Low-dose methotrexate in myeloproliferative neoplasm models

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In recent years gain-of-function driver mutations in JAK2, MPL and CALR have been identified that constitutively activate JAK/STAT pathway signaling.\textsuperscript{1,2} Collectively, these mutations lead to the development of the vast majority of myeloproliferative neoplasms (MPNs), a group of related diseases including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (MF). Patients with MPNs have higher mortality rates, primarily due to cardiovascular complications, infections and transformation to other hematological malignancies such as leukaemias.\textsuperscript{3} They also suffer significant constitutional symptoms including pruritus, headaches, weight loss, loss of appetite, fatigue and night sweats. Current MPN therapeutics include venesection to control blood counts in PV, aspirin to reduce the risk of thrombosis in both PV and ET and cytoreductive agents such as hydroxycarbamide to reduce blood counts. However, these treatments do not slow disease progression and provide little relief from sometimes debilitating constitutional symptoms. Recently, the JAK1/2 inhibitor ruxolitinib (rux) has emerged as a molecularly targeted therapy option. In trials, rux delivers significant survival benefits to MF patients, as well as a decrease in spleen size and constitutional symptoms.\textsuperscript{4} Similarly, randomized trials in advanced PV patients also showed significant decreases in constitutional symptoms, spleen size and control of hematocrit in those receiving rux.\textsuperscript{5} However, while effective, access to rux remains restricted and is not available to all patients that might benefit. Given these limitations, access to more affordable JAK/STAT pathway inhibitors would potentially address a significant unmet clinical need.
We have previously identified methotrexate (MTX) as a dose-dependent inhibitor of STAT phosphorylation in JAK2 V617F positive erythroleukemia-derived HEL cells (Fig 1A, Ref 6). Originally developed as an anti-folate and chemotherapy agent, MTX was repurposed during the 1980's at doses around 1% of chemotherapy levels and is now widely used to treat multiple autoimmune and inflammatory diseases including rheumatoid arthritis. Given that JAK/STAT pathway signaling is fundamental to both immune regulation and inflammatory responses we hypothesize that the effectiveness of MTX in these diseases is likely to be a consequence of JAK/STAT pathway suppression in vivo. As such, we reasoned that other diseases featuring inappropriate pathway activation may also respond to low-dose MTX treatment.

Consistent with its role in disease, transgenic mice whose endogenous JAK2 locus has been replaced by the human JAK2 V617F allele develop ET-like disease when heterozygous and PV-like symptoms, including erythrocytosis and splenomegaly, when homozygous (Fig S1, Ref 9). Both homozygotes and heterozygotes also have increased white cell counts (Fig S1). We therefore treated wild type and hJAK2 V617F age-matched littermates with either MTX or vehicle control (PBS) for 28 days using a low-dose regime previously shown to reduce rheumatoid arthritis-like symptoms in mouse models. Compared to controls, spleens of homozygous hJAK2 V617F mice contain increased levels of pSTAT5 and pSTAT3 (Fig 1B) while mRNA levels of the JAK2/STAT5 target gene PIM1 is also increased (Fig 1C). Strikingly, and consistent with in vitro results, homozygotes treated with MTX have reduced levels of pSTAT5 and pSTAT3 (Fig 1B) and express significantly lower levels
of the pathway target gene PIM1 (Fig 1C) suggesting that low-dose MTX also inhibits the JAK/STAT pathway in vivo.

We next tested hJAK2 V617F-expressing mice treated with MTX or rux as a positive control.\textsuperscript{12} Increased hemoglobin levels in both heterozygous and homozygous hJAK2 V617F mice are significantly reduced following treatment with MTX (Fig 1D), as are red blood cell counts and hematocrit levels (Fig S2A, B). Importantly, despite being as effective than rux under these conditions, MTX treatment does not result in the general myelosuppression of wild type controls (Fig 1D, Fig S2) and both controls and MTX treated mice continued to gain weight throughout the course of the experiment (not shown). While platelet numbers and mean corpuscular volume are not significantly affected (Fig S2C, D), MTX treatment is sufficient to normalize white cell counts (WCC) in both heterozygous and homozygous hJAK2 V617F mice, but does not affect the differentiation or relative levels of individual white blood cell subtypes (Fig S3). Consistent with these findings, histological analysis of bone marrows (Fig 1E and S4) shows no reduction in cellularity or differentiation in any of the three hematopoietic lineages in MTX-treated animals. Homozygous animals do however have erythrocytic and megakaryocytic hyperplasia and polylobated nuclei in a proportion of megakaryocytes, as previously described\textsuperscript{9}, phenotypes that are subjectively reduced in MTX-treated animals. As such, bone marrow morphology is consistent with a disease-specific effect of MTX, rather than non-specific myelosuppression.

Another MPN-related phenotype, and cause of considerable constitutional symptoms in patients, is splenomegaly.\textsuperscript{13} By 10-11 weeks of age homozygous
hJAK2 V617F mice have spleens around 3 times larger than wild type littermates, an enlargement that is strongly reduced by treatment with both MTX and rux (Fig 2A-B). Histological analysis of these spleens shows that homozygotes have erythroid hyperplasia with red pulp expansion and loss of normal architecture, with occasional megakaryocyte clusters\(^9\), features which are mitigated by MTX treatment which show reduced erythroid infiltration and a correspondingly improved morphology of white pulp (Fig 2C and S5).

Although MTX treatment reduces spleen weight and reticulocyte numbers in JAK2 V617F homozygous mice (Fig S6A-B), we also observed modest increases in reticulocyte numbers and spleen size and in wild type littermates (Fig S6A,B). Although the precise basis of these phenotypes is unclear, it is possible that splenic enlargement in wild type mice is associated with hypersplenism and increased red cell destruction. In this scenario, the elevated reticulocyte count is notable as it supports a model in which methotrexate is not causing myelosuppression, with the marrow being able to increase erythropoiesis and mount an appropriate reticulocyte response.

Intriguingly, we also observed slight increases in pSTAT5 and PIM1 mRNA levels in MTX-treated wild type animals (Fig S6C,D), changes that are not mirrored in JAK2 V617F homozygotes. While a detailed molecular analysis of the interactions between MTX and wild type JAK2 will be needed to elucidate the basis of these observations, it is possible that this observation may be relevant to the rare cases of MTX-induced lymphoma previously described in RA patients.\(^{14}\)

Finally, in order to gain insight into the potential mechanistic basis of MTX-mediated effects we observed in vivo, we undertook an in silico study in which
a high resolution structure of the human JAK2 JH1 kinase domain\textsuperscript{15} was used as a target onto which to dock MTX and ATP (Fig 2D-G, Table S1). Although such studies can only ever be indicative of potential interactions, it is intriguing to note that MTX is predicted to occupy the ATP binding pocket of the kinase with a binding affinity higher than ATP itself, suggesting that MTX may potentially be acting directly as a Type 1 kinase inhibitor \textit{in vivo}.

Taken together, our results show that low-dose MTX not only acts as an inhibitor of JAK/STAT signaling \textit{in vivo}, but also strongly reduces the hematological phenotypes and splenomegaly associated with this \textit{hJAK2 V617F}-based mouse model of human MPNs. Moreover, these effects are not a consequence of drug-induced myelosuppression. While JAK/STAT pathway activity has clearly been reduced in these JAK2 V617F mice, it remains a possibility that at least some of the disease-related responses observed are a consequence of reduced systemic inflammation mediated by MTX. Interestingly, previous studies have also demonstrated striking reductions in the levels of pro-inflammatory biomarkers such as C-Reactive protein, TNF\textalpha and IL6 in human MF patients treated with ruxolitinib.\textsuperscript{4} However, the link between MPN-related phenotypes and inflammatory markers remains to be elucidated.

Although not curative, ruxolitinib is clinically valuable in multiple MPN patient populations, reducing mortality, normalizing hematological values and strongly reducing constitutional symptoms.\textsuperscript{4,5,13} However, concerns regarding cost-effectiveness have limited availability even in well-funded healthcare systems. By contrast, MTX is a low cost generic on the World Health Organization list of essential drugs. It is routinely prescribed for millions of patients worldwide
and has a well understood toxicology and safety profile. Here we have shown that low-dose MTX suppresses JAK/STAT pathway activity in vivo and is able to normalize hematological and splenic hyperplasia in mouse models of human MPNs. Consistent with our results, a recent case study has demonstrated significant hematological and symptomatic improvements in two Italian MPN patients following low-dose MTX treatment. In the light of these results, we suggest that clinical trials should be undertaken to assess the safety and efficacy of low-dose MTX as a JAK/STAT inhibitor in human myeloproliferative neoplasms. If results are promising, repurposing MTX has the potential to provide a new, molecularly targeted treatment for MPN patients within a budget accessible to healthcare systems throughout the world - a development that may ultimately provide substantial clinical and health economic benefits.

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Authorship Contributions

KC, ST, SL, MH, JD & DH devised and undertook experiments and interpreted data. AG, JS, AC, KC, JRS and MZ interpreted data and wrote the manuscript.

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Figure Legends

Figure 1: Methotrexate is a JAK/STAT inhibitor that reduces hJAK2 V617F-induced erythrocytosis in vivo

(A & B) Western blots of the indicated total (t) and phosphorylated (p) STAT proteins in extracts from HEL cells (A) or the spleens of 10-11 week old mice of the indicated genotypes (B) following treatment with the indicated concentrations of methotrexate and ruxolitinib. β-Actin serves as a loading control and apparent molecular weights are indicated in kDa.

(C) Levels of PIM1 mRNA expressed by spleen cells harvested from mice of the indicated genotype and drug treatments. Results are expressed as a fold change following normalization to β-actin mRNA and PBS treated wild type mice.

(D) Hemoglobin concentration in blood from individual 10-11 week old mice of the indicated genotypes treated with either phosphate buffered saline carrier control (PBS, grey), methotrexate (MTX, green) or ruxolitinib (rux, orange) for 28 days. Individual values, mean and standard deviations are shown. Samples were compared by one-way Anova.

(E) Hematoxylin and eosin stained sections through decalcified tibia from mice of the indicated genotypes treated with the indicated compounds for 28 days. No marrow fibrosis was observed in either treated or untreated homozygous animals. Scale bar is 50μm. Images were obtained from a Zeiss Axioskop 2 with a 20x/0.5NA objective, a MicroPublisher 5.0 RTV camera and Qimaging v3.1.3.5 software. Brightness and contrast were adjusted in
Figure 2: Methotrexate reduces splenomegaly

(A) Weight of individual spleens from 10-11 week old mice of the indicated genotypes shown relative to wild type controls treated with phosphate buffered saline carrier control (PBS, grey), methotrexate (MTX, green) or ruxolitinib (rux, orange) for 28 days. Samples were compared by one-way Anova.

(B) Representative spleens from mice of the indicated genotypes and treatments immediately after dissection showing differences in size and reduction in spleen size following MTX treatment. Scale bar is 5mm

(C) Hematoxylin and eosin stained sections through formalin fixed spleens from mice of the indicated genotypes treated with the indicated compounds for 28 days. Images were obtained from a Zeiss Axioskop 2 with 5x/0.15NA (top row, scale bar is 1mm) and 20x/0.5NA (lower row, scale bar is 100μm) objectives, a MicroPublisher 5.0 RTV camera and Qimaging v3.1.3.5 software. Brightness and contrast were adjusted in Photoshop CS5

(D-G) Predicted interactions of methotrexate with JAK2 occludes ATP-binding site. (D) Cartoon representation of the human JAK2 JH1 (kinase) domain (from 5TQ8.pdb). Methotrexate (magenta sticks) is predicted to bind between the N and C lobes of the kinase in the ATP-binding site (cyan sticks). (E) Molecular surface of kinase with ligands bound in the cleft between N and C lobes (carbon grey, nitrogen blue and oxygen atoms shown in red). (F) MTX
showing predicted H-bonding (orange dashes) to labelled residues within the binding site and ion-pair interaction with lysine 882. Residues shown in sticks are within 5 Å of the bound ligand. (G) View of bound MTX as in (F) rotated 90° about the Y axis.