Supplementary Figure S1

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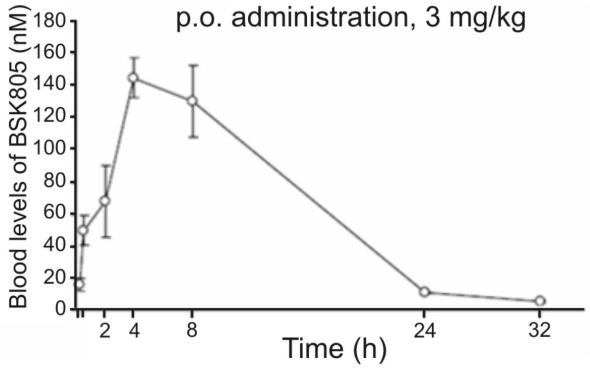

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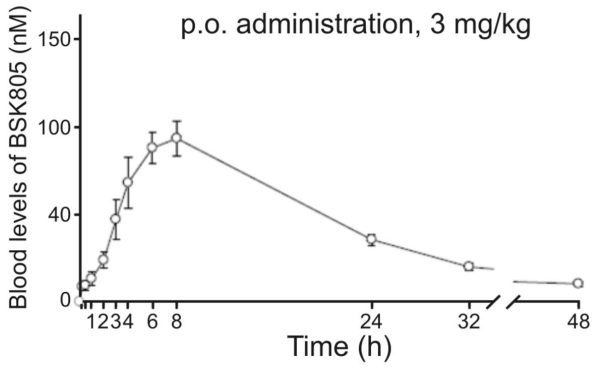
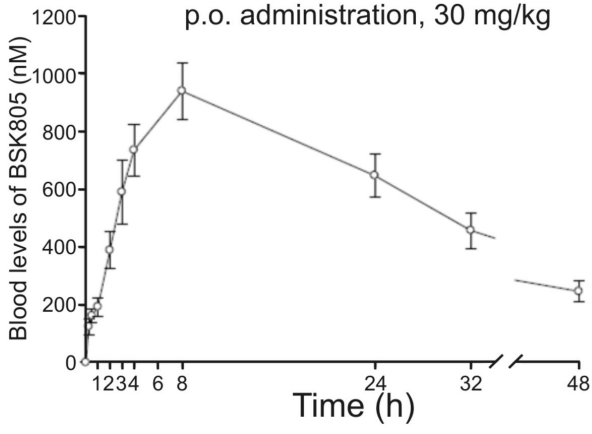




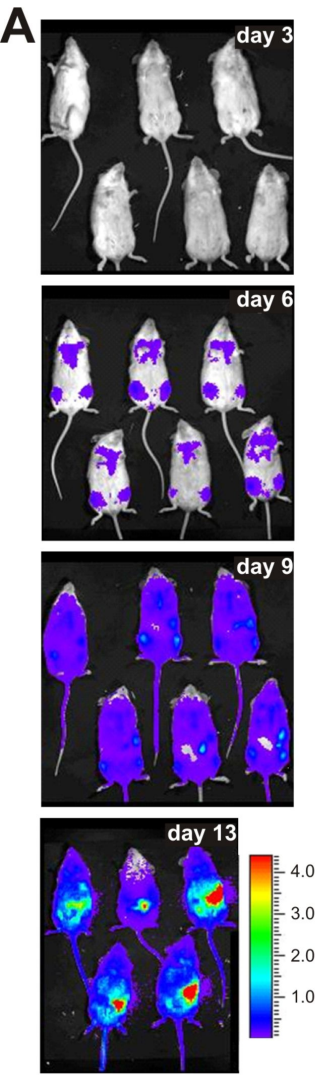
C

Mouse PK	
CL ($ml \cdot min^{-1} \cdot kg^{-1}$)	22
Vss (l/kg)	5.9
$t_{1/2term.}$ (h)	5.5
AUC ($nmol \cdot h \cdot l^{-1}$) i.v., d.n.	1573
AUC ($nmol \cdot h \cdot l^{-1}$) p.o., d.n.	711
C_{max} (nM) p.o., d.n.	48
T_{max} p.o. (h)	4
abs. oral bioav. (%)	45

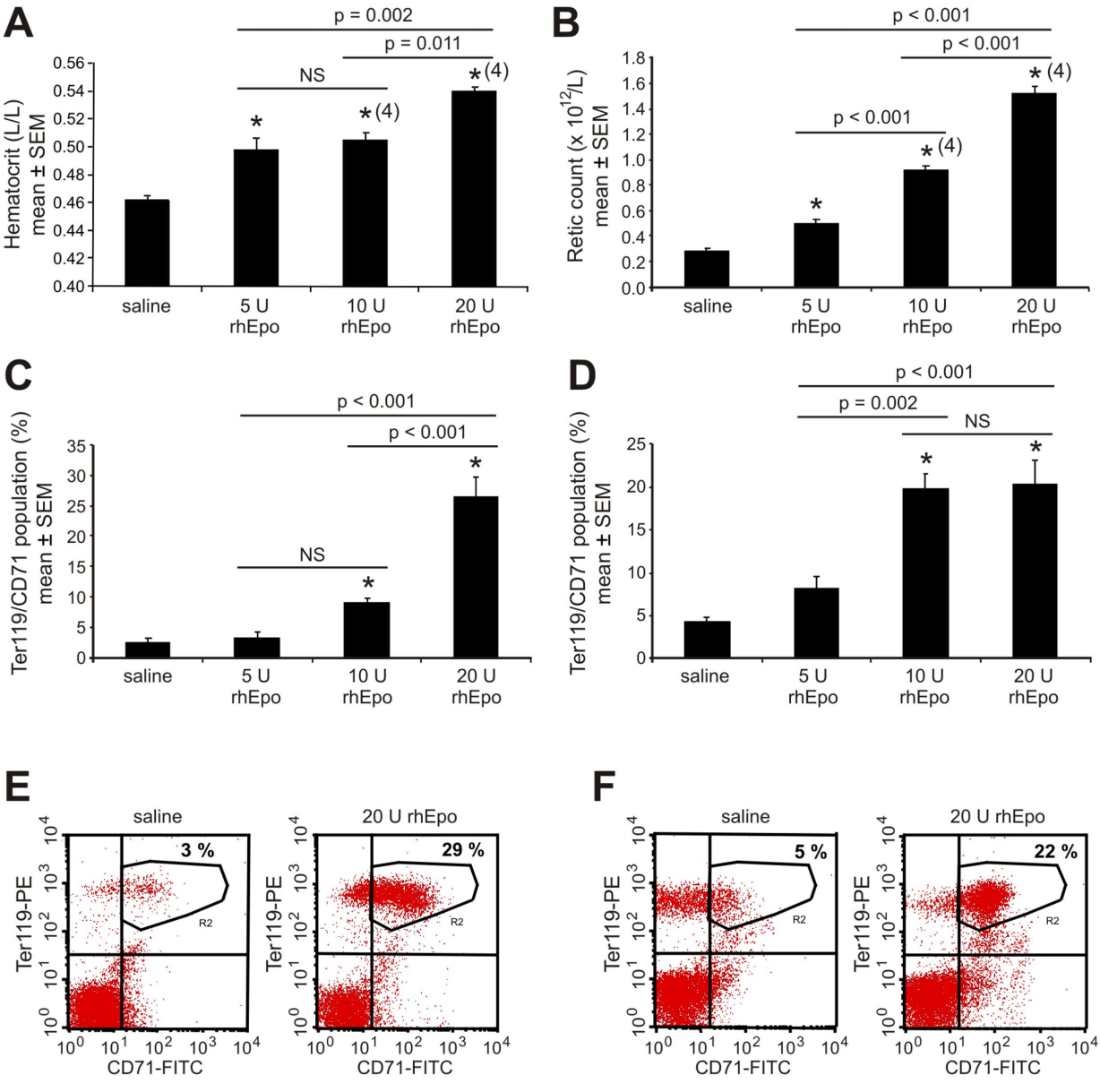
Rat PK	
CL ($ml \cdot min^{-1} \cdot kg^{-1}$)	23
Vss (l/kg)	33
$t_{1/2term.}$ (h)	18.4
AUC ($nmol \cdot h \cdot l^{-1}$) i.v., d.n.	1470
AUC ($nmol \cdot h \cdot l^{-1}$) p.o., d.n.	742
C_{max} (nM) p.o., d.n.	31
T_{max} p.o. (h)	7
abs. oral bioav. (%)	50

p.o. data is dose-normalized to 1 mg/kg

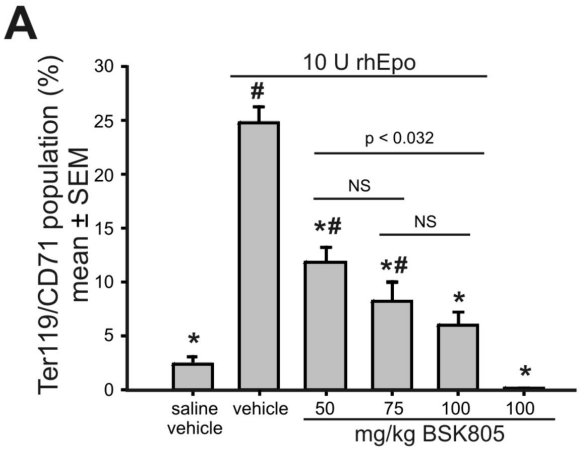
Supplementary Figure S2

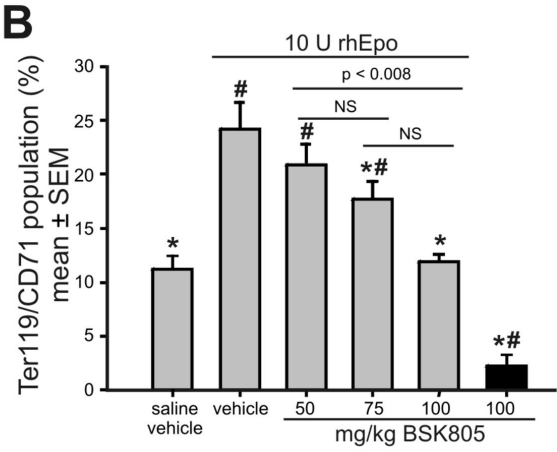


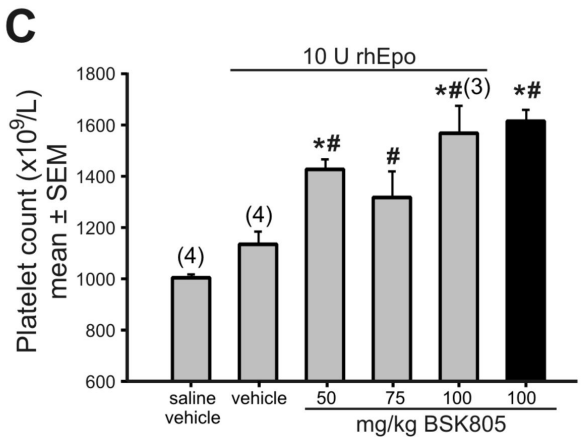
Supplementary Figure S3



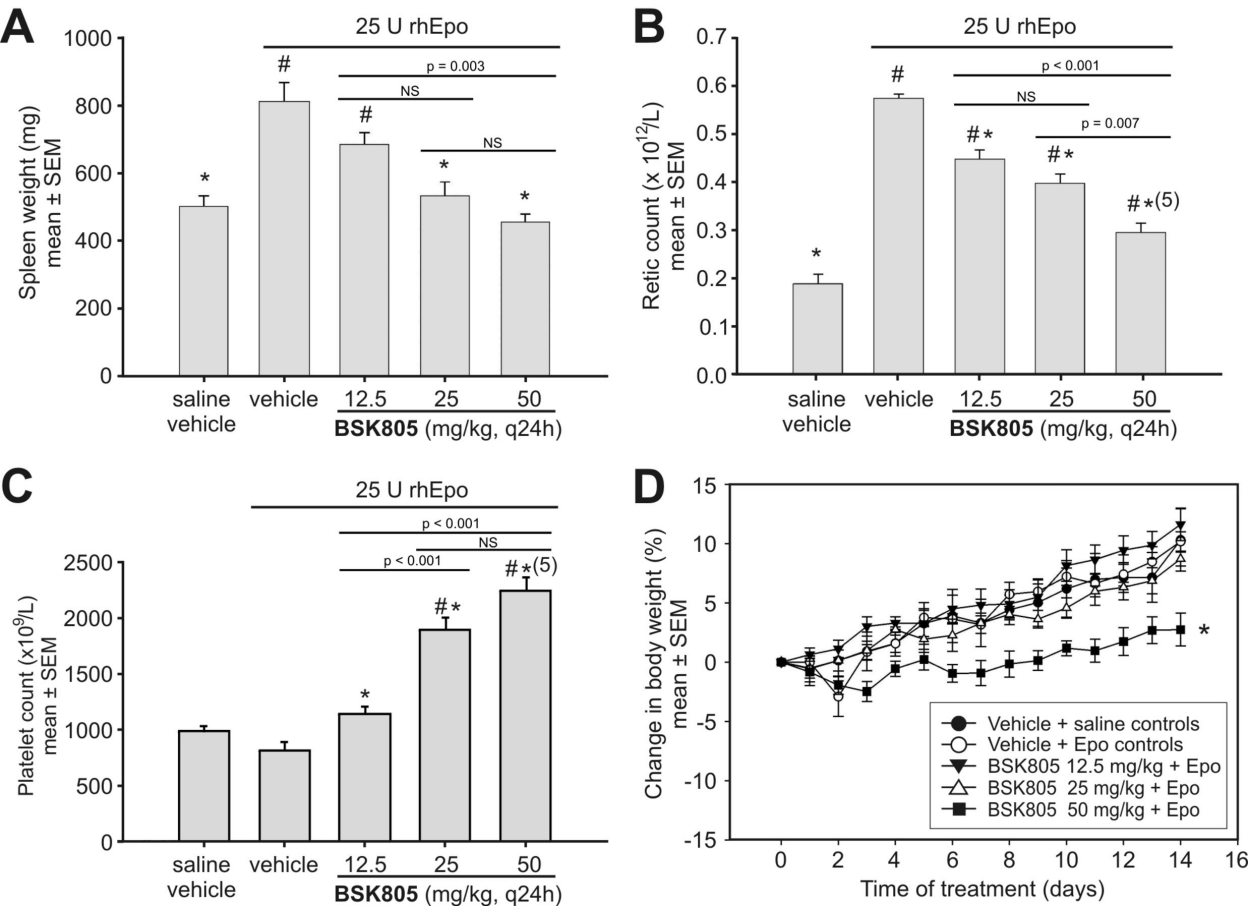
Supplementary Figure S4







Supplementary Figure S5



Supplementary information

Potent and selective inhibition of polycythemia by the quinoxaline JAK2 inhibitor NVP-BSK805

Supplementary materials and methods

Enzymatic assays

The human JAK2 kinase domain (amino acids 840-1132) was contained in plasmid construct pAcG2TtevJAK2. The plasmid constructs for JAK3 (813-1124), TYK2 (888-1187), and JAK1 (866-1154) were designed accordingly. The generation of the recombinant baculoviruses with BD BaculoGold™ Bright linearized DNA, plaque assay, and virus amplification from single plaques was performed according to the manual (BD Biosciences Pharmingen). Janus kinase domains were expressed in Sf9 cells in 400 mL shake flasks with 100 mL ExCell420 culture medium (JRH Biosciences Ltd) with penicillin/streptomycin solution (Sigma) for 48 h at 27 °C. Suspension culture cells were infected at a density of $1x10^6$ and the multiplicity of infection (MOI) for each virus was optimized for yield of soluble protein. The kinase domain of human JAK2 and of JAK1, JAK3, and TYK2 was expressed at an MOI of 1 and 0.5, respectively. Time of expression at 27 °C was 48 h for JAK2 and 48 h or 72 h for JAK1, JAK3, and TYK2. Forty-eight or seventy-two hours post-infection, the cells from a 100 mL expression culture were harvested by centrifugation at 3000 x g for 5 minutes and lysed with 12 mL lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1 mM sodium orthovanadate, 1 % Triton X-100, 10 % glycerol, 1 x EDTA-free complete protease inhibitor cocktail (Roche Diagnostics), and 12.5 U/mL Benzonase for 30 minutes at 4 °C, followed by centrifugation at 14,000 x g for 45 minutes to pellet insoluble material. For GST-tag affinity purification of kinase domain proteins all steps were performed at 4 °C. The cleared lysates were incubated with 0.2 mL of a 50 % slurry of washed Glutathione Sepharose 4B for 2 h at 4 °C, followed by 5 washes with 1 mL of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % Triton X-100, 1 mM DTT, and 10 % glycerol. Bound protein was eluted in 5 aliquots each starting with a 10 minutes incubation with 0.25 mL elution buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 % Triton X-100, 1 mM DTT, 10 % glycerol, 10 mM reduced L-glutathione). Eluates were concentrated about 5-fold with Amicon Ultra-4 spin columns (Millipore, 10’000 molecular weight cut-off). After addition of Brij35 to 0.1 % final concentration the protein was snap frozen in small aliquots and stored at -80 °C. In these conditions, kinase activities were stable for at least 6 months. The JAK kinase

domain enzymes were incubated for 30 minutes at room temperature in a medium containing 0.1 µM [γ33P]-ATP, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 30 µM of synthetic peptide substrate EQEDEPEGDYFEWLE, 1 mM DTT, 1 % DMSO, 50 µg/mL BSA, 0.01 % Brij35, and 50 mM Tris-HCl pH 7.5. The ATP concentration is below the K_m for all proteins. Curves were fitted by non-linear regression using the logistic equation and the global fit function of XLfit® (model 205). Expression and characterization of full-length wild type and V617F mutant JAK2 as well as kinase assay conditions have been described elsewhere (1). Kinase selectivity of NVP-BSK805 was assessed in an internal kinase panel: In the Caliper assays, kinase reactions were carried out with peptide substrates that migrate with different velocities in an electrical field when phosphorylated. The peptides carry a fluorescent label in order to allow the detection and quantification of the peptides in a capillary system. Peptide fluorescence intensities were quantified using the LC3000 instrument (Caliper Life Sciences, Hopkinton, MA, USA). In the Kinase Glo luminescent kinase assay (Promega, Madison, WI, USA) kinase activity is measured by quantifying the amount of ATP remaining in solution following a kinase reaction. In the LanthaScreen™ TR-FRET kinase assays (Invitrogen, Carlsbad, CA, USA), terbium is used as the lanthanide chelate combined with an antibody directed against the phosphorylated substrate.

RNA interference and JAK immunoprecipitation

The following stealth™ RNAi oligonucleotides (validated stealth™ RNAi DuoPak; Invitrogen, Carlsbad, CA, USA) were used; JAK2: duplex 1 5'-GCAACAGAGCCUAUCGGCAUGGAAU-3' and duplex 2 5'-GCAGCAAGUAUGAUGAGCAAGCUUU-3'; JAK1: duplex 1 5'-GCA CAAGAAGACGGAGGAAAUGGUAU-3' and duplex 2 5'-GCUUAAGGAAUAUCUUCC AAAGAA-3'; JAK3: duplex 1 5'-GCCAUGGGUCCUUCACCAAGAUUUA-3' and duplex 2 5'-GGGCCAUAGACAUGUAUCUGCGAAA-3'; TYK2: 1 5'-CCCAGAGAUGCAAGCCUG AUGCUAU-3' and duplex 2 5'-CCAUUCUGAAGACAGUCCAUGAGAA-3', and a non-targeting control stealth™ RNAi oligo 5'-GAUGAAGGGAGGGUGUACCAACUUA-3'. Cells were transfected with RNAi oligonucleotides using Nucleofactor™ Solution V (Amaxa GmbH, Cologne, Germany) and the Amaxa system according to the instructions of the manufacturer. In the case of CMK cells a second round of transfection was carried out after 24 hours. SET-2 cells were extracted after 48 hours and CMK as well as MB-02 cells were extracted after 72 hours as described above. After determination of protein concentrations, lysates were adjusted to 0.5 mg

total protein input in 200 µL of lysis buffer. For immunoprecipitation of JAK1, JAK2, JAK3 and TYK2 samples were incubated with the respective antibodies for 2 hours at 4 °C on a rotating device. The following antibodies were used for immunoprecipitation; 4 µL anti-JAK2 antibody (Cell Signaling Technology), 2 µg anti-JAK1 antibody, 1 µg anti-JAK3 antibody or 1 µg anti-TYK2 antibody (all from Santa Cruz Biotechnology). Then, 20 µL of Protein A Sepharose CL-4B beads (Amersham Biosciences, Buckinghamshire, United Kingdom) or 25 µL of UltraLink Immobilized Protein A/G beads (Pierce, Rockford, IL, USA) were added and samples were incubated for 1 hour with rotation at 4 °C. After washing, the bound fraction was released by adding 15 µL sample buffer and heating for 3 minutes at 95 °C. Samples were resolved by 8 % SDS-PAGE. JAK family members, levels of phospho-STAT5 and total STAT5 were determined by Western blotting as described above.

MOLM-13 and MV4;11 cell proliferation assays

FLT3-ITD (FMS-like tyrosine kinase-internal tandem duplication) mutant acute myeloid leukemia cell lines MOLM-13 (DSMZ, Braunschweig, Germany) and MV4;11 (ATCC, Manassas, VA, USA) were seeded in triplicate at a density of 20'000 cells per well in 90 µl of culture medium in 96-well plates. Cells were then treated with 10 µl of the compound ranging in concentration from 10 µM to 0.5 nM. Two controls were used, one with wells containing medium with 0.1 % DMSO and one with wells containing cells treated with the vehicle DMSO. Cells were incubated for 3 days at 37 °C. To assess the relative proliferation of the cells, 10 µl of the tetrazolium salt WST-1 (catalogue number 1644807, Roche Diagnostics GmbH, Penzberg, Germany) were directly added to the 100 µl of medium in each well and incubated for 20 minutes to 1 hour at 37 °C. Following incubation, absorbance was read with a spectrophotometer at 450 nm using 630 nm reference wavelength. Of each triplicate treatment the mean was calculated and these data were plotted in XLfit 4 (XLfit 4 curve fitting software for Microsoft Excel, ID Business Solutions Ltd, Guildford, Surrey, United Kingdom) to determine the respective GI_{50} values.

AlphaScreen Surefire phospho-STAT5 assay

SET-2 cells were pretreated for 30 minutes with an 8 point concentration range of the inhibitor and then stimulated for 10 minutes with 5 U/mL rhEpo (CellSciences, Canton, MA, USA).

STAT5 phosphorylation was assessed with the SureFire pSTAT5 Assay Kit according to the manufacturer's instructions (Perkin Elmer, Waltham, MA, USA). Briefly, cells were lysed by addition of AlphaScreen SureFire 5x lysis buffer. Cleared cell lysates were then incubated in the dark with a mix of Activation and Reaction Buffers containing AlphaScreen Acceptor Beads conjugated to the phospho-STAT5 antibody for 2 hours and subsequently for 3 hours with Donor Beads with conjugated STAT5 antibody (Perkin Elmer). Samples were read using a BMG-Labtech PheraStar microplate reader, using standard AlphaScreen settings.

IFN-γmediated STAT1-GFP nuclear translocation assay in HT1080 cells

To assess inhibition of IFN-γ driven, JAK1/JAK2-dependent, STAT1 cytoplasmic to nuclear translocation, HT1080 fibrosarcoma cells (ATCC, Manassas, VA, USA) were transfected with a STAT1-GFP construct using Fugene 6 Transfection Reagent (catalogue number 1815091, Roche Diagnostics GmbH, Penzberg, Germany) following the manufacturers protocol (3 µl of Fugene:1 µg of DNA). 24 hours after transfection the medium was replaced and selected in 1 mg/ml Geneticin. Selection was considered to be complete when all untransfected cells were dead. Cells were routinely cultured in alpha Modified Eagle Medium containing 10 % FCS and 400 µg/ml Geneticin. HT1080 STAT1-GFP cells were plated at a density of 10,000 cells per well in clear-bottom black 96-well Packard View-Plates™ (catalogue number 6005182, Packard Instrument Company, Meriden, CT, USA). 24 hours later, cells were pre-treated for 30 minutes with a 8 point concentration range of the inhibitor and then stimulated for 2 hours with 100 ng/ml IFN-γ (catalogue number 285-IF, R&D Systems, Minneapolis, MN, USA) Then, cells were washed twice in pre-warmed PBS and fixed in 200 µl of pre-warmed fixation solution (PBS, 3.7 % formaldehyde) for 10 minutes. The plates were washed twice in 200 µl PBS and incubated, protected from light, in 100 µl of DNA-staining solution (PBS, 0.5 µg/ml Hoechst-33342) for 1 minute. The plates were then washed once in PBS and 200 µl PBS were added per well. The plates were read on a Cellomics® ArrayscanII automated fluorescence microscope plate reader (Cellomics Inc., Pittsburgh, PA, USA) equipped with a Mercury-Xenon white light illumination source and a Zeiss Axiovert inverted microscope, using the XF100 dichroic/emission filter cube and matching excitation filters, 10x magnification, and a 0.3 numerical aperture objective. Image acquisition and analysis was performed using a customized protocol based on the "NuclearTranslocation" Bioapplication. IC_{50} values were calculated using Excel Fit software

(XLfit 4 curve fitting software for Microsoft Excel, ID Business Solutions Ltd, Guildford, Surrey, United Kingdom).

Phospho-STAT5 immunohistochemistry on spleen sections

Spleen pieces were immersion-fixed in neutral buffered formalin 10 % (v/v) at 4 °C for 24 hours. After fixation, tissue samples were rinsed in PBS and placed in 70 % ethanol until processing into paraffin on the TPCduo 15 automated tissue processor (Medite, Switzerland). 3 µm thick sections were cut on a sliding or rotary microtome (Mikrom International AG, Switzerland), spread in a 45 °C water-bath, mounted on microscope slides (Polysine, VWR International, Leuven, Belgium) and air-dried in an oven at 37 °C overnight. Thereafter, the sections were immediately used for anti-phospho-STAT5 immunohistochemistry. Sections were deparaffinized by three changes of fresh xylene for 3 x 5 minutes at room temperature, followed by hydration (with agitating from time to time) of the sections in absolute ethanol for 5 minutes, in 95 % ethanol for 5 minutes, in 70 % ethanol for 5 minutes, and finally in PBS (pH 7.4, w/o Ca^{2+} and Mg^{2+}), for 2 x 5 minutes. Heat-induced antigen retrieval was performed in TRS 6.1 buffer, pH 6.1 (DAKO, North America Inc.,USA), for 10 minutes at 98 °C using a temperature controlled microwave (Micromed T/T MEGA, Medite, Switzerland). Sections were allowed to cool for 20 minutes and were washed in PBS for 2 x 5 minutes. The endogenous peroxidase was blocked with freshly prepared 3 % H_2O_2 in methanol for 30 minutes, followed by rinsing in PBS for 2 x 5 minutes. Unspecific absorption was reduced by incubation of the sections with 10 % NGS (normal goat serum) in PBS for 30 minutes. For immunohistochemical detection, primary rabbit monoclonal anti-phospho-STAT5 (Tyr694) antibody (clone C11C5, Lot number 1, Cell Signaling Technologies, USA) was diluted 1:50 in Antibody diluent (DAKO, North America Inc., USA) and incubated overnight at 4 °C. Sections were rinsed twice in PBS for 5 minutes and incubated with DAKO EnVision™+ HRP-labelled polymer anti-rabbit secondary antibody (catalogue number K4003, DAKO, North America Inc., USA) for 30 minutes at room temperature. Thereafter, sections were rinsed twice in PBS for 5 minutes and incubated with freshly prepared liquid DAB+ substrate-chromogen (DAKO, North America Inc., USA) for 6 minutes. The reaction was stopped by rinsing sections in MilliQ water and counterstaining was performed in hematoxylin (HistoLab, Göteborg, Sweden) for 30 seconds. Sections were blued in tap water for 5 minutes, dehydrated by serial transfer through 70 %, 95 % and 100 % ethanol (with agitating

from time to time) followed by two rinses in xylene for 2 minutes each . Finally, sections were coverslipped with xylene-based mounting medium (Pertex, HistoLab, Göteborg, Sweden) using an automated coverslipper (Meisei Coverslipping Machine RCM 7000, Medite, Switzerland) and air-dried at room temperature.

Flow cytometry

Cultured cells were collected after treatments, washed once with PBS, and resuspended in propidium iodide buffer (1 mM sodium citrate (pH 4.0), 1.5 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP40, 4 µg of propidium iodide/mL, and 80 µg/mL of RNaseA in PBS). After 30 minutes of incubation in the dark on ice, cellular DNA content was measured with a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The percentage of cells with less than 2N DNA content (sub-G1 fractions) at each dose and time point was determined from four independent experiments. The *t*-test or, if not applicable, the Mann-Whitney rank sum test was conducted to determine statistical significance between two groups. The significance level was set at $p < 0.05$. Statistical analysis was performed using SigmaStat v3.1 (Jandel Scientific, San Rafael, CA, USA).

For analysis of murine erythroblasts, single cell suspensions from spleen samples were obtained using a 70 µm cell strainer. Bone marrow was obtained by centrifugation at 2'000 rpm of both tibias and aggregates were dispersed by flushing through a 23G needle. Cell suspensions from spleen or bone marrow were stained with conjugated antibodies for CD71 (FITC-conjugated) and TER119 (PE-conjugated) after RBC lysis. Briefly, cells were centrifuged at 2'000 rpm for 5 minutes and then erythrocytes were lysed with 500 µL (bone marrow) to 1 mL (spleen) of lysis buffer (Sigma) for 10 minutes at room temperature. Cells were then resuspended in 300 µL of PBS containing 2 % BSA, counted and stained. Cells were incubated first with 2.5 µL of Fc-Block for 30 minutes at room temperature. Then, 2.5 µL of each antibody was added followed by 30 minutes incubation at room temperature. Cells were washed twice in PBS containing 2 % BSA and subsequently analyzed by flow cytometry using the Cell Quest software (BD Biosciences). For the analysis, an R2 gate was drawn around the double positive TER119/CD71 population and the number of positive cells was expressed as percentage of total cells.

Quantitative Western blotting

Snap-frozen spleen samples were homogenized in RIPA lysis buffer (50 mM Tris-HCl pH 7.2, 120 mM NaCl, 1 mM EDTA, 6 mM EGTA pH 8.5, 1% NP-40, 20 mM NaF) supplemented with 0.1 % SDS, 2 mM sodium vanadate, 10 mM Na pyrophosphate and one protease inhibitor cocktail tablet per 50 mL (Complete Mini, Roche, Roche Diagnostics) using a Polytron homogenizer (Ultra-Turran T25, IKA Labortechnik), keeping samples on ice during the homogenization. After 1 hour on ice, two volumes of ice cold PBS were added to the samples prior to centrifugation at 2,000 rpm (15 minutes, 4 °C). Homogenates were filtered through glass-fiber filters and 40 µg of total protein from each sample were resolved by SDS-PAGE followed by transfer to Millipore Immobilon-P membranes. Membranes were blocked in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) and incubated with primary anti-phospho-STAT5 antibody (BD Biosciences) overnight at 4 °C. After several washes, the membranes were incubated at room temperature with goat-anti-mouse IRDye™ antibody conjugated secondary antibody (Infra Red Dye 800, Rockland, Gilbertsville, PA, USA) for 1 hour in the dark. The membrane was washed again and the immunoreactive signals read on the LI-COR Odyssey near infrared imaging system in the 800 nm channel using a resolution of 169 µm. To assess levels of total STAT5, the same membrane was stripped. After blocking for 1 hour at room temperature in Odyssey blocking buffer, the membrane was incubated with primary anti-STAT5 antibody as described above. After several washes membranes were incubated for 45 minutes at room temperature with goat anti-rabbit Alexa fluor 680 conjugated secondary antibody (Invitrogen) for 1 hour in the dark. The membrane was washed again and the immunoreactive signals read on the LI-COR Odyssey system in the 700 nm channel using a resolution of 169 µm.

Pharmacokinetic analysis

NVP-BSK805 blood concentrations were determined by HPLC/MS-MS using an internal standard method. Following addition of 25 µL of internal standard (1 µg/mL) to analytical aliquots (25 µL) of blood the proteins were precipitated by the addition of 200 µL acetonitrile. The supernatants were transferred in a fresh vial. After evaporation to dryness the samples were re-dissolved in 35 µL acetonitrile/water (1/1 v/v). An aliquot (5 µL) of this solution was separated on a RESECT™ Ultra Phenyl reverse-phase HPLC column (column size 50 x 1 mm, particle size 3µm, preceded by a guard column: Phenomenex™ AJO-4350 Phenylpropyl, size 4 x

2 mm) with a mobile phase consisting of a mixture of 0.2 % formic acid in water (solvent A) and 0.2 % formic acid in acetonitrile (solvent B). Gradient programming was used with a flow rate of 60 µL/min. After equilibration with 95 % solvent A, 5 µL of sample was injected. Following a latency period of 0.75 min, the sample was eluted with a linear gradient of 5 - 100 % solvent B over a period of 2.75 minutes followed by a 3.5 minutes hold. The column was prepared for the next sample by re-equilibrating over 3 minutes to the starting conditions. The column eluent was directly introduced into the ion source of the triple quadrupole mass spectrometer Quattro Ultima™ (Micromass, Manchester, UK) controlled by Masslynx™ 4.0 software. Electrospray positive ionization (ESI +) multiple reaction monitoring was used for the MS/MS detection of the analyte. Areas under the blood concentration versus time curves (AUC) were calculated from the mean values with linear trapezoidal rule, and further relevant parameters by using a non-compartmental model for extravascular dosing (WinNonlin® Professional Version 5.2, Pharsight corp., CA, US). The concentrations of Cmax and the last data point (Clast) were determined by inspection of the data.

Rat rhEpo-induced polycythemia model

For oral application in rat, NVP-BSK805 was formulated freshly in 50 mM citrate buffer, pH 3, and applied by gavage. The oral application volume was 5 mL/kg. Concomitantly with NVP-BSK805 treatment, rats received daily subcutaneous injections (500 µL, 2.5 mL/kg) of 25 U rhEpo (CellSciences, Canton, MA, USA) for 14 consecutive days. Controls were injected corresponding volumes of saline. Rats were sacrificed 24 hours after the final dose and total blood, spleen and bone marrow were taken for further analysis. Female Wistar rats were obtained from RCC, Switzerland. Animals were 10-12 weeks of age at treatment start (180-220g bodyweight). Animals were housed under optimized hygienic conditions in filter top cages with free access to food and water. The animals were fed food and water ad libitum. All animal experiments were performed in strict adherence to the Swiss law for animal protection. The experimental protocols were approved by the Swiss Cantonal Veterinary Office of Basel-Stadt.

Supplementary references

1. Erdmann D, Allard B, Bohn J, De Pover A, Floersheimer A, Fontana P, et al. Kinetic Study of Human Full-Length Wild-Type JAK2 and V617F Mutant Proteins. The Open Enzyme Inhibition Journal 2008;1:80-4.
2. Lacronique V, Boureux A, Della Valle V, Poirel H, Quang CT, Mauchauffe M, et al. A TEL-JAK2 Fusion Protein with Constitutive Kinase Activity in Human Leukemia. Science 1997;278:1309-12.

Supplementary figure and table legends

Supplementary Figure S1. Pharmacokinetics of NVP-BSK805 in mice and rats. At predetermined time points, groups of animals were bled and the resulting blood samples were analyzed for NVP-BSK805 using LC/MS-MS. **A,** Concentration versus time curve of NVP-BSK805 in female OF-1 mice following oral administration of 3 mg/kg. **B,** Concentration versus time curves of NVP-BSK805 in female OFA rats following oral administration of the compound at 3 mg/kg and 30 mg/kg, respectively. **C,** NVP-BSK805 pharmacokinetic parameters in mice and rats as determined by administering 1 mg/kg intravenously and 3 mg/kg orally by gavage. Note, that p.o. data is dose normalized to 1 mg/kg. NVP-BSK805 was formulated as a solution in 30 % PEG200 / 7 % Solutol HS 15 / 63 % water (v/v).

Supplementary Figure S2. Characterization of the Ba/F3 cell-based JAK2^{V617F}-driven mouse leukemia model. A, Ba/F3 JAK2^{V617F}-luc cells were injected i.v. in the tail vein of female SCID beige mice. Leukemic burden was monitored on days 3, 6, 9 and 13 by measuring whole body light emission after i.v. injection of D-luciferin. The pseudocolor gradient of each image has been adjusted to the same scale. Mice succumbed to leukemic disease by day 13. **B,** Macroscopic analysis of spleens of SCID beige mice 4, 7 and 10 days post-tail vein injection of Ba/F3 JAK2^{V617F} cells. Control: spleen from naïve mouse. The respective spleen weights are indicated. **C,** Histopathological assessment of spleen samples shown in B. Fixed and paraffin-embedded spleen sections were stained with hematoxylin and eosin. **D,** Higher magnification (observed with 90 x) of spleens sections from animals on day 10 post Ba/F3 JAK2^{V617F} cell injection reveals complete disruption of spleen architecture by infiltrating cells. The Ba/F3 JAK2^{V617F} cells are characterized by their relatively large cell size, moderate polymorphism and a distinct nucleus

with a prominent nucleolus. A spleen section of a naïve control SCID beige mouse is shown as a reference of the splenic substructures normally seen in this strain.

Supplementary Figure S3. Effects of rhEpo on erythropoiesis in female Balb/c mice. Mice received saline or 5, 10 and 20 U rhEpo (s.c.) every 24 hours for 4 consecutive days. N=5 per group. Animals were sacrificed 24 hours after the final rhEpo administration for analysis of complete blood counts. **A,** hematocrit, **B,** reticulocyte counts. The Ter119/CD71 erythroblast population in spleen (**C**, **E**) and bone marrow (**D**, **F**) was determined by flow cytometry. Data are presented as mean ± SEM. Numbers in parentheses depict number of samples per group amenable to analysis of the respective parameter (i.e. no flags due to clotting). *$p < 0.05$ versus saline treated animals (One-way ANOVA followed by post hoc Dunnett's). The post hoc Tukey's test was used for inter-group comparison.

Supplementary Figure S4. NVP-BSK805 suppresses rhEpo-induced Ter119/CD71 erythroblast expansion in Balb/C mice and elevates platelet count. Balb/C mice received daily subcutaneous injections of 10 U rhEpo for four consecutive days. Concomitantly, mice were orally dosed either with vehicle or with NVP-BSK805 at 50, 75 and 100 mg/kg. Control animals received subcutaneous injections of saline and oral administration of vehicle or NVP-BSK805 dosed at 100 mg/kg (dark bar). Animals were sacrificed 24 hours after the final treatment for analysis (N=5 per group, except group dosed NVP-BSK805 alone (N=3)). Ter119/CD71 erythroblast populations in spleen (**A**) and bone marrow (**B**) were assessed by flow cytometry. **D,** platelet count. Numbers in parentheses depict number of samples per group amenable to analysis of the respective parameter, if smaller than number of animals per group (e.g. flags due to clotting or clumped platelets). *$p < 0.05$ versus rhEpo-treated animals, $^{\#}p < 0.05$ versus saline-treated animals (One-way ANOVA followed by post hoc Dunnett's). The post hoc Tukey's test was used for inter-group comparison.

Supplementary Figure S5. Efficacy of NVP-BSK805 in a rat model of rhEpo-induced polycythemia. A, Female Wistar rats received either saline/vehicle, 25 U rhEpo (s.c.) or NVP-BSK805 administered once daily p.o. at 12.5, 25 mg/kg or 50 mg/kg simultaneously with 25 U rhEpo (s.c.) every 24 hours for 14 consecutive days. Animals were sacrificed 24 hours after the final administration of saline/vehicle, rhEpo/vehicle or rhEpo/NVP-BSK805. N=6 per group. The

figure depicts **A,** spleen weight, **B,** reticulocyte count **C,** platelet count and **D,** body weight. Numbers in parentheses depict number of samples per group amenable to analysis of the respective parameter, if smaller than number of animals per group (e.g. flags due to clotting or clumped platelets). *$p < 0.05$ versus rhEpo-treated animals, (One-way ANOVA followed by post hoc Dunnett's or ANOVA on ranks followed by Dunn's test for bodyweight). #$p < 0.05$ versus saline-treated animals (One-way ANOVA followed by Tukey's test).

Supplementary Table S1. Profiling of NVP-BSK805 against a panel of Ser/Thr/Tyr protein and lipid kinases. Kinases were assessed either in Caliper, Kinase Glo, or LanthaScreen assay formats. The following kinases were tested in the Caliper assay: BTK, CDK2A, JAK1, JAK2, JAK3, TYK2, MK2, MK5, PDK1, PKA, PKBα, GSK. PI3Kα, PI3Kβ, PI3Kδ, PI3Kγ, PI4Kβ and VPS34 were assessed in the Kinase Glo assay format. The remaining kinases in the panel were screened with LanthaScreen TR-FRET assays.

Supplementary Table S1

Assay	IC_{50} (μM)	Assay	IC_{50} (μM)
ALK	>10	MK5	>10
BTK	4.2	PDGFRα	8.1
CDK2A	>10	PDK1	>10
EphA4	1.7	PI3Kα	>9.1
EphB4	0.92	PI3Kβ	>9.1
FGFR-4	>10	PI3Kδ	>9.1
FGFR3K	8.9	PI3Kγ	>9.1
FLT-3	1.2	PI4Kβ	>9.1
HCK	>10	PKA	>10
HER1	>10	PKBα	>10
HER2	>10	RET	4.1
IGF1R	>10	SYK	>10
INS1R	>10	VPS34	>9.1
JAK1	0.069	cABL	0.08
JAK2	<0.003	cABL T315	0.54
JAK3	0.95	cKIT	>10
TYK2	0.097	cMET	5.8
KDR	0.72	cSRC	7.2
LCK	1.1	mTOR	>9.1
MK2	>10	GSK3 beta	>10