometry. Specific
human cell subsets were detected by staining with antibodies to human CD34-PE-Cy7 (#25-0349-42, eBioscience), CD33-PE (#347787, BD Bioscience), and CD19-efluoro450 (#48-0199-42, eBioscience). Human CD45+ cells were selected by immunomagnetic column selection. To assess engraftment of malignant BCR-ABL expressing cells, CD45+ selected cells were evaluated for BCR-ABL mRNA levels by qRT-PCR using custom made primers and probes for both BCR-ABL (forward primer GGGCTCTATGGGTTTCTGAATG; reverse primer CGCTGAAGGGCTTTTGAACT; probe CATCGTCCACTCAGCCACTGGATTTAAGC) and BCR (forward primer CCTTCGACGTCAATAACAGGAT; reverse primer CCTGCGATGGCGTTCAC; probe TCCATCTCGCTCATCATAACCGA) as the reference gene.

**FACS and western blotting**

FACS for intracellular protein staining and western blot analysis were performed as previously reported using the following antibodies: p-JAK2Tyr1007/1008 (#1477-1) (Epitomics), p-STAT5Tyr694 (#9351), p-CrKLTyr207 (#3181), p-c-AblTyr245 (#2868) and betatubulin (#2128) (New England BioLabs), SH-PTP2 (#sc-280) (Santa Cruz Biotechnology).

**RNA extraction, cDNA synthesis and qRT-PCR**

Total RNA was isolated from pellets using the RNeasy Mini kit (Qiagen Ltd, UK) according to the manufacturer’s instructions. cDNA was generated using the High Capacity cDNA Reverse Transcription kit and specific target amplification performed using the TaqMan PreAmp Mastermix kit (Life Technologies) according to manufacturer’s protocol. qRT-PCR was performed using the Fluidigm BioMark HD System with the following TaqMan validated gene expression assays: BCL-XL (Hs01067345_g1); BCL6 (Hs00277037_m1); Cyclin D1 (Hs00765553_m1); Cyclin D2 (Hs00277041_m1); Cyclin D3 (Hs00426901_m1); CDKN1B/p27 (Hs00153277_m1); GAPDH (Hs99999905_m1); ID1 (Hs03676575_s1); MYC (Hs00905030_m1); PTEN (Hs03673482_s1); TBP (Hs99999910_m1).

**Statistical analysis**

All data from independent experiments are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using the paired Student t-test for matched
samples, the Mann-Whitney test for unpaired samples and the one-way ANOVA with post-hoc testing for multiple comparisons. A $P$ value $\leq 0.05$ was considered statistically significant.

**Results**

The combination of NL and RUX further reduces JAK2 and STAT5 activities compared to NL alone, with no off-target effects on BCR-ABL kinase

To assess the respective activities of JAK2 and STAT5 in CML CD34$^+$ cells following treatment with NL or RUX or both, levels of p-JAK2 and p-STAT5 were measured by flow cytometry over 72 hours of culture in medium supplemented with physiological GFs to mimic microenvironmental signalling$^{33}$. Interestingly, while both p-JAK2 and p-STAT5 levels were reduced by NL treatment, which infers that both are partially regulated by BCR-ABL kinase, the addition of RUX caused a faster and deeper reduction in the level of both phosphoproteins (Figures 1A, B and supplemental Figure 1A). Further evidence of this more pronounced inhibition of STAT5 activity by the combination treatment was derived by gene expression analysis of several STAT5 target genes. Treatment with the combined agents, as compared to NL or RUX alone, caused further downregulation of genes known to be positively regulated by STAT5, such as the anti-apoptotic gene $BCL-X_L$, the positive cell cycle regulators $Cyclin D1, D2, D3$, $MYC$ and the transcription factor $ID1^{34}$, which plays a key role in HSC self-renewal and differentiation$^{35-36}$. Conversely genes normally negatively regulated by STAT5 were upregulated, including the negative regulator of the AKT pathway, $PTEN$, the negative regulator of cell cycle, $CDKN1B/p27$ and the transcription factor $BCL6$, which has been reported to be important for the maintenance of CML LSC$^{37}$ (Figure 1C). When these cultures were repeated in the absence of paracrine GFs the enhanced STAT5 inhibition with the combination was no longer observed (supplemental Figure 1B).

Analysis of the phosphorylation levels of BCR-ABL and its kinase substrate CrKL demonstrated that full inhibition of BCR-ABL kinase activity had been achieved upon treatment with NL. No significant changes in neither p-BCR-ABL nor p-CrKL levels were observed when cells were treated with RUX as a single agent (Figure 2A, B). These data eliminate any concerns that the additive effects on STAT5 signalling of the combined inhibitor treatment were secondary to RUX-induced off-target effects on BCR-ABL kinase.
activity, as had been suggested for other JAK2 inhibitors and for RUX at higher concentrations (>20µM)\textsuperscript{27}.

Overall these data showed that the combination of NL and RUX caused a more rapid and profound inhibition of p-STAT5 activity as compared to single agents and that this was not secondary to off-target effects of the JAK2 inhibitor on BCR-ABL kinase activity.

**The combination of NL and RUX increases kill and reduces CFC output of human CML CD34\textsuperscript{+} cells, while sparing normal CD34\textsuperscript{+} cells in vitro**

Given the additive effects of NL in combination with RUX on STAT5 signalling, the biological effects of these agents against primary CML CD34\textsuperscript{+} cells were investigated in vitro. Following preliminary experiments to establish the IC\textsubscript{50} of RUX in this cell population (supplemental Figure 2), a concentration of 200nM was selected. This concentration was commensurate with that used in published preclinical studies of cell lines harbouring the JAK2\textsuperscript{V617F} mutation\textsuperscript{29} and below the maximal concentration of 1000nM achieved in the plasma of patients treated with currently licensed doses of RUX\textsuperscript{38}. Moreover this concentration was 100-fold less than the RUX concentration reported to produce off-target effects on BCR-ABL kinase\textsuperscript{27}. While either NL or RUX showed limited effects on CML CD34\textsuperscript{+} cell viability, their combination induced higher levels of apoptosis (Figure 3A). Similarly, while RUX had no effect and NL reduced CML CD34\textsuperscript{+} CFC output by 47% relative to starting CFC output (baseline), their combination produced a further 49% reduction in the total number of CFC produced relative to NL as a single agent (Figure 3B). Similar effects were seen for both erythroid and granulocyte-macrophage colonies (Figures 3C, D) and NL and RUX combined were also noted to reduce the size of the colonies (Figure 3E). Interestingly these effects were not observed when CML CD34\textsuperscript{+} cells were grown in the absence of any GFs (supplemental Figure 3). A similar series of experiments was then performed using normal CD34\textsuperscript{+} cells to determine if there was a selectivity towards CML CD34\textsuperscript{+} cells over normal SPC for the drug combination. Following combination treatment, apoptosis levels were only mildly and not significantly increased for the combination versus each single agent and this did not translate to a significant reduction in normal CFC output (Figures 3F, G). Overall these data showed that the combination of NL and RUX was more effective than NL treatment alone in eradicating CML CD34\textsuperscript{+} cells, including CFC, in short term culture assays. These effects were observed only in the presence of GFs. Although a
degree of toxicity towards mature normal progenitors was observed this was less marked than for CML CD34⁺.

**The combination of NL and RUX increases kill of human CML CD34⁺ CD38⁻ and CFSEmax cells while sparing primitive normal CD34⁺ cells in vitro**

In order to assess the effects of the NL and RUX combination on a more primitive population of CML and normal SPC, cell division tracking experiments using CFSE staining were performed. These confirmed a marked effect on CML CD34⁺ cell proliferation with the combination treatment, with a reduction in undivided (CFSE\text{max}), slowly proliferating (division 1) and the total number of CD34⁺ cells recovered (Figures 4A, C). These effects were paralleled by an increase in apoptosis levels within the CFSE\text{max} population with the combination treatment compared to either single agent (Figure 4E). Conversely similar experiments on normal CD34⁺ cells confirmed a slight anti-proliferative effect for the combination treatment, however in contrast to results for CML there was no significant reduction in the total number of normal CD34⁺ cells recovered over 72 hours and in particular no significant reduction in the CFSE\text{max} population (Figures 4B, D). Nor was there a significant increase in apoptosis levels within the CFSE\text{max} population (Figure 4F). Finally, similar additive effects were also observed following NL and RUX combination treatment on sorted populations of CML \((BCR-ABL)⁺ by FISH\) CD34⁺CD38⁻ cells which are known to be enriched for the most primitive, quiescent population\(^{39}\) (Figure 4G). Taken together these data showed that NL and RUX in combination caused a more profound effect on the quiescent stem cell compartment compared to either agent alone while appeared to spare the normal quiescent fraction.

**The combination of NL and RUX selectively eliminates NSG repopulating CML cells**

The effects of NL and RUX, alone and combined, were compared against primary CML and normal SPC in NSG transplantation assays (Figure 5A). CD34⁺ cells treated with NL, RUX, or the combination were injected into NSG mice and engraftment was evaluated. We chose this ex vivo treatment approach since CML CP cells demonstrate relatively poor engraftment and do not develop leukaemia in NSG mice which limits the feasibility of in vivo drug treatment studies to evaluate the effects of treatment on leukaemia development and survival. Following 72 hours drug exposure reconstitution of human CD45⁺ haemopoiesis by CML
CD34+ cells was significantly reduced by either RUX or NL alone, however the combination of NL with RUX induced a more profound reduction of short-term (6 weeks) and long-term (18 weeks) engraftment of CML cells in NSG mice (supplemental Figure 4 and Figure 5B). In particular both CD34+ cell engraftment and myeloid engraftment were significantly reduced by the combination treatment (Figure 5C). Moreover qRT-PCR analysis showed that the reduction in human CD45+ cell engraftment correlated with a reduction of BCR-ABL levels in residual cells (Figure 5D). Similar experiments performed using CD34+ cells derived from normal human CB showed that both NL alone and the NL with RUX combination reduced the reconstitution of total human CD45+ haemopoietic cells (supplemental Figure 5). However subset analysis confirmed that the reduction in engraftment was restricted to CD19+ B cells with no such effect on either CD34+ or CD33+ myeloid cell engraftment. The reduction in B cell engraftment was driven by NL and not further affected by the addition of RUX (Figure 5E).

Overall these results showed that the combination of NL and RUX reduced the transplantable leukaemogenic activity of CML CD34+ cells more effectively than either agent alone while having no significant effects on normal human CD34+ and myeloid cell engraftment.

**Discussion**

The development of JAK2 inhibitors available for clinical investigation has opened up an exciting potential avenue to explore in CML, however further clarification of the role of JAK2 in this disease is required before pursuing it as a legitimate therapeutic target. In this study we have provided preclinical evidence for effective targeting of JAK2 in CML CD34+ cells, using the inhibitor RUX, by showing a consistent reduction in viability, colony output and proliferation of CML CD34+ cells, including the quiescent fraction, *in vitro* and reduced engraftment of CML CD34+ cells *in vivo*, when treated with NL and RUX compared to NL treatment alone. Interestingly the effects of RUX as a single agent were modest, but became more dramatic when combined with NL, suggesting that the role of JAK2 is particularly prominent during TKI therapy and that CML CD34+ cells become more reliant on JAK2 kinase when BCR-ABL is fully inhibited. These findings are consistent with those published recently by other research groups, mainly using non-clinically developed JAK2 inhibitors40-43, but are contrary to those reported by Hantschel *et al*, showing that BCR-ABL is still able to transform murine BM cells in which JAK2 has been deleted, both *in vitro* and *in vivo*27.
Moreover a recent report suggested that JAK2 deletion might accelerate CML development in mouse models by preferentially causing elimination of normal HSC which are more dependent on JAK2 signalling compared to CML LSC where BCR-ABL is active. This discrepancy might be explained by the fact that the role of JAK2 may indeed be dispensable when BCR-ABL is fully active, but becomes critical when BCR-ABL kinase is inhibited as has also been shown for other potential targets. Only upon inhibition of BCR-ABL do these targets become relevant, thus explaining the need to continue TKI together with any novel therapeutic agent to eradicate CML LSC, despite the evidence that CML LSC are not BCR-ABL kinase dependent for their survival. Moreover in Hantschel’s report a different model was employed (respectively BCR-ABL transduced murine BM cells versus primary CP CML cells, with the former likely expressing higher levels of BCR-ABL compared to the latter) and most of their in vitro work was performed in the absence of exogenous GFs. We also noted that when RUX and NL were combined in CML cells grown in the absence of GFs, the additional effects of the JAK2 inhibitor were abolished, suggesting that one of the main roles of JAK2, independent of BCR-ABL kinase and in the presence of NL, is to relay survival signals from exogenous GFs which can be effectively inhibited by RUX, as also previously reported. This further helps to resolve the discrepancy between our findings and those of Hantschel et al. Our data therefore suggested that in the absence of GFs, JAK2 signals were either absent or under the direct control of BCR-ABL, hence completely abrogated by the high doses of NL used in our experiments. Based on our data, one of the putative mechanism of action of the NL and RUX combination in the presence of exogenous GFs was a more profound inhibition of JAK2/STAT5 activity, as shown by the correlative changes in both p-JAK2 and p-STAT5 levels, with combined treatment, associated with correlative changes in STAT5 target genes. This was not owing to off-target inhibition of BCR-ABL kinase by RUX, as has been shown for other JAK2 inhibitors, and we were careful to use RUX at concentrations well below those previously shown to potentially cause off-target inhibition of BCR-ABL kinase, i.e. more than 20μM. Moreover achieving further inhibition of BCR-ABL kinase activity by adding RUX was highly unlikely in our experimental setting as the NL concentration used was shown to achieve maximal BCR-ABL kinase inhibition. In summary we conclude that enhanced inhibition of STAT5 activity by the NL and RUX combination was one of the major mechanisms underlying the effects seen.

Alternative modes of action for the NL and RUX combination independent of STAT5 activation are however possible. Although we deliberately chose NL for this study as the
most specific BCR-ABL kinase inhibitor available, we cannot exclude that additional NL targets are important and required for the observed additive effects. Similarly JAK2 has also been shown to have other targets than STAT5. It directly phosphorylates BCR-ABL on its key residue tyrosine 17720 (which is central for its transforming capacity through its ability to activate both the RAS/MAP kinase and the PI3 kinase pathways), it functions as a histone kinase and chromatin modifier while also being able to activate MYC and β-catenin. This last effect is particularly relevant given the central role of β-catenin in CML LSC self-renewal and might explain the effects of RUX on the most primitive population, including CFSEmax, CD34+CD38- and NSG repopulating cells, which support those already reported by others using different, non clinically developed JAK2 inhibitors. Finally it should also be considered that RUX is a potent inhibitor of JAK1 and other oncogenic kinases at the concentrations used and therefore some of the effects seen could be mediated independently of JAK2. In this respect, other reports on the efficacy of RUX (and other JAK inhibitors) in BCR-ABL positive cells have shown inhibition of STAT3 activity (a downstream target of JAK1/2) as a possible mechanism of action for these agents. All the above might explain why the effects seen in functional assays are greater than the ones seen on the JAK2/STAT5 pathway when using RUX alone. Future work will clarify if these or other unknown mechanisms of action of JAK2 are present in CML SPC which might account for the effects seen here in functional assays.

One of the main concerns for the use of JAK2 inhibitors in CML is their potential toxicity to normal BM, following a report showing toxicity of several JAK2 inhibitors towards normal haemopoiesis. Despite its apparently narrow therapeutic window, our in vitro and in vivo data reassuringly showed that a strategy combining RUX and NL preferentially eradicated CML compared to normal CD34+ cells, when a carefully selected concentration of RUX was chosen. The reason behind the increased sensitivity of TKI treated CML versus normal SPCs to JAK2 inhibition is currently unclear. We speculate that in CML SPCs, the prior expression of BCR-ABL may alter signalling networks such that cells are dependent on higher levels of GFs signalling activity, possibly due to upregulation or enhanced activity of cytokine receptors as shown already in both CML and acute myeloid leukaemia models. Upon BCR-ABL inhibition these cells may therefore be dependent on higher levels of GF mediated JAK2/STAT5 activation for continued viability as compared to normal cells. Moreover it is reassuring that RUX has been used as a single agent for the treatment of patients with myelofibrosis, with no severe BM toxicity experienced, especially for patients with good
BM reserve, which is the prevailing situation in CP CML patients with minimal residual disease (MRD) on TKI. This suggests that the BM toxicity secondary to JAK2 inhibition in adults might be less than that observed during embryonic development in JAK2 KO mice\textsuperscript{12-14}. This is to be expected given that chemical inhibition is less complete than gene deletion and evidence that JAK2 scaffolding functions, which would not be targeted by a JAK2 kinase inhibitor, might be important for its activity\textsuperscript{56}. In conclusion, our work supports a role for JAK2 in primary CML SPC survival, and provides further preclinical evidence for studies combining TKI with RUX in CML patients. We also provide evidence of a role for JAK2 in activating STAT5 dependent survival signals in CML CD34\textsuperscript{+} cells, upon BCR-ABL kinase inhibition and in the presence of GFs, thus explaining the observed efficacy of JAK2 inhibitors. We therefore believe that in CML patients with MRD who have optimally responded to TKI therapy, additional therapeutic strategies targeting JAK2 in combination with TKI warrant investigation in an attempt to achieve disease eradication.
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Authorship Contributions

P.G. designed and performed research, analysed and interpreted data and wrote the manuscript; A.C., S.R., H.G.J., L.H., H.W. performed research, analysed data and reviewed the manuscript; A.D.W., H.G.J., R.B. and T.L.H. designed research, interpreted data and reviewed the manuscript.

Conflict of Interest Disclosures

P.G. has previously received travel grants from Bristol-Myers Squibb, R.B. has previously served in advisory boards and received honoraria from Novartis, Bristol-Myers Squibb, Pfizer and Teva and T.L.H. has previously received research funding from Novartis and Bristol-Myers Squibb.
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Figure legends

Figure 1. Effects of the NL and RUX combination on JAK2/STAT5 signalling

CML CD34+ samples (n=3) were either left untreated (UT) or treated with NL (5µM) or RUX (200nM) or their combination. After 24 hours of incubation in suspension cultures p-STAT5Tyr694 (A) and p-JAK2Tyr1007/1008 (B) levels were measured by intracellular flow-cytometry. Levels of phosphorylation of both proteins were expressed as a ratio of the mean fluorescence intensity (MFI) of p-STAT5Tyr694 (A) and p-JAK2Tyr1007/1008 (B) antibody stained cells over the MFI of cells stained with a matched isotype control. The average of UT values was normalised to 100% and changes following treatment expressed as % change from UT.

(C) Candidate STAT5 target genes mRNA expression changes were measured in CML CD34+ samples (n=5) following 8 hours in suspension culture with NL (5µM) or RUX (1000nM) or their combination. Differences in gene expression levels following treatment were calculated using the 2^{-\Delta\Delta C_{t}} method after normalisation within each sample of candidate gene expression levels against the expression levels of the reference genes (GAPDH and TBP). Relative quantitation (RQ) of candidate genes mRNA expression following NL and NL and RUX treatment was then plotted as log_{2} of the 2^{-\Delta\Delta C_{t}} values (with the RUX treated cells having a value of 0 in the graph being the calibrator).

All data from independent experiments are presented as mean ± standard error of the mean (SEM). Significance values; *, P<0.05; †, P<0.01; ‡, P<0.001; ns, not significant.
Figure 2. Effects of the NL and RUX combination on BCR-ABL kinase activity

CML CD34⁺ cells were either left UT or treated for 8 hours with NL (5µM), RUX (1000nM) or their combination prior to protein extraction and measurement of (A) p-BCR-ABL (p-c-AblTyr²⁴⁵) (n=2) and (B) p-CrKL^{Tyr²⁰⁷} (n=4, 2 representative blots shown) levels by western blot. SH-PTP2 (A) and Beta-tubulin (B) were used as loading controls.
Figure 3. Effects of the NL and RUX combination on CML and normal CD34\(^+\) cell viability and CFC output

(A) CML CD34\(^+\) cells (n=3) were either left UT or treated with NL (5\(\mu\)M) or RUX (200nM) or their combination and cultured. At 48 hours apoptosis levels were measured by Annexin-V/7AAD staining.

CML CD34\(^+\) cells (n=3) were cultured as in (A) for 72 hours before drug washout and plating in methylcellulose progenitor assays. Total CFC output (B) was recorded after 12 days culture and compared to the CFC output for each sample prior to the start of the culture (baseline) (note that the number of colonies following 72 hours culture was adjusted for the expansion of CD34\(^+\) cells in vitro in each arm relative to baseline). CFC frequency based on their morphology – erythroid-burst forming unit (BFU-E) and erythroid-colony forming unit (CFU-E) (C) and granulocyte/macrophage-colony forming unit (CFU-GM) (D) – was also recorded and again compared to baseline. Representative pictures of the size and morphology of recovered CFC in each treatment arm is shown in panel E.

Normal CD34\(^+\) cells (n=3) were either left untreated UT or treated with NL (5\(\mu\)M) or RUX (200nM) or their combination. (F) At 48 hours apoptosis levels were measured by Annexin-V/7AAD staining. Normal CD34\(^+\) cells (n=3) were cultured as in (A) for 72 hours before drug washout and plating in methylcellulose progenitor assays. Total CFC output (G) was recorded after 12 days culture and compared to the CFC output at baseline as explained in panel B.

All data from independent experiments are presented as mean ± SEM. Significance values; *, \(P<0.05\); †, \(P<0.01\); ‡, \(P<0.001\); ns, not significant.
Figure 4. Effects of the NL and RUX combination on primitive and quiescent (CFSE\textsuperscript{max}) CML and normal CD34\textsuperscript{+} cells

CML (A) and normal (B) CD34\textsuperscript{+} cells (n=3) were stained with CFSE and either left UT or treated with NL (5µM) or RUX (200nM) or their combination and cultured. At 72 hours the percentage of starting CD34\textsuperscript{+} cells recovered within each division in each treatment arm was calculated by recording the number of viable cells seeded initially in each culture and their number following different treatment conditions and using levels of CFSE fluorescence to measure the percentage of cells within each division as explained elsewhere\textsuperscript{32}. Percentage of total number of starting CML (C) and normal (D) CD34\textsuperscript{+} cells recovered in each arm following treatment was also recorded at 72 hours. Percentage of apoptotic cells within the undivided (CFSE\textsuperscript{max}) population was measured by gating on the population double positive for maximal CFSE expression and Annexin-V staining at 72 hours for both CML (E) and normal (F) CD34\textsuperscript{+} cells. (G) Sorted CML, BCR-ABL\textsuperscript{+} (by FISH) CD34\textsuperscript{+} CD38\textsuperscript{−} cells (n=3) were cultured as in panel A for 72 hours. Percentage of viable cells was measured by gating on the double negative population following Annexin-V/DAPI staining. The results were then normalised against UT within each sample. All data from independent experiments are presented as mean ± SEM. Significance values; *, $P<0.05$; †, $P<0.01$; ‡, $P<0.001$; ns, not significant.
Figure 5. Effects of NL and RUX combination on the transplantable leukaemogenic activity of CML CD34+ cells and on normal CB CD34+ cell engraftment

(A) Experimental design for the in vivo experiments. CML CD34+ cells (from 2 patients) or human cord blood CD34+ cells (from 2 subjects) were cultured for 72 hours with either NL (1µM), RUX (500nM), their combination or media control. Following treatment, recovered cells were washed and injected intravenously into 8-week old, sublethally irradiated (300 cGy) NSG mice (CML, 5 mice/per group, cord blood 4 mice for controls, 4 for RUX, 5 for NL and 5 for NL+RUX). The levels of human CML CD45+ cells (B) and CD34+, CD33+ and CD19+ cells (C) regenerated in the BM of mice transplanted with cells treated in different conditions were measured at 18 weeks. The levels of BCR-ABL mRNA within the human CML CD45+ selected cells obtained from mouse BM at 18 weeks were evaluated by qRT-PCR and normalised to BCR mRNA expression levels (D). The levels of human CB CD34+, CD33+ and CD19+ cells regenerated in the BM of mice transplanted with cells treated in different conditions were measured at 16 weeks (E). All data from independent experiments are presented as mean ± SEM. Significance values; *, P<0.05; †, P<0.01; ‡, P<0.001; ns, not significant.
Figure 1

(A) CML CD34+

- %p-STAT5+ relative to UT
- %p-JAK2+ relative to UT

(B) CML CD34+

- %p-STAT5+ relative to UT
- %p-JAK2+ relative to UT

(C) CML CD34+

- RQ log2

**Figure 1**
Figure 2

**A**

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**B**

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</table>
Figure 4
Figure 5

CML or CB CD34+ cells

72h RUX (500nM), NL (1 µM), Combination, or media control

Wash cells

IV injection NSG mice

16 weeks (CB) 18 weeks (CML)

Check human cell engraftment in BM at 16-18 weeks

HUMAN CML CELLS IN BONE MARROW

% Human CD45+ cells

DMSO
RUX
NL
NL+RUX

†
*

HUMAN CML CELLS RECOVERED FROM BONE MARROW

BCR-ABL mRNA EXPRESSION IN HUMAN CML CELLS RECOVERED FROM BONE MARROW

2^-Delta CT (Ratio to BCR)

UT NL RUX NL+RUX

† ns *

HUMAN CORD BLOOD CELLS IN BONE MARROW

% of Human CD45+ cells

CD34+ CD33+ CD19+

DMSO RUX NL NL+RUX

† ns *

Figure 5