Low-dose methotrexate in myeloproliferative neoplasm models

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Supplementary Information

Methods

Mice
Groups of 6-7 week old male and female wildtype, heterozygote or homozygote JAKV617F mice were treated for 4 weeks with either vehicle (PBS), 5 mg/kg MTX (Sigma-Aldrich) given 3 times a week by intraperitoneal injection or 90 mg/kg Rux (SelleckChem, Houston, Texas, USA) given 5 times a week by oral gavage. All treatment protocols involving animals were approved by the UK Home Office (project licence PPL 70/8799/3-M).

Blood and histological analysis
Peripheral blood was taken by cardiac puncture from isoflurane sedated mice into EDTA coated tubes. Total and differential blood cell counts were measured by an automated Sysmex XN-10 FBC analyser. For histological analysis, tibiae and spleens were fixed in 10% formalin and processed for hematoxylin and eosin staining before imaging using a Zeiss Axioskop microscope and Q-imaging camera system.

Western Blots
Cells or mouse spleens were lysed in RIPA buffer containing proteinase and phosphatase inhibitors and processed for western blot analysis as previously shown. Antibodies for pSTAT5, tSTAT5, pSTAT3, tSTAT3 (Cell signalling) and Actin (Abcam) were used.

Cell culture
Erythroleukaemia-derived JAK2 V617F-homozygous HEL cells were cultured and treated as described previously.

Real-time PCR
Quantitative real-time PCR was performed with a Biorad CFX96 as previously described, using the \( \Delta\Delta C_t \) formula with actin as the housekeeping control gene.

Statistics and graphical representation
Graphs and the indicated statistical analyses were generated in Prism Version 5.01 (GraphPad software). Significance was determined using one-way Anova.

in silico modelling
We used protein structure 5TQ8 from the Protein Database as the target for docking using Autodoc Vina software implemented from the PyRx interface. The protein structure was first prepared using pdbcur from the CCP4 suite. Ligand structures were obtained from the RCSB website and processed with obabel v2.3.1 with correct protonation for pH 7. An exhaustiveness level of 16 was used for docking of all ligands.
References


Figure Legends

Figure S1: Baseline blood cell numbers in hJAK2 V617F mouse.

(A-D) Hemoglobin (A), hematocrit (B), platelet number (C) and white blood cell counts (D) of blood from individual 10-11 week old mice of the indicated genotypes. Individual values, mean and standard deviations are shown. Samples were compared by one-way Anova.

Figure S2: Effect of MTX on blood counts from hJAK2 V617F mice

Red blood cell count (A), hematocrit (B), platelet numbers (C) and mean corpuscular volume of individual 10-11 week old mice of the indicated genotypes treated with either phosphate buffered saline carrier control (PBS, grey dots), methotrexate (MTX, green dots) or ruxolitinib (rux, orange dots) for 28 days.

Figure S3: Effect of MTX on white blood cell counts from hJAK2 V617F mice

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

(B-C) Stacked bar graphs for each individual mouse of the indicated genotype and drug treatment. Graphs show the % (B) and absolute numbers (C) of the indicated white blood cell types where BAS=basophil, EO=eosinophil, MONO=monocyte, NEUT=neutrophil, LYMPH=lymphocyte. The mean total of all white blood cells is shown by the blue bar in C.

Supplemental Figure S4: Bone Marrow Histology

Haematoxylin and eosin stained sections through de-calcified and formalin fixed tibia from mice of the indicated genotypes treated with the indicated compounds for 28 days. Scale Bar= 50μm

Supplemental Figure S5: Spleen Histology

Haematoxylin and eosin stained sections through formalin fixed spleens from mice of the indicated genotypes treated with the indicated compounds for 28 days. Scale bars in larger sub-panels =200μm and in smaller high magnification panels 50μm

Figure S6: Effect of MTX on wild type mice

(A) Spleen weights of 10-11 week old mice of the indicated genotypes and drug treatments shown as a % of body mass. The increased mass of MTX
treated wild type and heterozygous spleens is not statistically significant. 

(B) Number of circulating reticulocytes in 10-11 week old mice of the indicated genotypes and drug treatments. The increase in MTX treated wild type and heterozygous spleens is statistically significant as determined by one-way Anova.

Western blot analysis of tSTAT5 and pSTAT5 (C) as well as Q-PCR analysis of the pathway target gene PIM1 mRNA (D) from spleens of mice of the indicated genotype and drug treatment. Low levels of pathway activation may be present in wild type mice treated with MTX.

Table S1: Binding of ligands to JAK2 kinase domain

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<th>Ligand</th>
<th>Binding Affinity</th>
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<tr>
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Comparison of predicted binding modes of ligands ranked by Autodock Vina scoring (kcal/mol). Methotrexate and ATP results are shown for the top nine scores of each.
Figure S1

A. Haemoglobin

B. Haematocrit

C. Platelets

D. White Blood Cells (WBC)
Figure S2

A) Red Blood Cells

B) Haematocrit

C) Platelets

D) Mean Corpuscular Volume
Figure S3

A

White Blood Cells (WBC)

![Graph showing WBC levels with drug and genotype groups.]

B

% of WBC

![Bar graph showing percentage of WBC with drug and genotype groups.]

C

WBC (x10^9/L)

![Scatter plot showing WBC levels with drug and genotype groups.]

Legend:
- PBS
- MTX
- wild type
- heterozygous
- homozygous
- BAS
- EO
- MONO
- NEUT
- LYMPH
- average
Figure S6

A. Spleen Weight (% body mass)

B. Reticulocytes

C. Protein expression levels:
- wild type
- homozygous

D. PIM1 mRNA levels

- wild type
- homozygous