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AT9283, a potent inhibitor of the Aurora kinases and Jak2, has therapeutic potential in myeloproliferative disorders

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Running Title: Inhibition of Jak2-dependent systems by AT9283

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Summary

Constitutive activation of Janus kinase (Jak) 2 is the most prevalent pathogenic event observed in the myeloproliferative disorders (MPD) suggesting that inhibitors of Jak2 may prove valuable in their management. Inhibition of the Aurora kinases has also proven to be an effective therapeutic strategy in a number of haematological malignancies. AT9283 is a multi-targeted kinase inhibitor with potent activity against Jak2 and Aurora kinases A and B, which is currently being evaluated in clinical trials. To investigate the therapeutic potential of AT9283 in the MPD we studied its activity in a number of Jak2-dependent systems. AT9283 potently inhibited proliferation and Jak2-related signalling in Jak2-dependent cell lines as well as inhibiting the formation of erythroid colonies from haematopoietic progenitors isolated from MPD patients with Jak2 mutations. The compound also demonstrated significant therapeutic potential *in vivo* in a TEL-Jak2 murine leukaemia model. Inhibition of both Jak2 and Aurora B was observed in the model systems used indicating a dual mechanism of action. Our results suggest that AT9283 may be a valuable therapy in patients with MPD and that the dual inhibition of Jak2 and the Aurora kinases may potentially offer combinatorial efficacy in the treatment of these diseases.

Keywords: Janus kinase 2, Aurora kinase, myeloproliferative disorder, kinase inhibitor, Jak-dependent signalling

Introduction

The human myeloproliferative disorders (MPD) represent a spectrum of clonal haematological disorders, with three main members: polycythaemia vera (PV), essential thrombocythaemia (ET) and idiopathic myelofibrosis (Campbell & Green, 2006). These diseases are all thought to reflect transformation of a multipotent haematopoietic stem cell (Adamson *et al*, 1976; Fialkow *et al*, 1981; Jamieson *et al*, 2006) and are characterised by cytokine-independent growth of haematopoietic progenitors and overactive haematopoiesis with increased production of one or more of the mature myeloid lineages. The molecular pathogenesis of the MPD has been illuminated by the recent identification of gain of function mutations in Janus kinase (Jak) 2 and/or MPL, which are present in the majority of patients with a MPD (Baxter *et al*, 2005; James *et al*, 2005; Kralovics *et al*, 2005; Levine *et al*, 2005; Pikman *et al*, 2006; Scott *et al*, 2007b) The most prevalent gain of function mutation in Jak2 is V617F, which is seen in over 97% of patients with PV, and in more than half of the patients with ET and idiopathic myelofibrosis. The few patients with PV who are Jak2V617F-negative harbour alternative gain of function mutations in Jak2, cumulatively known as Jak2-exon 12 mutations (Scott *et al*, 2007a).

Several lines of evidence, including murine models, indicate that gain of function mutations in Jak2 are both sufficient and necessary to re-capitulate many of the features seen in the myeloproliferative diseases (Jamieson *et al*, 2006; James *et al*, 2005; Levine *et al*, 2005; Scott *et al*, 2007b). The principal clinical complication in PV and ET is thrombosis, although haemorrhage can also occur. In the longer term a minority of patients with PV and ET may develop myelofibrosis or acute myeloid leukaemia (Campbell & Green, 2006; Levine *et al*, 2007). Current available treatments for the MPDs are primarily centered around non-specific cytoreductive

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3 agents aimed at preventing the thrombo-haemorrhagic sequale of these malignancies
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5 (Campbell & Green, 2006; Levine *et al*, 2007). The choice of agent is often dictated
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7 by balancing therapeutic efficacy, as monitored by blood counts, versus limiting side-
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9 effects. Whilst there is some evidence that patients with the Jak2V617F mutation may
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11 respond differently to these treatments (Campbell *et al*, 2005), this has not been a
12
13 major influence in directing therapy.
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17 The success of targeted therapy against oncogenic tyrosine kinases in other
18
19 haematological malignancies such as chronic myeloid leukaemia and subsets of chronic
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21 eosinophilic or myelomonocytic leukaemia has provided a new paradigm for
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23 pathogenesis-directed therapies (Apperley *et al*, 2002; Cools *et al*, 2003; Druker *et al*,
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25 2001). The interest in Jak2 as a target for MPD has led to the investigation of the Jak2
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27 inhibitory activity of a number of kinase inhibitors including CEP-701 (Hexner *et al*,
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29 2008), TG101209 (Pardanani *et al*, 2007), TG101348 (Wernig *et al*, 2008) and LS104
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31 (Lipka *et al*, 2008). Studies have demonstrated the proof of principle that Jak2
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33 inhibitors have moderate therapeutic efficacy in murine models of the MPD and several
34
35 of these inhibitors have now entered clinical trials for MPD (Pardanani, 2008). What
36
37 remains uncertain is whether selective Jak2 inhibition, which at best provides a modest
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39 therapeutic window between Jak2 wildtype and Jak2V617F-positive cells, is a better
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41 therapeutic approach than non-selective inhibition with small molecules that target other
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43 oncogenic kinases in addition to Jak2.
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47 AT9283 is a multi-targeted kinase inhibitor, discovered using a fragment-based
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49 approach, with potent activity against Jak2, Jak3, Aurora kinases, Flt3 and Abl (T315I)
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51 (Howard *et al*, 2009) and currently being evaluated in clinical trials. Previously we
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53 described the Aurora B kinase inhibitory activity of this compound, which predominates
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55 in a number of solid tumour cell lines (Curry *et al*, 2009). The merit in targeting the
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Aurora kinases in the haematological malignancies has recently been recognised (Giles *et al*, 2007; Huang *et al*, 2008; Shi *et al*, 2007) and here we describe the inhibitory activity of AT9283 against Jak2 and Aurora B in a range of Jak2-dependent systems.

For Peer Review

Materials and methods

Materials

AT9283, (1-Cyclopropyl-3-[3-(5-morpholin-4-ylmethyl-1H-benzimidazol-2-yl)-1H-pyrazol-4-yl]-urea) was synthesized at Astex Therapeutics (Howard *et al*, 2009). Antibodies against phospho-Stat5^(Tyr694), Stat5, phospho-Erk1/2^(Thr202/Tyr204), Erk, phospho-S6^(Ser240/244), S6, phospho-Akt^(Ser473), Akt, phospho-Jak2^(Tyr1007/1008), phospho-histone H3^(Ser10), phospho-histone H3^(Ser28), histone H3 and cleaved PARP (Asp214) were from Cell Signalling Technology, Danvers, MA, USA. The antibody against actin was from AbCam, Cambridge, UK. All other reagents were purchased from Sigma unless otherwise stated.

Cell lines

The human erythroleukaemia (HEL), SET-2, CMK, TF-1 and Ba/F3 wildtype cell lines were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

TEL-Jak2 fusion clones were constructed by polymerase chain reaction (PCR)-mediated amplification of the oligomerization domain of murine TEL (amino acids 43-154) (Poirel *et al*, 1997) and the 298 COOH-terminal residues of human Jak2 (Harpur *et al*, 1992). TEL-Jak2 cDNA was subcloned into pCDNA3.1 vector. Interleukin (IL)-3 independent Ba/F3 cell lines were obtained by electroporating (Bio-Rad Gene Pulser) 5x 10⁶ Ba/F3 cells with 20 µg of TEL-Jak2 plasmid DNA and then selecting stably transfected TEL-Jak2 Ba/F3 cells in the presence of 1 mg/ml G418 (Calbiochem, Darmstadt, Germany).

All cell lines were cultured at 37°C in RPMI 1640 medium supplemented with 10-20% fetal bovine serum (Invitrogen, Paisley, UK) and maintained in a humidified atmosphere

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3 of 5% CO₂ in air. Ba/F3 wildtype and TF-1 cell culture medium was supplemented with
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5 2 ng/ml murine interleukin (IL)-3, Ba/F3 TEL-Jak2 cell culture medium was
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7 supplemented with 500 µg/ml G418 and interferon regulatory factor-1 (*irf1*)-*bla* HEL
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9 cell culture medium was supplemented with 1% non essential amino acids, 1 mM
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11 sodium pyruvate, 100 iu/ml penicillin, 100 µg/ml streptomycin and 2 µg/ml blasticidin
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13 (all from Invitrogen).
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20 *In vitro kinase assays*

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22 Assays for obtaining enzyme IC₅₀ values were performed as described previously
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24 (Howard *et al*, 2009).
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30 *Cell proliferation assays*

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32 Between 2.5 and 5 x 10³ cells per well were seeded into 96-well plates and incubated
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34 overnight before incubation with compound in 0.1% dimethylsulphoxide (DMSO) for
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36 approximately 3 times the doubling time of the cell lines. Alamar Blue™ (Biosource
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38 International, Camarillo, CA, USA) was then added and fluorescence measured as
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40 previously described (Squires *et al*, 2009). IC₅₀ values were calculated using a
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42 sigmoidal dose response equation (Prism GraphPad Software).
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50 *Irf1-bla HEL Cellsensor® assay*

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52 The *irf1-bla* HEL Cellsensor® assay kit was from Invitrogen. The CellSensor® *irf1*-
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54 *bla* HEL cell line contains a beta-lactamase reporter gene under control of the *irf1*
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56 response element stably integrated into HEL cells. *Irf1-bla* HEL cells were
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58 resuspended at 1x10⁶ cells/ml in culture medium without blasticidin, seeded at 8x10⁴
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60 cells/well into 96-well plates and incubated with compound in 0.1% DMSO for 16

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3 hours. Cells were then loaded with LiveBLAzer™-FRET B/G substrate for 4 h and
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6 fluorescence measured at $\lambda(\text{ex})=460$ nm and $\lambda(\text{em})=530$ nm.
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10 *In-cell western*

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12 HEL cells were treated for 4 h with sodium orthovanadate and then with compound
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14 for 2 h. Cells were then fixed with 4% formaldehyde in PBS, washed in PBS and
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16 permeabilised by incubating with ice-cold methanol for 5 min. Levels of
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18 phosphorylated Stat5 were determined using a monoclonal antibody followed by a
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20 secondary antibody (Goat anti-rabbit IgG) conjugated with an infra-red fluorophore
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22 (800CW) (LI-COR Bioscience, Lincoln, NE, USA). Infra-red fluorescence was
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24 measured using the Odyssey infra-red imaging system (LI-COR). Data were
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26 normalised to cell number using the fluorescent DNA stain, DRAQ5 (Biostatus,
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28 Shepshed, UK).
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36 *Western blot analysis*

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38 All cells, except TF-1, were treated with compound, harvested, lysed and samples
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40 processed as described previously (Curry *et al*, 2009). TF-1 cells were serum starved
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42 overnight, resuspended in serum-free medium with 2 mM glutamine containing
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44 indicated concentrations of AT9283 and, following 2 h incubation, stimulated with 5
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46 ng/ml IL-3. Samples were then incubated for 20 min, harvested and processed as above.
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48 Samples were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis
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50 (SDS-PAGE) and immunoblotted with the indicated antibodies followed by either infra-
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52 red dye labeled anti-rabbit or anti-mouse antibodies (LI-COR). Blots were scanned to
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54 detect infra-red fluorescence on the Odyssey infrared imaging system.
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Cell cycle analysis of HEL cells

HEL cells were seeded into T150 tissue culture flasks for mid-log phase cultures and then incubated with AT9283 or vehicle control as described previously (Curry *et al*, 2009). At the indicated times samples were removed, centrifuged, fixed in 2 ml 70% ethanol and stored at 4°C for 1-7 days. Cells were then washed and re-suspended in PBS containing 50 µg/ml propidium iodide and 0.1 mg/ml RNase, incubated and analysed by flow cytometry as previously described.

Murine Ba/F3 TEL-Jak2 model

Male athymic BALB/c mice (nu/nu) were obtained from Harlan UK (Bicester, UK) and were given food and water *ad libitum*. The care and treatment of experimental animals were in accordance with the United Kingdom Coordinating Committee for Cancer Research guidelines.

Groups of (n= 8) male BALB/c mice were injected into the tail vein with Ba/F3 TEL-Jak2 cells (5×10^6) in PBS on day 1. Animals were randomized and treated intraperitoneally with vehicle or AT9283 (10 mg/kg twice a day) formulated in 10% DMSO, 20% water, 70% 2-hydroxypropyl-β-cyclodextrin (25% w/v aq.) on days 2-5 and 8-12. Groups of (n=4) animals injected with PBS instead of cells were treated with vehicle or AT9283 as additional controls. The number of Ba/F3 TEL-Jak2 cells in peripheral blood was determined as follows. Blood was collected on the days indicated from the saphenous vein and total white blood cells counted under the microscope. Genomic DNA was isolated from each blood sample and quantitative PCR performed, using genomic DNA of cultured Ba/F3 TEL-Jak2 cells as standard, to determine the number of Ba/F3 TEL-Jak2 positive cells in the total white blood cell population: the amount of Ba/F3 DNA was determined using primers designed to

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3 detect the TEL-Jak2 junction and total amount of template with primers specific to
4 GJA5 gene. Spleen and liver weights were recorded on day 12 of the experiment. All
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6 results are represented as mean +/- SEM of between 4 and 8 animals.
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10 11 12 13 *Patient accrual and sample collection*

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15 The research involving patient samples was approved by Addenbrookes NHS Trust
16 local Research Ethics Committee and was carried out in accordance with the
17 principles of the Declaration of Helsinki.
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22 23 24 *Primary cell clonogenic assay*

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26 Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation of
27 whole blood through a Ficoll density gradient. Clonogenic assays were set up in
28 triplicate. Freshly isolated PBMCs (3×10^5) were plated in a methylcellulose-based,
29 semisolid medium (MethoCult media, Stem Cell Technologies, Vancouver, Canada)
30 containing various concentrations of AT9283 with erythropoietin (EPO) (3 iu/mL)
31 (R&D systems) to support colony growth. Plates were incubated at 37°C in 5% carbon
32 dioxide in air for 12–14 days. Colonies were scored using standard morphological
33 criteria. IC₅₀ values were calculated as described above. Cytospun erythroid colonies
34 were stained with a Romonovsky stain for morphological assessment and photographs
35 were taken using an Olympus BX-40 microscope using a x 60 magnification lens and
36 a mounted DP-12 digital camera (Olympus, Tokyo, Japan).
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53 54 55 *Cell cycle analysis of BFUe colonies*

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57 Individual erythroid burst-forming unit (BFUe) colonies derived from the clonogenic
58 assays were harvested after 12–14 days. At least 20 BFUe colonies were plucked and
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3 pooled from each test condition. Cells were fixed in 70% (v/v) ethanol in PBS and
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5 incubated on ice for 30 min. RNase treated samples (10 µg RNase/mL for 30 min at
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7 37°C) were stained with propidium iodide (5 µg/mL) (Sigma) at 37°C for at least 30
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9 min. Cell cycle parameters were assessed by FACS analysis using a Becton
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11 Dickinson FACS Calibur™ system.
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17 *Jak2V617F mutant allele quantification*

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20 BFUe colonies were genotyped for Jak2V617F and assigned as homozygous,
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22 heterozygous or wildtype based on the mutant allele burden determined by either
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24 quantitative real-time PCR or pyrosequencing as previously described (Beer *et al*,
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26 2008; Levine *et al*, 2006).
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Results

AT9283 inhibits Jak2

AT9283 is a multi-targeted kinase inhibitor (Fig 1A) (Howard *et al*, 2009). Its potent inhibitory activity of the Jak family members, Jak2 and Jak3 (1.2 nM, 1.1 nM), prompted the investigation of the effects of AT9283 in Jak-dependent systems. AT9283 was shown to inhibit the proliferation of a number of Jak2-dependent cell lines (Table I), including lines with the Jak2V617F mutation (HEL, SET-2), a cell line with an engineered constitutively active Jak2 (Ba/F3 TEL-Jak2) and cells stimulated by IL-3 through wildtype Jak2 (Ba/F3, TF-1), with IC₅₀ values in the range 16-110 nM. Similar inhibition of proliferation was seen in cell-lines dependent on Jak3 such as CMK Jak3 A572V mutant cells (Table I).

To investigate the direct inhibition of Jak2 in Jak2-dependent cell lines the phosphorylation of its substrate, Stat5, was monitored by in-cell western in HEL cells, which are homozygous for the Jak2V617F mutation. AT9283 inhibited this phosphorylation with an IC₅₀ of 200 nM (Fig 1B); similar to the IC₅₀ for inhibition of proliferation of this cell line of 110 nM. The inhibition of Stat5-activated transcription was measured in an *irf-bla* cell sensor reporter gene assay using an engineered HEL cell line that contains a beta-lactamase reporter gene and the IC₅₀ determined (100 nM) (Fig 1C) correlated well with both inhibition of Stat5 phosphorylation and inhibition of proliferation.

AT9283 inhibits Jak-dependent signalling

Jak2 not only phosphorylates Stat5 and so activates Stat5-regulated transcription but also upregulates the Erk and Akt pathways through its effects on the Gab2 signalling complex (Hibi & Hirano, 2000). We investigated the effects of AT9283 inhibition on

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3 signalling through these pathways in a number of Jak2-dependent cell lines, HEL, TF-1
4 and Ba/F3 TEL-Jak2. In all cell lines exposure to 1 μ M AT9283 caused a decrease in
5 phosphorylated Stat5 one hour after the start of treatment confirming inhibition of Jak2
6 (Fig 2). Phosphorylation of Erk and S6 was also inhibited in a dose-dependent manner
7 when treated with AT9283 at concentrations similar to those which caused inhibition of
8 Stat5 phosphorylation (Fig 2A) indicating an inhibition of signalling in the Erk and Akt
9 pathways. Further studies in HEL cells, exposed for up to 72 h to AT9283 (Fig 2B),
10 indicated that phospho-S6 levels appeared to take longer to decrease compared with the
11 levels of phospho-Stat5 and phospho-Erk, which were reduced by 30 min after AT9283
12 treatment. Stat5 and Erk phosphorylation were returning to control levels after 16 h
13 exposure, but phospho-S6 remained ablated at this time point. However, cleaved PARP
14 levels were increasing after 16 h treatment indicating that cells were already committed
15 to apoptosis and by 48 and 72 h after the start of treatment a significant proportion of
16 cells had died.

17
18 AT9283 also inhibited wildtype Jak2 signalling in IL-3-stimulated TF-1 cells (Fig 2C).
19 Stimulating signalling through Jak2 in this cell line caused phosphorylation of Stat5, Erk
20 and S6 to increase and this was inhibited by AT9283 again in a dose-dependent manner.
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23 *HEL cells treated with AT9283 show a double block in their cell cycle*

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25 AT9283 also inhibits the Aurora kinases A and B with *in vitro* IC₅₀ values of 3 nM.
26 Inhibition of phosphorylation of histone H3S10ph, an Aurora B kinase substrate, was
27 seen on treatment of Ba/F3 TEL-Jak2 cells (Fig 2A) and HEL cells (Fig 3A) at
28 concentrations of 100 nM AT9283 indicating that Aurora B kinase is inhibited to a
29 similar extent to Jak2 in these cells. To establish which, if either, of these inhibitory
30 activities predominates in Jak2-dependent cell lines we investigated the effects of
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3 AT9283 treatment on the cell cycle profile of HEL cells. Asynchronous HEL cells were
4 treated with 1 μ M AT9283 for up to 72 h (Fig 3B). After 24 h treatment a population of
5 cells appeared with 8N DNA indicative of the endoreduplication generally caused by an
6 Aurora B kinase inhibitor in p53-checkpoint compromised cells (Curry *et al*, 2009). G₁
7 and G₂/M cell populations could also be seen at this time point. After 48 h of treatment
8 the G₁ and 8N populations of cells were still present, but the G₂/M population had
9 significantly decreased. The G₁ population was still present after 72 h treatment
10 **suggestive** of a G₁ block in the cell cycle such as has been observed when Jak2 is
11 inhibited (Apperley *et al*, 2002), but the population of cells with 8N DNA had
12 disappeared as they continued to endoreduplicate (data not shown). The sub-G₁
13 population increased after 24 h treatment confirming that cells were undergoing
14 apoptosis by this time-point as suggested by the increase in cleaved PARP shown above
15 (Fig 2B). These data suggest that both the Jak2 and Aurora B kinase inhibitory activities
16 of AT9283 play a part in the compound's activity on HEL cells.
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39 *AT9283 is efficacious in a Jak2-dependent murine model*

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41 The efficacy of AT9283 was investigated in a Jak2-dependent murine leukaemia model.
42 Recombinant Ba/F3 TEL-Jak2 cells with constitutively active Jak2, constructed as
43 described in Materials and methods, were injected intravenously and subsequent
44 proliferation was measured by monitoring circulating tumour cells and by increases in
45 spleen and liver size (Fig 4). Treatment with AT9283 commenced the following day and
46 significantly suppressed the proliferation of circulating Ba/F3 TEL-Jak2 cells in the
47 mice over the 12 day dosing period compared with vehicle control treatment (Fig 4A).
48 Spleen weights, which increased significantly in the Ba/F3 TEL-Jak2 leukaemia model,
49 were reduced by over 50% in the AT9283-treated mice when assessed at 12 days
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3 following initiation of treatment (Fig 4B) and liver weights, which also increased in the
4 untreated model, were reduced to almost normal levels (Fig 4C). These data confirm
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6 that AT9283 is efficacious in a Jak2-driven animal model.
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12 *AT9283 potently inhibits erythroid colony formation from patients with a*
13 *myeloproliferative disease*
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17 To assess the activity of AT9283 in primary patient samples clonogenic assays were
18 performed using PBMCs derived from normal volunteers and a spectrum of patients
19 with a well characterized MPD harboring gain-of-function mutations in Jak2. The
20 PBMCs were plated in a semi-solid medium and differentiated to erythroid colonies in
21 presence of EPO and increasing concentrations of AT9283. Colony growth in normal
22 volunteers was markedly inhibited by 4 nM of AT9283 and virtually no erythroid
23 colonies were seen at concentrations higher than 10 nM (Fig 5A). This pattern of
24 inhibition was recapitulated in patients with PV harbouring Jak2V617F or Jak2-exon 12
25 mutations (Fig 5B and C, respectively) and ET patients with Jak2V617F (Fig 5D).
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39 IC₅₀ values of AT9283 in BFUe colony assays performed at a fixed concentration of
40 EPO (3 iu) were similar in normal volunteers (IC₅₀: 3.2-4.6 nM) and patients with
41 Jak2V617F positive PV (IC₅₀: 3.6-5.0 nM). When compared to the untreated control
42 sample, (Fig 5E), BFUe colonies grown in the presence of 10 nM AT9283 demonstrated
43 a preservation of nuclear architecture and morphological appearance (Fig 5F). In
44 addition to the quantitative reduction in BFUe colony number there was an appreciable
45 reduction in the size of individual colonies associated with increasing concentrations of
46 AT9283 (data not shown).
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3 To assess the effects of AT9283 on the cell cycle of primary cells, BFUe colonies grown
4 in the presence of EPO and increasing concentrations of AT9283 from normal
5 volunteers and patients with PV were stained with propidium iodide and analysed by
6 flow cytometry. Data shown are from a normal volunteer, but are representative of all
7 samples tested. These results suggest that AT9283 causes a progressive increase in the
8 G_0/G_1 fraction and a concomitant decrease in the G_2/M fraction of primary erythroid
9 progenitors (Fig 5G-H). These data are consistent with inhibition of Jak2 by AT9283
10 and suggest a similar requirement for active Jak2 signalling in primary cells to drive the
11 cell cycle through the G_1/S interface as that previously shown in cell lines (Walz *et al*,
12 2006). Checkpoint-compromised cells have been shown to respond differently to
13 Aurora inhibition compared to checkpoint-competent cells (Curry *et al*, 2009; Soncini *et*
14 *al*, 2006; Carpinelli *et al*, 2007), with checkpoint-compromised cells showing enhanced
15 endoreduplication in response to treatment. Although no evidence of polyploidy was
16 observed here, we therefore cannot exclude the possibility that inhibition of Aurora
17 kinases also contributes to the inhibition of colony growth.

AT9283 inhibits growth of both Jak2V617F positive and wildtype erythroid colonies

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20 It has been suggested that some Jak2 inhibitors show specificity for the Jak2V617F
21 mutation over wildtype Jak2, (Pardanani *et al*, 2007; Wernig *et al*, 2008; Geron *et al*,
22 2008; Lasho *et al*, 2008) however, interpretation of this data is complicated by
23 comparisons made between colonies grown in the presence and absence of cytokines,
24 and that much of the data in support of this claim has been derived from pooled
25 colonies where the Jak2V617F allele burden has been represented as percentages with
26 no reference to the number of colonies remaining after treatment.

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3 To systematically assess if AT9283 may show a selective inhibition of progenitors
4 that are either homozygous or heterozygous for Jak2V617F in comparison to Jak2
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To systematically assess if AT9283 may show a selective inhibition of progenitors that are either homozygous or heterozygous for Jak2V617F in comparison to Jak2 wildtype progenitors we chose seven Jak2V617F positive PV patients, including two that have been extensively characterized and demonstrated to harbor prominent homozygous or heterozygous Jak2V617F clones. PBMCs from these patients were plated with 3 iu of EPO and increasing concentrations of AT9283, individual BFUe colonies were plucked and genotyped. As demonstrated in Fig 6A & B, AT9283 does not selectively inhibit either homozygous or heterozygous Jak2V617F progenitors. Moreover, the importance of genotyping sufficient colonies to accurately address the issue of selectivity is underlined by patients 1, 3 and 5 in Fig 6C. These data, if represented as a percentage, may suggest that AT9283 does indeed show selectivity for Jak2V617F positive progenitors at higher concentrations. However, the low number of colonies present at this concentration statistically precludes such a claim.

Discussion

The discovery of the V617F and exon 12 Jak2 mutations has demonstrated that constitutive activation of Jak2 signalling is strongly associated with MPD. Moreover, recent evidence would suggest that aberrant Jak/Stat signaling is more ubiquitous than previously appreciated and is likely to underpin many haematological malignancies (Bercovich *et al*, 2008; Kotecha *et al*, 2008; Lacronique *et al*, 1997), highlighting Jak2 as a key therapeutic target in haematological neoplasms. In addition, the Aurora kinases have long been appreciated to be dysregulated in tumourigenesis (Keen & Taylor, 2004) and more recently their role as bona fide oncogenes in haematological cancers has also been demonstrated (Giles *et al*, 2007; Huang *et al*, 2008; Ikezoe *et al*, 2007). AT9283, a multi-targeted kinase inhibitor, offers potent inhibitory activity against both Jak2 and Aurora kinases. This compound was investigated in a number of Jak2-dependent cellular systems, patient samples and *in vivo* in a murine animal model.

AT9283 inhibited both the proliferation of cell-lines that are Jak2-dependent and the growth of erythroid colonies from patient samples. *In vivo* the compound was efficacious in a murine model of Jak2-driven disease, suppressing the proliferation of Jak2-driven tumour cells and reducing spleen and liver weights. These data indicate that the compound has inhibitory effects on Jak2-driven cells both *in vitro* and *in vivo*.

AT9283 is a multi-targeted kinase inhibitor and could exert its activity through inhibition of a number of targets. In order to ascertain whether the inhibitory activity we observed could be linked to the direct inhibition of Jak2 we investigated the effects of the compound on Jak2 signalling in a number of systems. Inhibition of phosphorylation of the direct Jak2 substrate Stat5 was observed in a number of cell lines as was the abrogation of phospho-S6 and phospho-Erk indicating inhibition of the Erk and Akt pathways, which are upregulated by Jak2 activity on the Gab2 complex (Hibi & Hirano,

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3 2000). Other kinases in these pathways such as Mek and p70S6 kinase, which directly
4 phosphorylate Erk and S6, and Akt are not effectively inhibited by AT9283 in enzyme
5 assays (Howard *et al*, 2009) suggesting that the pathway inhibition demonstrated can be
6 attributed to direct inhibition of Jak2 rather than to inhibitory effects on other parts of
7 the signalling cascade. AT9283 did not appear to be selective for mutated Jak2 over the
8 wildtype enzyme in cell lines where similar levels of inhibition of proliferation were
9 seen regardless of whether cells were wildtype for Jak2 (TF-1) or heterozygous or
10 homozygous for the V617F mutation (SET-2, HEL respectively). Similarly in patient
11 samples, where cell populations were grown in an identical cytokine milieu, AT9283
12 was equally efficacious at inhibiting erythroid colony formation of haematopoietic
13 progenitors from normal volunteers and patients with heterozygous or homozygous Jak2
14 mutations. This is not unexpected since AT9283 targets the conserved kinase domain
15 rather than the pseudokinase domain where the mutations are located (Baxter *et al*,
16 2005; Saharinen *et al*, 2003). It has been suggested that some Jak2 inhibitors selectively
17 inhibit mutant Jak2 erythroid progenitors (relative to Jak2 wildtype erythroid
18 progenitors) (Pardanani *et al*, 2007; Wernig *et al*, 2008; Geron *et al*, 2008; Lasho *et al*,
19 2008). The apparent discrepancy between the published data and those presented here
20 may reflect differences in methodology. Most of the published data claiming specificity
21 rely on comparisons made between colonies grown in the presence and absence of EPO.
22 Furthermore, the genotyping data presented in these manuscripts are from an unspecified
23 number of pooled colonies and not derived from individually genotyped colonies.
24 Moreover, it remains mechanistically difficult to reconcile how small molecule ATP
25 analogs that bind to a conserved catalytic site can offer this degree of specificity.
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27 AT9283 has other kinase inhibitory activities and inhibition of phosphorylation of
28 histone H3, an Aurora B kinase substrate, was observed in HEL cells treated with
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3 AT9283. Cell cycle analysis suggested that a proportion of HEL cells treated with
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5 AT9283 became polyploid, also indicative of Aurora B inhibition. A further fraction of
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7 treated cells, however, appeared to arrest in the G₁ phase of the cell cycle, which is an
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9 effect more indicative of Jak2 inhibition (Walz *et al*, 2006). This suggests that both the
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11 Aurora B kinase and Jak2 inhibitory activities of AT9283 may contribute to the overall
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13 inhibition of proliferation of Jak2-dependent cells. The predominating mechanism for
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15 any single cell may depend on its position in the cell cycle at the start of treatment, the
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17 Jak2 inhibitory activities taking effect for cells that reach the G₁ phase first whilst the
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19 Aurora B kinase inhibitory activities dominating in those which first encounter G₂/M.
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21 There was no evidence of a polyploid phenotype in the erythroid colonies grown from
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23 patient samples after 10-14 days treatment with AT9283 but checkpoint-competent cells
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25 have been shown to arrest rather than endoreduplicate (Curry *et al*, 2009; Carpinelli *et*
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27 *al*, 2007; Carpinelli *et al*, 2007) after treatment with Aurora kinase inhibitors and the
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29 longer timescales of this assay could also have obscured observations of Aurora-
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31 inhibitory events.
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39 The advantages we predict for a multi-targeted inhibitor may be offset by disadvantages
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41 related to a broader spectrum of kinase inhibition. One such issue that has been raised
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43 with respect to Jak2 inhibitors is the potential effect of inhibition of Jak3 (Pardanani,
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45 2008) which plays a key role in the immune system (Nosaka *et al*, 1995; Thomis &
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47 Berg, 1997). We observed inhibition of Jak3 by AT9283 in cells, however, AT9283,
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49 like CEP701, (Hexner *et al*, 2008) has been well tolerated in the clinic with no currently
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51 reported adverse effects related to Jak3 associated immunological dysfunction.
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55 AT9283 is currently in clinical trials for the treatment of acute leukaemia and its potent
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57 Jak2-inhibitory activity indicates that it is also an excellent clinical candidate for further
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59 testing in patients with Jak2-mediated haematological neoplasms. Taken together our
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3 data demonstrate that AT9283 inhibits Jak2 and Aurora B kinase in several Jak2-
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6 dependent systems. A compound with combinatorial oncogenic kinase inhibition has the
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8 potential to provide more effective disease control and may also be less susceptible to
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10 development of resistance, which has been observed to rapidly arise against kinase
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12 inhibitors such as imatinib (O'Hare *et al*, 2007). AT9283, therefore represents, the first
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14 clinically applied kinase inhibitor that effectively targets two kinases, Jak2 and Aurora
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16 B, which have a prominent role in the development and perpetuation of haematological
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18 malignancies.
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For Peer Review

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Table I: Inhibition of proliferation of Jak-dependent cell lines by AT9283

Cell Line	Origin	Mutation or Receptor stimulation	Inhibition of Proliferation IC ₅₀ (nM)
HEL	Erythroleukaemia AML	Jak2 V617F mut/mut	110
SET2	Essential Thrombocythaemia AML M7	Jak2 V617F mut/wt	57
Ba/F3 TEL-Jak2	Murine haematopoietic	Constitutively active Jak2 kinase domain	16
Ba/F3 wildtype	Murine haematopoietic	IL-3 stimulated through IL-3R and wt Jak2	17
TF-1	Erythroleukaemia	IL-3 stimulated	40
CMK	AMKL	Jak3 A572V mut	26

IC₅₀ values are the mean of at least two independent experiments

Figure Legends

Figure 1. AT9283 potently inhibits Jak2. (A) X-ray crystal structure showing AT9283 bound to the active site of the Jak2 kinase domain (Howard *et al*, 2009). (B) AT9283 inhibited phosphorylation of the Jak2 substrate Stat5 with an IC₅₀ of 200 nM. Stat5 phosphorylation was measured in HEL cells by in-cell western. Representative of 2 independent experiments. (C) AT9283 inhibited Stat5-dependent transcription with an IC₅₀ of 100 nM. Stat5-dependent transcription was measured using a CellSensor® assay to monitor transcription of a beta-lactamase reporter gene under control of an irf1 response element in an engineered HEL cell line. Representative of 5 independent experiments.

Figure 2. AT9283 inhibits Jak2 signalling. Cells were treated with AT9283 and samples run by SDS-PAGE and immunoblotted with the indicated antibodies (A) TEL-Jak2 engineered Ba/F3 cells were treated with the indicated concentrations of AT9283 for 1 h. C are cells treated with 0.1% DMSO for 1 h (B) HEL cells were treated with 1 µM AT9283 for the indicated times. C are cells treated with 0.1% DMSO for 1 h (C) TF1 cells were treated with the indicated concentrations of AT9283 for 2 h and then with IL-3 to stimulate the Jak2 pathway.

Figure 3. Effects of AT9283 treatment in HEL cells indicate a mixture of Jak2 and Aurora B kinase inhibition (A) Inhibition of phosphorylation of the Aurora B kinase substrate, histone H3^(Ser10), was measured by treating HEL cells with the indicated concentrations of AT9283 for 2 h and then running samples by SDS-PAGE and immunoblotting with the indicated antibodies. C are cells treated with 0.1% DMSO for 2 h. (B) Cell cycle profiles of propidium iodide stained HEL cells after treatment with 1

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μ M AT9283 for the indicated times show endoreduplication, G₁ block and increasing apoptosis.

Figure 4. AT9283 is efficacious in a Jak2-dependent murine leukemic model. Mice were injected intravenously with TEL-Jak2 engineered Ba/F3 cells on Day 1 and then treated intraperitoneally with vehicle or AT9283 (10mg/kg bid) on days 2-5 and 8-12. (A) Number of Ba/F3 TEL-Jak2 cells circulating in untreated and AT9283-treated mice up to 15 days after initiation of treatment. The number of Ba/F3 TEL-Jak2 cells was determined using quantitative PCR. (B) Spleen weights of untreated and AT9283-treated mice 12 days after initiation of treatment. (C) Liver weights of untreated and AT9283-treated mice 12 days after initiation of treatment. All results are represented as mean +/- SEM of between 4 and 8 animals.

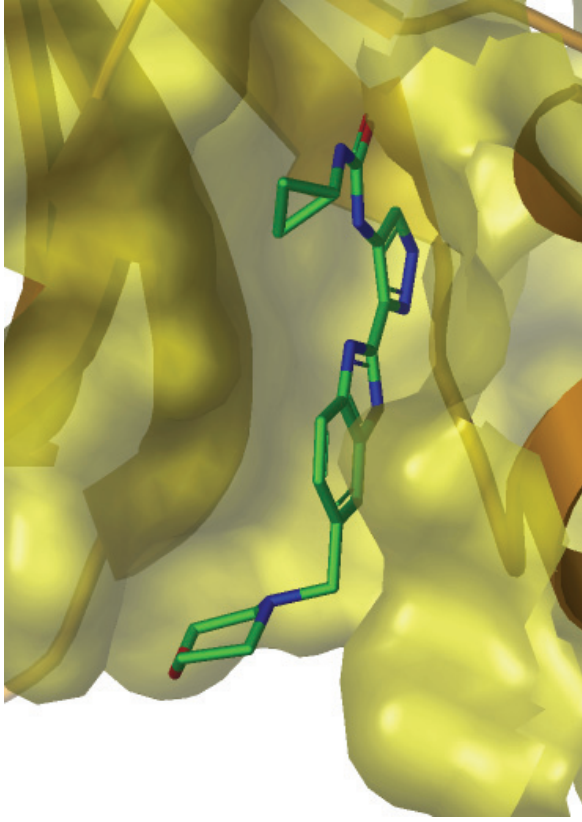
Figure 5. AT9283 inhibits erythroid colony formation in MPD patients. BFUe colonies were grown in triplicate from peripheral blood mononuclear cells plated at a concentration of 3×10^5 per plate in the presence of EPO at 3iu/ml and the listed concentrations of AT9283. The number of colonies from the three plates at each concentration was counted and the average number plotted. Each bar graph is a representative example of at least three individuals who were (A) normal volunteers or (B) patients with Jak2V617F positive PV, (C) Jak2 exon 12 positive PV or (D) Jak2V617F positive ET. AT9283 abolishes BFUe growth at low nanomolar concentrations. (E) Romanovsky stain of cytopun BFUe colonies grown in the absence of AT9283 from a normal volunteer demonstrates the full spectrum of erythroid development from early pronormoblasts to late haemoglobinised nucleated red cells. (F) These morphological features are unchanged with no discernible

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4 evidence of polyploidy in BFUe colonies grown at 10nM, the maximum concentration
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6 of AT9283 at which colonies are able to grow. These images are representative of
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8 those seen in both normal volunteers and Jak2V617F/exon 12 positive patients.
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10 Images were captured at x 60 magnification on an Olympus BX-40 microscope and a
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12 mounted DP-12 digital camera (Olympus, Tokyo, Japan). (G) Twenty individual
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14 BFUe colonies at each of the indicated drug concentrations were pooled, stained with
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16 propidium iodide and analysed by flow cytometry. Cell cycle distribution
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18 demonstrates that AT9283 leads to an incremental increase in cell cycle arrest at the
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20 G_0/G_1 interface with a concomitant decrease in cells at the S and G_2/M phases of the
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22 cell cycle. (H) The percentage of cells in each phase was quantified and charted. The
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24 cell cycle analysis presented is of a normal volunteer and is representative of
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26 experiments performed in both normal volunteers and PV patients on at least three
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28 occasions. The error bars represent the standard deviation of measurements made
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30 from biological duplicates.
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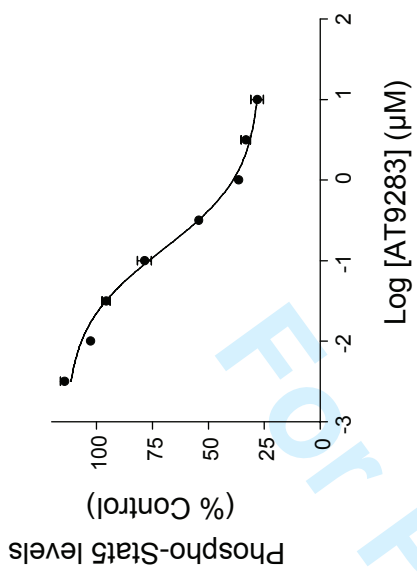
40 **Figure 6. AT9283 affects wildtype, heterozygous and homozygous V617F erythroid**
41 **clones equally.** BFUe colonies grown at a fixed EPO concentration in the presence of
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43 AT9283 at the indicated concentrations were plucked and individually genotyped to
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45 determine if AT9283 selectively inhibits Jak2V617F positive progenitors. Bar charts
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47 demonstrating the percentage of homozygous (Hom), Heterozygous (Het) and wildtype
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49 (WT) colonies grown at the indicated concentrations in patients harboring a prominent
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51 (A) homozygous and (B) heterozygous Jak2V617F positive clone. These data
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53 demonstrate that when sufficient (>10) individual colonies are genotyped AT9283 does
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55 not demonstrate selective inhibition of the Jak2V617F positive progenitors. (C) Five
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57 further PV patients also analysed by individually genotyping colonies as above further
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3 demonstrate that when analysed at a fixed EPO concentration AT9283 has equal
4 efficacy against wildtype, and Jak2V617F heterozygous / homozygous progenitors. PV-
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8 6 and PV-7 are the same patients depicted in panels (A) and (B) respectively.
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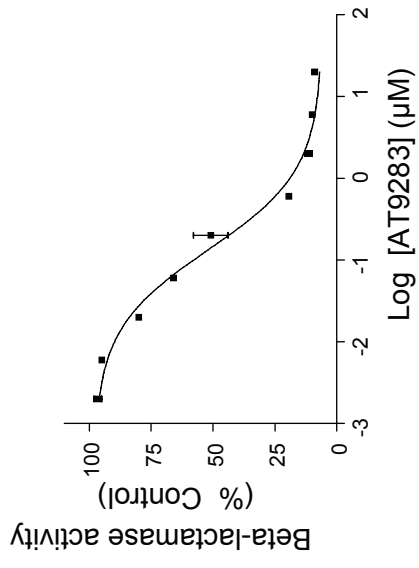
For Peer Review



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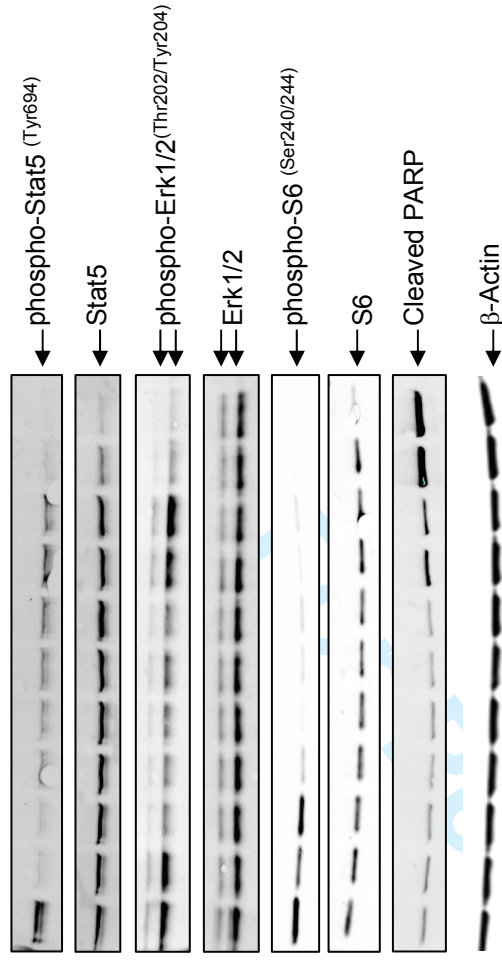


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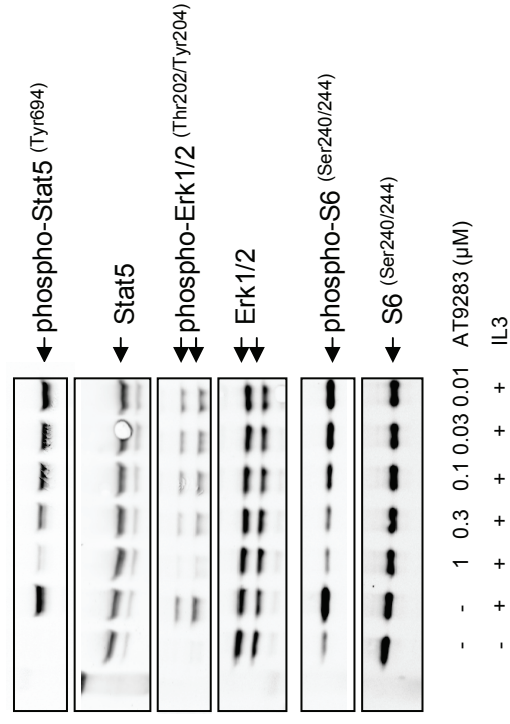


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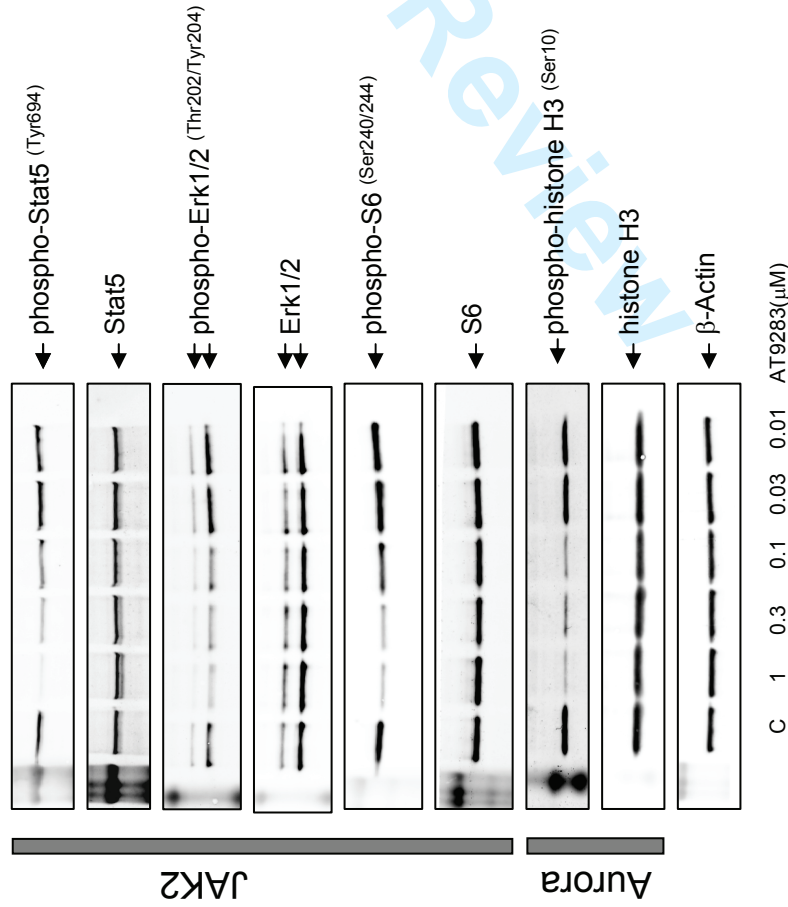
B. HEL cells



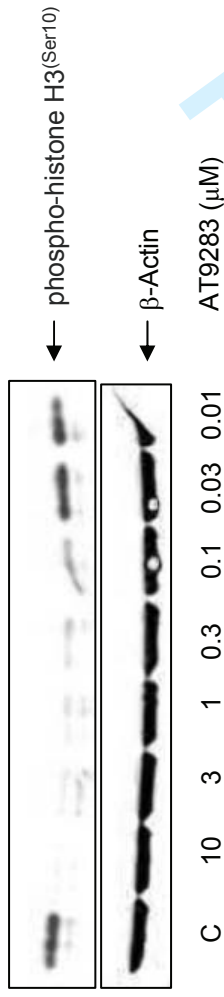
C. TF-1 cells



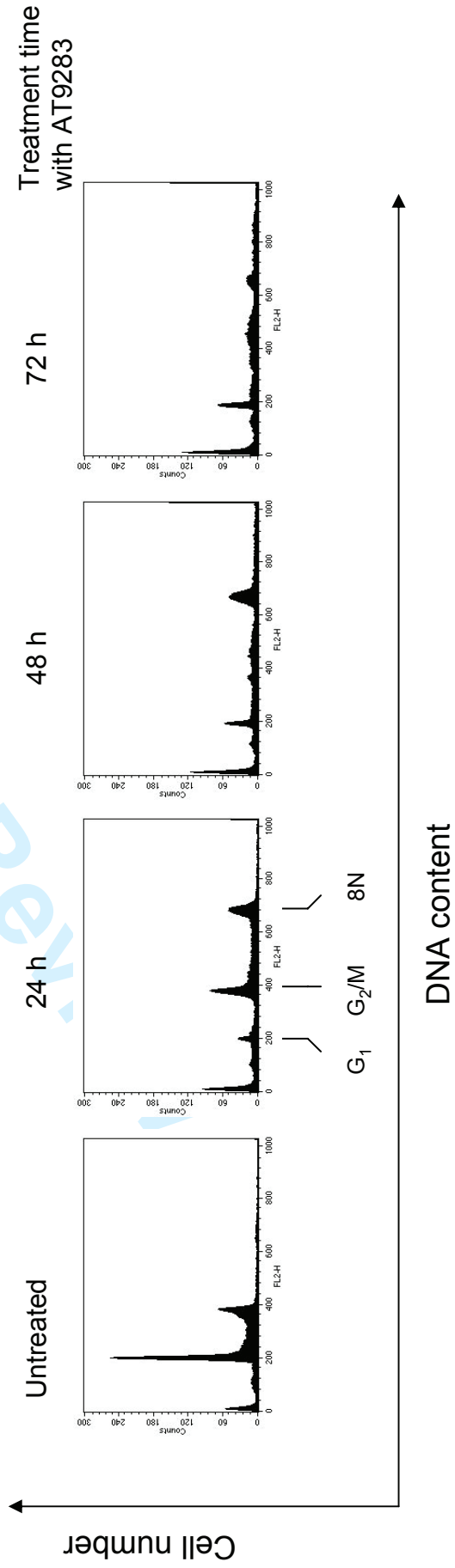
A. Ba/F3 TEL-JAK2



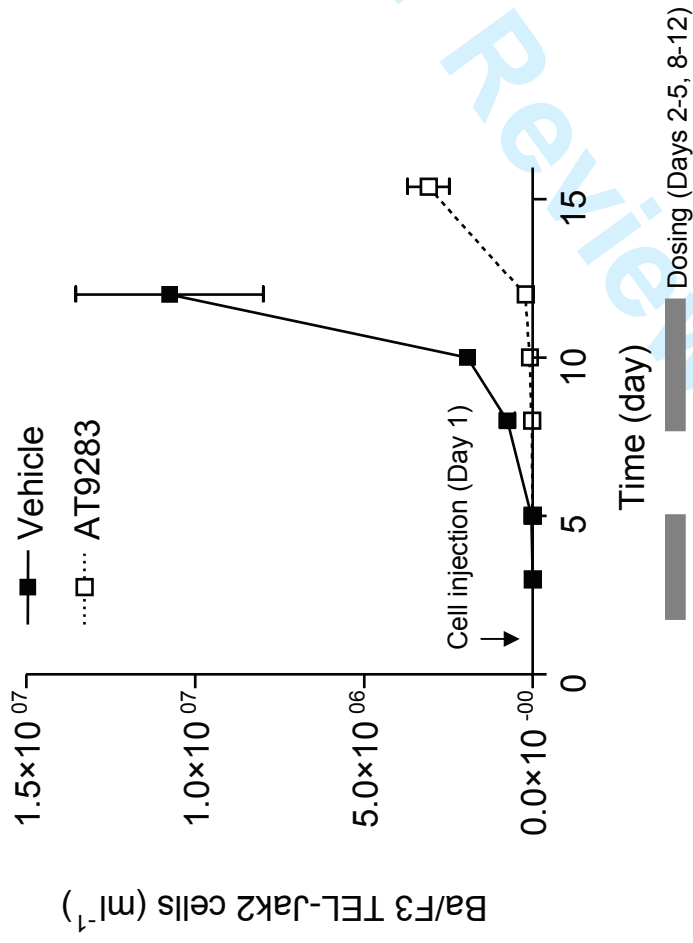
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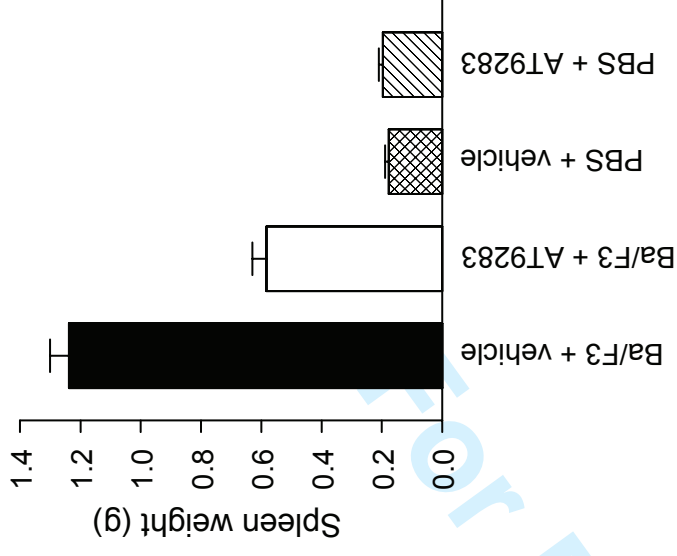
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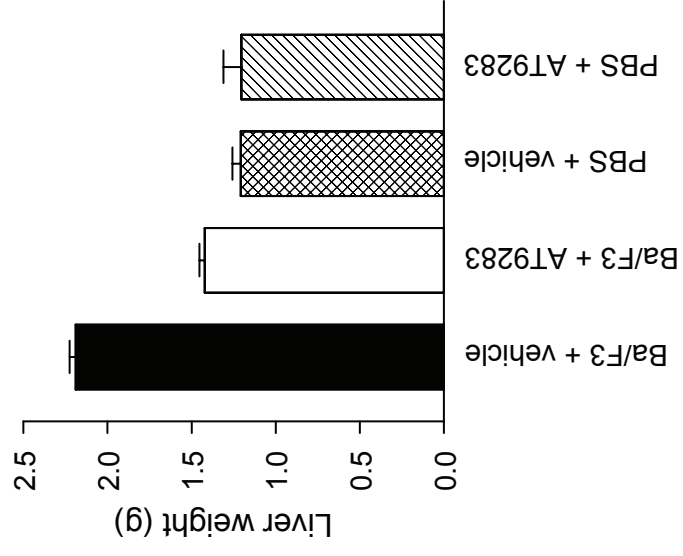
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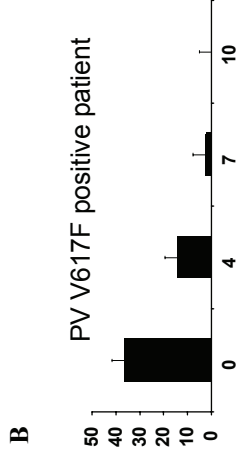
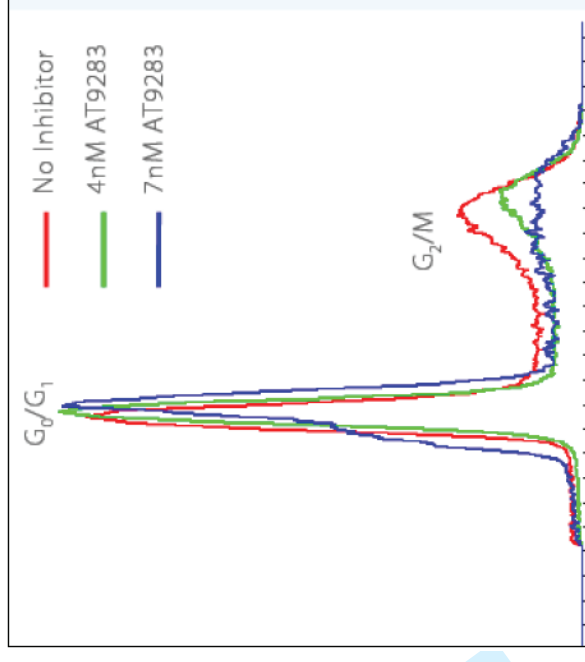
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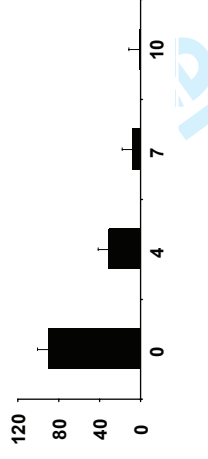


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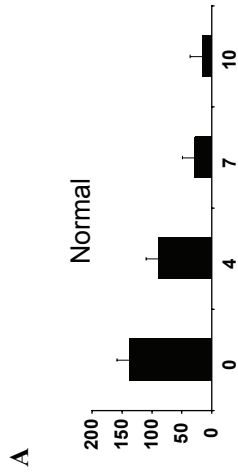
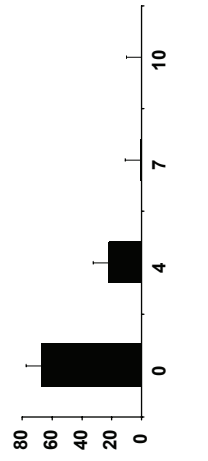
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ET V617F positive patient



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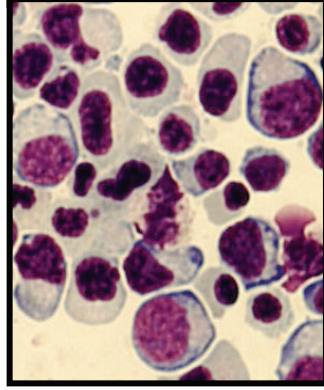
PV exon-12 positive patient



Colony number

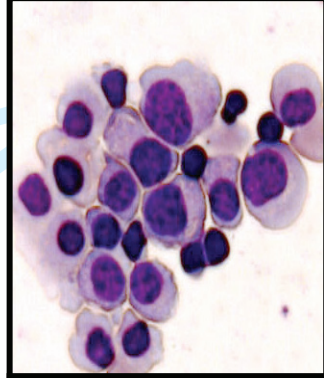
AT9283 concentration (nM)

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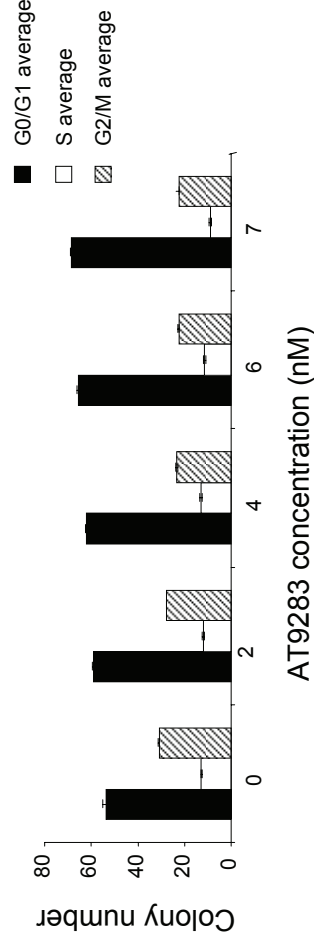
Untreated

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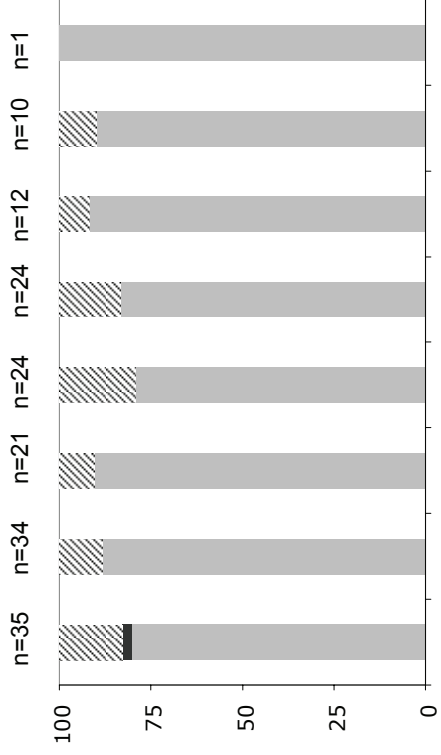


10 nM AT9283

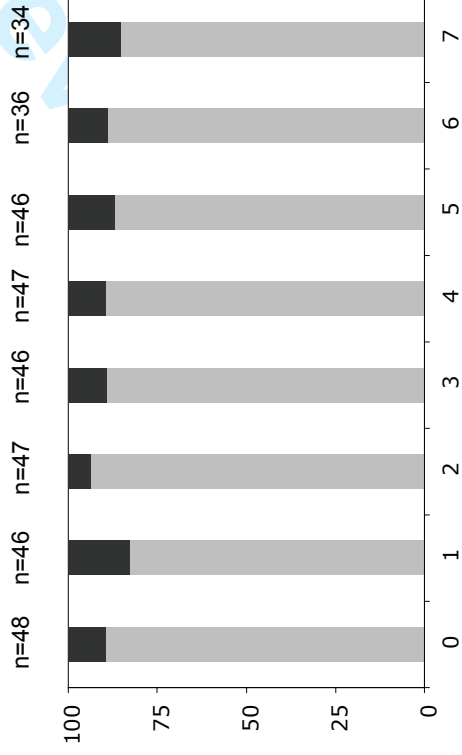
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A PV V617F patient with prominent homozygous clone



B PV V617F patient with prominent heterozygous clone



C

Patient	Mutation	Total number of colonies genotyped	Mutation load at various concentrations of AT9283 (% of Hom/Het/WT colonies genotyped) (n = number of colonies)		
			0nM	4nM	7nM
PV-1	V617F	104	17/3/80 (n=50)	17/0/83 (n=50)	0/0/100 (n=4)
PV-2	V617F	126	0/10/90 (n=50)	0/11/89 (n=50)	0/15/85 (n=26)
PV-3	V617F	103	2/15/83 (n=50)	0/19/81 (n=50)	0/0/100 (n=3)
PV-4	V617F	34	6/6/88 (n=20)	0/33/67 (n=14)	N/A
PV-5	V617F	20	20/70/10 (n=10)	0/50/50 (n=8)	0/0/100 (n=2)
PV-6	V617F	161	6/1/28 (n=35)	4/0/20 (n=24)	0/0/1 (n=1)
PV-7	V617F	350	0/5/43 (n=48)	0/5/42 (n=47)	0/5/29 (n=34)

Hom %
 Het %
 WT %

Het %
 WT %

% of erythroid colonies with described genotype

AT9283 concentration (nM)