PRECLINICAL STUDIES

Preclinical characterization of atiprimod, a novel JAK2 AND JAK3 inhibitor

Alfonso Quintás-Cardama · Taghi Manshouri · Zeev Estrov · David Harris · Ying Zhang · Amos Gaikwad · Hagop M. Kantarjian · Srdan Verstovsek

Summary We herein report on the activity of the JAK2/JAK3 small molecule inhibitor atiprimod on mouse FDCP-EpoR cells carrying either wild-type ($JAK2^{WT}$) or mutant ($JAK2^{V617F}$) JAK2, human acute megakaryoblastic leukemia cells carrying $JAK2^{V617F}$ (SET-2 cell line), and human acute megakaryocytic leukemia carrying mutated JAK3 (CMK cells). Atiprimod inhibited more efficaciously the proliferation of FDCP-EpoR $JAK2^{V617F}$ (IC_{50} 0.42 μM) and SET-2 cells (IC_{50} 0.53 μM) than that of CMK (IC_{50} 0.79 μM) or FDCP-EpoR $JAK2^{WT}$ cells (IC_{50} 0.69 μM). This activity was accompanied by inhibition of the phosphorylation of JAK2 and downstream signaling proteins STAT3, STAT5, and AKT in a dose- and time-dependent manner. Atiprimod-induced cell growth inhibition of $JAK2^{V617F}$–positive cells was coupled with induction of apoptosis, as evidenced by heightened mitochondrial membrane potential and caspase-3 activity, as well as PARP cleavage, increased turnover of the anti-apoptotic X-linked mammalian inhibitor of apoptosis (XIAP) protein, and inhibition of the pro-apoptotic protein BCL-2 in a time- and dose-dependent manner. Furthermore, atiprimod was more effective at inhibiting the proliferation of peripheral blood hematopoietic progenitors obtained from patients with $JAK2^{V617F}$-positive polycythemia vera than at inhibiting hematopoietic progenitors from normal individuals ($p=0.001$). The effect on primary expanded erythroid progenitors was paralleled by a decrease in $JAK2^{V617F}$ mutant allele burden in single microaspirated BFU-E and CFU-GM colonies. Taken together, our data supports the clinical testing of atiprimod in patients with hematologic malignancies driven by constitutive activation of JAK2 or JAK3 kinases.

Keywords Atiprimod · JAK inhibitor · JAK2 mutation · FDCP cells · Megakaryoblastic cells · Polycythemia vera · Myeloproliferative neoplasia

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Introduction

The myeloproliferative neoplasias (MPNs) polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), constitute a heterogeneous group of clonal disorders that arise from the malignant transformation of a hematopoietic stem cell [1]. The Janus kinases (JAK) JAK1, JAK2, TYK2, and JAK3 are cytoplasmic protein tyrosine kinases that engage with membrane-bound cytokine receptors and convey signals to the cell nucleus via phosphorylation of signal transducer and activator of transcription (STAT) proteins, MAP kinase/ERK, and the PI3K/AKT pathway [2–4]. JAK2-mediated phosphorylation of STAT monomers is followed by STAT dimerization and translocation to the nucleus where they modulate gene transcription. STAT5 targets include anti-apoptotic genes such as BCL-X [5]. A dominant gain-of-function G-to-T mutation at nucleotide 1,849 of the JAK2 gene involving the substitution of valine for phenylalanine at position 617 of the JAK2 protein ($JAK2^{V617F}$) can be identified in a

large proportion of patients with MPNs [2, 6–9]. $JAK2^{V617F}$ has been reported in approximately 50% of patients with PMF or ET, and in almost all patients with PV by using allele-specific polymerase chain reaction (PCR) on granulocytes from patients with MPNs [6, 8]. Mutations within exon 12 of *JAK2* have been found in $JAK2^{V617F}$-negative MPNs [10].

$JAK2^{V617F}$ kinase is constitutively activated in patients with MPNs [9]. Murine FDCP cells expressing the erythropoietin receptor (FDCP-EpoR) acquired the ability of growing in an Epo-independent fashion upon retroviral transduction of $JAK2^{V617F}$ [2, 8]. Retroviral overexpression of $JAK2^{V617F}$ in mice recapitulates the PV phenotype observed in humans including erythrocytosis and progression to myelofibrosis [2, 11–13]. Hence, $JAK2^{V617F}$ represents an attractive target with potentially important therapeutic implications for patients with MPNs. JAK2 inhibitors are being evaluated in clinical trials and some have shown activity in patients with MPNs [14–17].

In this study, we report that inhibition of JAK2 kinase activity using novel JAK kinase inhibitor atiprimod results not only in antiproliferative and proapoptotic effects on $JAK2^{V617F}$–bearing cell lines such as FDCP-EpoR (murine) and SET2 (human), but also on the JAK3 mutant cell line CMK. Similar activity was observed when ex vivo expanded erythroid bone marrow progenitors obtained from patients with $JAK2^{V617F}$-positive PV were exposed to atiprimod. These effects were accompanied by strong inhibition of JAK2-dependent downstream signaling targets and a marked reduction of the $JAK2^{V617F}$ allele burden.

Materials and methods

Cell lines, antibodies, and chemicals

Mouse FDCP-EpoR cells transduced with retroviral vectors carrying human $JAK2^{WT}$ or $JAK2^{V617F}$ were maintained as previously described [18]. Both cell lines were cultured at 37°C in a humidified 5% CO_2 atmosphere in RPMI 1,640 medium supplemented with 10% fetal calf serum (FCS), and 5% WEHI conditioned media (WEHI-CM). The human ET (at megakaryoblastic leukemic transformation) cell line carrying the $JAK2^{V617F}$ mutation (SET-2, ACC608) and the mutant JAK3-positive human acute megakaryocytic leukemia carrying the mutation A572V within the JAK3 pseudokinase (CMK, ACC393) were purchased from DSMZ (Braunschweig, Germany) and maintained in RPMI 1,640, supplemented with 20% (SET-2) or 10% FBS (CMK). Atiprimod (Callisto Pharmaceutical, New York, NY, USA) was dissolved in phosphate-buffered solution at a final concentration of 8 mM. The stock solution was kept at 4°C and further diluted in tissue cultured medium as needed.

Antibodies used for immunoblotting were as follows: rabbit antibody specific for JAK2 (06-255), JAK3 (04-011), mouse anti-phosopho-STAT3 (05-485), phospho-STAT5 (06-553), anti-phosphotyrosine clone-4G10 (05321), rabbit anti total-STAT3 (06-596), and STAT5 (05-533), all purchase from Upstate Biotechnology (Lake Placid, NY, USA); anti-v-akt murine thymoma viral oncogene homology (AKT) (550747), and anti-AKT (610876) antibodies were obtained from BD Pharmingen (San Diego, CA, USA); monoclonal mouse anti-poly (ADP-ribose) polymerase (PARP) antibody (4338-MC-50) was purchased from Trevigen Inc. (Gaitherburg, MD, USA); anti-X-linked inhibitor of apoptosis protein (XIAP) rabbit antibody (2042) was obtained from Cell Signalling Technology (Beverly, MA, USA); monoclonal anti-Caspase-3 (60-6663) was purchased from eBioscience (San Diego, CA, USA) and mouse anti-β-actin antibody (A5441) was purchased from Sigma-Aldrich (St. Louis MO, USA). Agarose conjugated protein A/G (SC-2003) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

FDCP-EpoR, SET-2, and CMK growth inhibition assays

Determination of growth inhibition by atiprimod was performed using identical culture conditions for both mouse FDCP-EpoR $JAK2^{WT}$ and $JAK2^{V617F}$ cell lines using 10% fetal calf serum (FCS), and 5% WEHI conditioned media. Human SET-2 cells were cultured in 20% FCS in RPMI1640 and the CMK cell line in 10% FCS in RPMI1640. Briefly, 10×10^4 cells per wells were cultured in 96-well flat-bottom plates at 37°C in a humidified 5% CO_2 atmosphere with different concentrations of atiprimod for 72 h. Growth inhibition assays were terminated by adding 20 μl of CellTiter96 One Solution Reagent (Promega, Madison, WI, USA). Plates were incubated for an additional 3 h at 37°C for color development. Absorbance was determined at 595 nm on a BioTek Synergy HT microplate reader (Bio-Tek, Winooski, VT, USA). Results represent the average ±standard deviation of three independent determinations.

Cell lysates and western blot analysis

Upon exposure to different concentrations of atiprimod, cell lysates were obtained as previously described [19]. Western blotting for p-STAT3, p-STAT5, bcl2, and XIAP was performed by loading 50 μg of cell lysate onto a NuPAGE® 4–12% Bis-Tris mini-gel (Invitrogen). After transferring and blocking with 5% non-fat milk for 5 h, the membrane was probed with 0.1 μL per mL of mouse anti-p-STAT3, and 1 μL per mL of p-STAT5 in 5% non–fat milk in 0.01% Tween 20 overnight at 4°C. After the membrane was washed with PBS containing 0.01% Tween 20, active bands were detected using conjugated HRP-sheep anti-mouse. Detection was performed using enhanced chemilumines-

cence as specified by the manufacturer (ECL™; Amersham). Upon detection of p-STAT3, p-STAT5, bcl2, and XIAP, membranes were stripped, blocked, and reprobed with 1 µl per ml (V/V) of total STAT3, and STAT5 antibody in 5% non-fat milk overnight at 4°C. After washing, active bands were detected using conjugated HRP-donkey anti-rabbit antibody. Finally, membranes were stripped and reprobed with mouse anti-β-actin antibody to check the equal loading of the protein.

JAK2 and JAK3 immunoprecipitation and western blotting

FDCP-EpoR, SET-2, and CMK cells (20×10^6 of each) were treated with increasing equipotent concentrations of atiprimod and harvested after 4 h. Cells were pelleted, washed three times with 50 ml of cold PBS, resuspended in 300 µl of lysis buffer (Roche, Indianapolis, IN, USA) and incubated on ice for 1 h. Cell lysates were centrifuged at 20,000 g for 30 min at 4°C and the clear supernatant was collected; 10 µl of rabbit anti-JAK2 or -JAK3 was then added to the lysates from treated and untreated cells followed by 1 h incubation on ice. Finally, 50 µl of protein A/G agarose slurry was added to the supernatant and incubated overnight at 4°C under constant rotation. The antibody-protein complex was washed three times, once with RIPA buffer (100 mM NaCl in 10 mM phosphate buffer, pH 7.2 containing 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS, 5 mM EDTA, and a cocktail of protease inhibitors), once with washing buffer (100 mM NaCl in 10 mM sodium phosphate, pH 7.2, and 0.1% Triton X-100), and finally, once with 50 mM Tris-HCl buffer pH 7.5. The immunoprecipitated complex was eluted from the agarose with 2X loading buffer and run on a NuPAGE® 4–12% Bis-Tris gel (Invitrogen). Western blotting was performed overnight at 4°C. After blocking the nitrocellulose membrane with 5% non-fat milk in PBS-0.1% Tween-20 for 3 h, membranes were incubated with mouse anti-phosphotyrosine antibody diluted in 5% non-fat milk (dilution 1:12,000) overnight at 4°C. Phosphorylated JAK2 and JAK3 bands were detected using conjugated HRP-sheep anti-mouse antibody. Detection was performed by enhanced chemiluminescence as specified by the manufacturer (ECL™, Amersham). Membranes were subsequently stripped and re-probed with rabbit anti-JAK2 (dilution 1:10,000) and rabbit anti-JAK3 antibodies (1 µg/ml) in 5% non-fat milk in 1X PBS- 0.1% Tween-20 overnight at 4°C. The active band for JAK2 was detected with HRP-donkey anti-rabbit antibody (Amersham).

Apoptosis assay

Apoptotic cells were detected by flow cytometry using recombinant human annexin-V conjugated with allophycocyamin (APC, CALTAG, Burlingame, CA). Briefly, after exposure of 2×10^5/ml of each cell lines to atiprimod for different periods of time, cells were washed in Ca^{2+}-free PBS and resuspended in 100 µl of binding buffer (10 mM 4-[2-hydroxyethyl]-1-piperazineethane-sulfonic acid, pH 7.4; 0.15 M NaC1; 5 mM KCl; 1 mM MgCl$_2$; 1.8 mM CaCl$_2$) to which annexin-V-APC had been previously added. Cells were then incubated for 20 min in the dark at room temperature and then washed and resuspended in 0.4 mL of binding buffer. Cells were analyzed in a FACSort flow cytometer (Becton Dickinson Systems, San Jose, CA) equipped with Cell Quest Pro software (Becton Dickinson).

Measurement of mitochondria transmembrane potential

Upon atiprimod treatment, cells were incubated with submicromolar concentrations of the MitoTracker Red probe (Invitrogen, Carlsbad, CA) to evaluate changes in mitochondrial membrane potential. Harvested cells were washed in Ca^{2+}-free PBS, stained with MitoTracker Red for 1 h at 37°C in the dark. Samples were washed and analyzed on a FACSCalibur flow cytometer using the Cell Quest Pro software.

In vitro expansion of erythroid progenitors

Peripheral blood samples from normal individuals and patients with PV were obtained in accord with an Institutional Review Board approved protocol upon signing of an informed consent. Peripheral blood mononuclear cells were separated by Histopaque (density 1.077) gradient centrifugation and used immediately in experiments. Expansion of progenitor cells from the mononuclear cell population was carried out in two steps. In the first step (days 0–7), 3×10^5/mL mononuclear cells were cultured in Stem Span Serum-Free Expansion Medium (StemCell Technology) in the presence of the cytokine cocktail CC110 (StemCell Technology) consisting of FLT3 (50 ng/ml), thrombopoietin (100 ng/ml), and stem cell factor (100 ng/ml). During the second step (day 8–12), cells from the first step were resuspended at a concentration of 5×10^5/ml Stem Span Serum-Free Expansion Medium with the addition of stem cell factor (50 ng/ml), insulin like growth factor-1 (IGF1 50 ng/ml), and erythropoietin (Epo 3 U/mL). On day 5 of phase 2, expanded progenitor cells were collected and treated with different concentration of atiprimod for 48 h [20].

Growth inhibition of expanded progenitor cells

At the end of phase 2, expanded progenitor cells were harvested and cultured in 96-well flat-bottom plates (2.5×10^4 cells/well) with different concentration of atiprimod for 48 h. Growth inhibition assays were terminated by adding

20 µl CellTiter96 One Solution Reagent, which was followed by 3-h incubation at 37°C for color development. Absorbance was determined at 595 nm on a BioTek Synergy_HT microplate reader.

BFU-E and CFU-GM colony culture assay

The burst-forming unit-erythroid (BFU-E) and colony forming unit-granulocyte-macrophage (CFU-GM) colony culture assay was performed as previously described [21]. Briefly, 2×10^5 mononuclear cells from the peripheral blood of patients with PV were cultured in 0.8% methylcellulose in IMDM supplemented with 10% FCS, 1.0 units/ml human erythropoietin (Amgen, Thousand Oaks, CA), 50 ng/ml stem cell factor (SCF) (Amgen), and 50 ng/ml GM-CSF. Next, 1 ml of the culture mixture was placed in 35-mm Petri dishes in duplicate and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. All cultures were evaluated after 14 days for the presence of BFU-E and CFU-GM using an inverted microscope. A BFU-E was defined as an aggregate of more than 500 hemoglobinized cells or three or more erythroid subcolonies, whereas a CFU-GM was defined as a cluster of more than 50 granulocyte and/or monocyte/macrophage cells.

Quantitation of the JAK2^{V617F} mutant T-allele

Single BFU-E and CFU-GM colonies from 3 patients with PV grown in the presence or absence of varying concentrations of atiprimod (0.25, 0.5, 1, 2 µM), were micro-aspirated and assayed for JAK2^{V617F} allele quantitation. Genomic DNA (gDNA) from each individual colony for each dose was extracted using the Puregene DNA purification reagents (Gentra, Minneapolis, MN) and total gDNA was used for PCR amplification with primers: *JAK2-Exon14-F* (GGACCAAAGCACATTGTATCCTC) and *JAK2-Exon14-R* (GGGCATTGTAACCTTCTACTT). The resulting 400 bp JAK2 PCR product was purified using the Qiagen PCR Purification Kit (Qiagen, Valencia, CA). Quantitative allele-specific suppressive PCR (ASS-PCR) was performed using the purified PCR product on a sequence detection system 7,000 platform (Applied Biosystems, Foster City CA) as previously described [22].

Results

Atiprimod inhibits the proliferation of cells carrying mutant JAK2 or JAK3

To establish the activity of atiprimod against cells harboring mutant JAK2 or JAK3 proteins, we first determined the viability of FDCP-EpoR cells transduced with human

JAK2^{WT} or JAK2^{V617F}, human SET-2 carrying mutant JAK2^{V617F} and human CMK cells expressing mutant JAK3 upon exposure to atiprimod using the MTT assay. Exposure to increasing concentrations of atiprimod resulted in dose-dependent inhibition of cellular proliferation (Fig. 1). The antiproliferative activity of atiprimod was higher on FDCP cells carrying mutant *JAK2*^{V617F} (IC$_{50}$ 0.42 µM) and SET-2 cells (IC$_{50}$ 0.53 µM) than on CMK cells carrying mutant JAK3 (IC$_{50}$ 0.79 µM) or FDCP-EpoR *JAK2*^{WT} cells (IC$_{50}$ 0.69 µM).

Atiprimod inhibits the phosphorylation of JAK2 and JAK3

Because atiprimod inhibited the proliferation of human mutant FDCP-EpoR JAK2^{V617F} reporter cells, SET-2 cells carrying JAK2^{V617F} and CMK cells expressing mutant JAK3, we next examined the activation status of JAK2 and JAK3 in untreated FDCP-EpoR *JAK2*^{WT} and FDCP-EpoR *JAK2*^{V617F} cells (Fig. 2a), as well as in SET-2 and CMK cells (Fig. 2b). Immunoprecipitation and western blot analysis, demonstrated the activation and phosphorylation of JAK2 only in FDCP-EpoR JAK2^{V617F} reporter cells and SET-2 cells but not in mutant JAK3-carrying CMK cells or in murine cells harboring human JAK2^{WT}, further emphasizing the constitutively active status of JAK2 in
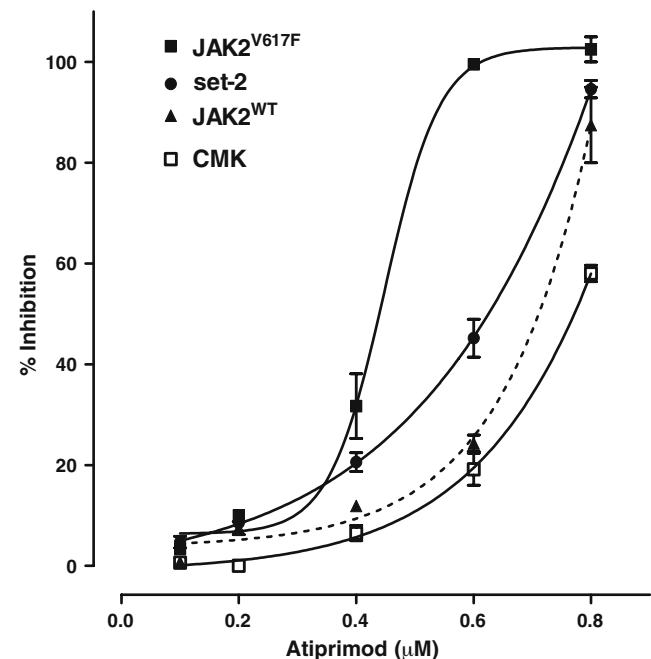

Fig. 1 Atiprimod inhibits the proliferation of cells harboring mutant JAK2^{V617F}. JAK2^{WT}- and JAK2^{V617F}-transduced FDCP-EpoR cells, human SET-2 and CMK cells were exposed to increasing concentrations of atiprimod for 72 h. (**a**) The MTS assay was used to evaluate cell proliferation. Data points are the mean ± SD from three independent determinations. (**b**) Non-linear regression model of the MTS results was used to estimate the inhibitory constants (IC) values for both cell lines

JAK2^{V617F}–carrying cell lines (Fig. 2). JAK3 phosphoryla-
tion was present in CMK cells but not in murine FDCP-
EpoR JAK2^{V617F} reporter cells (data not shown) or human
SET-2 cells (Fig. 2b). Notably, exposure to atiprimod
resulted in decreased phosphorylation of JAK2^{V617F} as
well as mutant JAK3 in a dose-dependent manner (Fig. 2b).

Atiprimod inhibits the JAK-STAT signaling pathway

In vitro expression of JAK2^{V617F} results in constitutive
activation of the STAT3/5 and PI3K/AKT signaling path-
ways, which are important for cellular proliferation and
survival [2]. Because atiprimod inhibited JAK2 and JAK3
phosphorylation, we reasoned that JAK-mediated phos-
phorylation of the downstream signaling effectors STAT3/
5 and PI3K/AKT should also be affected. At baseline,
phosphorylation of STAT3/5 and PI3K/AKT were not
detectable in murine cells harboring human JAK2^{WT},
further emphasizing the constitutively active status of
mutant JAK2^{V617F}. However, cells carrying mutant iso-
forms of JAK2 or JAK3 exhibited marked phosphoryla-
tion of STAT3/5 and PI3K/AKT. Upon treatment with
atiprimod, a dose- and time-dependent inhibition of
phosphorylation of STAT3 and STAT5 was observed in
all mutant JAK2 or JAK3-bearing cells without appreciab-
ble changes in total levels of STAT3, STAT5, or AKT
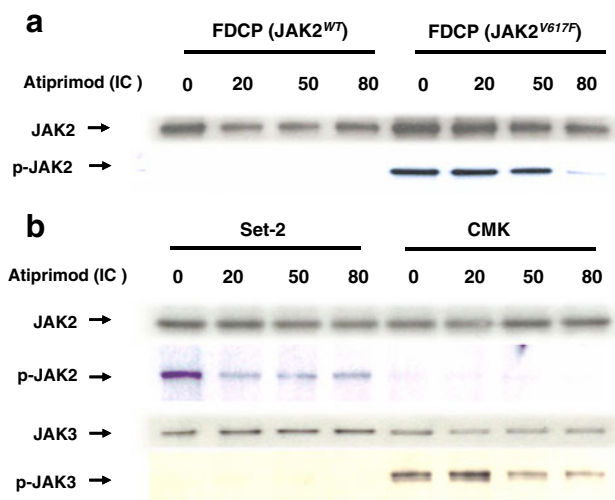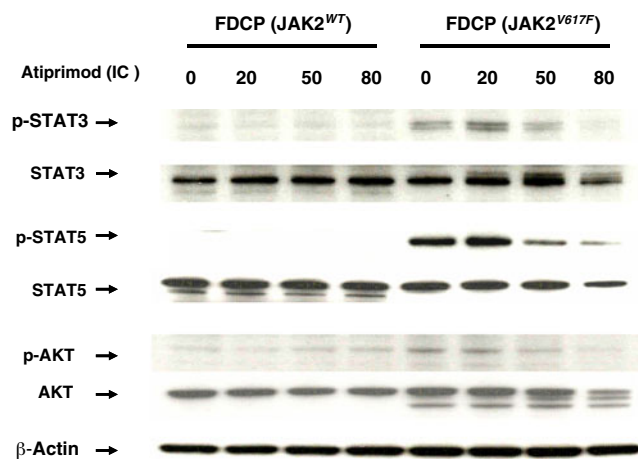(Figs. 3 and 4).

Fig. 3 Atiprimod inhibits the phosphorylation of STAT3, STAT5, and
AKT in FDCP-EpoR cells expressing mutant JAK2^{V617F}. FDCP-EpoR
cell lines carrying JAK2^{WT} or JAK2^{V617F} were treated with equipotent
doses (IC$_{20}$, IC$_{50}$, and IC$_{80}$) of atiprimod for 24 h. Extracted cell
lysates were separated on a 4–12% SDS-PAGE gel, and western
blotting was performed using mouse monoclonal antibodies against
the specified proteins. After stripping, membranes were reprobed with
rabbit anti-total STAT3, STAT5 and AKT. β-actin served as loading
control

Atiprimod induces apoptosis of mutant
JAK2- and JAK3-bearing cells

Next, we investigated whether atiprimod-mediated growth
inhibition of FCDP-EpoR JAK2^{V617F}, SET-2 and CMK
cells was associated with drug-induced apoptosis. To this
end, we performed a series of experiments in which cell
lines were exposed to equipotent doses of atiprimod. An
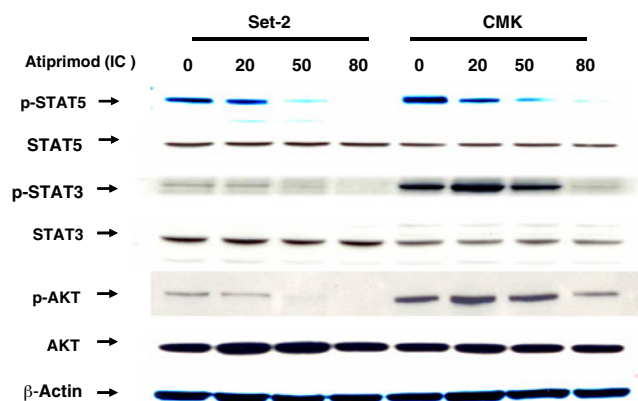increase in the fraction of apoptotic cells was observed in

Fig. 2 Atiprimod inhibits the phosphorylation of JAK2, and JAK3.
FDCP-EpoR cell lines carrying JAK2^{WT} or JAK2^{V617F}, human SET2
and CMK cells were treated with equipotent doses (IC$_{20}$, IC$_{50}$, and
IC$_{80}$) of atiprimod for 4 h. Cells were lysed and whole lysates were
immunoprecipitated with anti-JAK2 and AK3 antibodies. (**a**) Atipri-
mod inhibits the phosphorylation of JAK2 in FDCP-EpoR cell lines
carrying constitutively active JAK2^{V617F} (**a**) and the phosphorylation
of JAK2 in human SET2 cells and JAK3 in CMK cells (**b**)

Fig. 4 Desphosphorylation of STAT3, STAT5, AKT in human SET2
and CMK cells. Human SET-2 and CMK cell lines carrying
JAK2^{V617F} or JAK2^{WT}, respectively, were treated with equipotent
doses (IC$_{20}$, IC$_{50}$, and IC$_{80}$) of atiprimod for 20 h. Whole cell lysates
were separated on a 4–12% SDS-PAGE gel, and western blotting was
performed using mouse monoclonal anti-p-STAT3, anti-p-STAT5, and
anti-p-AKT. After stripping, the membrane was reprobed with rabbit
anti-total STAT3, anti–total STAT5, and anti-total AKT. The quantity
of protein was confirmed by probing the membranes with β-actin

Table 1 Induction of apoptosis (A) and mitochondrial potential damage (B) by atiprimod at equipotent concentrations (IC_{50} and IC_{80}) in FDCP-EpoR cell lines transduced with either $JAK2^{WT}$ or $JAK2^{V617F}$

		FDCP-EpoR JAK2^{WT}			FDCP-EpoR JAK2^{V617F}		
		0	IC_{50}	IC_{80}	0	IC_{50}	IC_{80}
A. Apoptosis (% Annexin V positive)	48 h	11 (±2)	17 (±3)	96 (±7)	14 (±2)	46 (±1)	89 (±5)
	72 h	18 (± 2)	33 (±5)	97 (±10)	17 (±7)	57 (±3)	95 (±9)
B. Mitochondrial membrane potential (% CMX negative)	48 h	16 (±2)	32 (±4)	99 (±6)	8 (±4)	18 (±6)	89 (±4)
	72 h	15 (±4)	44 (±4)	99 (±4)	14 (±6)	23 (±8)	99 (±7)

human JAK2^{V617F}–bearing cell lines in a time- and dose-dependent manner (Table 1a). A significant increase in the fraction of apoptotic cells expressing wild-type human JAK2 was observed only at the highest atiprimod concentration tested (Table 1a). A similar differential effect was observed when assessing mitochondrial membrane potential damage (Table 1b). Atiprimod also induced apoptosis and mitochondrial membrane potential damage in SET-2 and CMK cells, although these effects were attenuated compared with those observed in FCDP-EpoR JAK2^{V617F} cells (Table 2). Collectively, these observations appear to indicate that atiprimod-induced inhibition of cell proliferation is coupled with induction of apoptosis, likely as a consequence of inhibition of the JAK/STAT pathway. Further supporting this hypothesis was the fact that proteolytic activation of caspase 3 and PARP, coupled with a decrease of BCL-2 and XIAP levels was observed as early as 24 h after exposure of mutant JAK2 and JAK3-bearing cells to increasing concentrations of atiprimod (Fig. 5b).

Effect of atiprimod on in vitro expanded human erythroid progenitor cells

Mononuclear cells obtained from three healthy volunteers and three patients with $JAK2^{V617F}$–positive PV, with $JAK2^{V617F}$ mutant allele frequencies ranging from 20 to 80%, were used for in vitro expansion and differentiation of erythroid progenitors as described in materials and methods. Growth inhibition of day 12 expanding progenitor cells from healthy controls and patients with PV was assessed by the MTT assay upon exposure to increasing concentrations of atiprimod for 48 h (Fig. 6). In keeping with the results obtained using FCDP-EpoR $JAK2^{WT}$ and FCDP-EpoR $JAK2^{V617F}$ cells, atiprimod induced significant inhibition of proliferation, with an IC_{50} estimated at 0.495 μM, independent of the JAK2^{V617F} allele frequency (Fig. 6). In contrast, atiprimod inhibited the growth of primary progenitor cells obtained from healthy controls with an IC_{50} of 0.825 μM (Fig. 6)

Atiprimod inhibits JAK2^{V617F}-positive BFU-E colony-forming cells

The in vitro effect of atiprimod in $JAK2^{V617F}$ mutant allele burden was investigated in single microaspirated BFU-E colonies grown from the peripheral blood mononuclear cells of three patients with PV carrying $JAK2^{V617F}$ mutant allele burdens ranging from 36 to 99%. Peripheral blood mononuclear cells were plated in semisolid media in the presence of optimal concentrations of Epo, GM-CSF, and SCF for 14 days, with or without increasing concentrations (ranging from 0.0 μM to 2.0 μM) of atiprimod. A decrease in $JAK2^{V617F}$ mutant allele frequency in surviving BFU-E ($p=0.001$) and CFU-GM ($p=<0.0001$) colonies was noted upon atiprimod treatment (Fig. 7).

Table 2 Induction of apoptosis (A) and mitochondrial potential damage (B) by equipotent (IC_{50} and IC_{80}) atiprimod concentrations on SET-2 and MCK cells

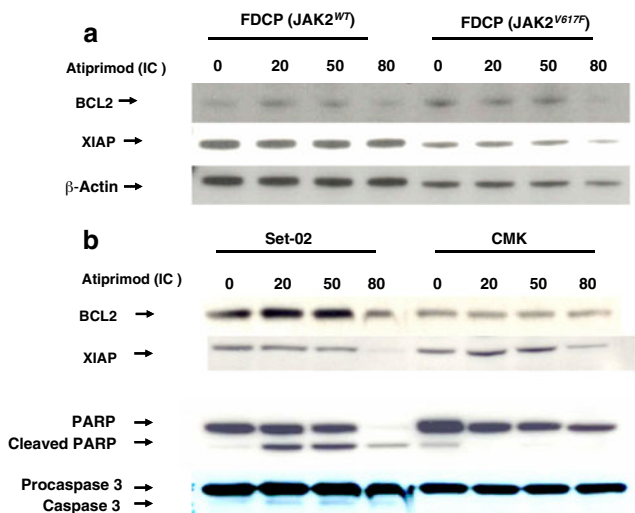|		Set-2			CMK		
		0	IC_{50}	IC_{80}	0	IC_{50}	IC_{80}
A. Apoptosis (% Annexin V positive)	48 h	12 (±4)	12 (±4)	36 (±6)	12 (±3)	36 (±9)	51 (±4)
	72 h	19 (± 3)	19 (±5)	47 (±11)	19 (±6)	42(±4)	80 (±10)
B. Mitochondrial membrane potential (% CMX negative)	48 h	9 (±2)	6 (±2)	24 (±5)	7 (±5)	15(±8)	23 (±5)
	72 h	10 (±5)	7 (±5)	53 (±5)	8 (±7)	40 (±7)	57 (±8)

Fig. 5 Atiprimod induces PARP and caspase-3 cleavage and decreases XIAP levels. (**a**) FDCP-EpoR cell lines carrying JAK2^{WT} or JAK2^{V617F} were treated with equipotent doses (IC$_{20}$, IC$_{50}$, and IC$_{80}$) of atiprimod for 24 h. Western blotting was performed using mouse anti-BCL2 and XIAP antibodies. β-actin served as loading control. (**b**) Human SET-2 and CMK cells were exposed to three different doses (IC$_{20}$, IC$_{50}$ and IC$_{80}$) of atiprimod for 24 h. Cell lysates were analyzed by western blot using mouse monoclonal anti-BCL2, XIAP, anti-PARP, and anti-caspase-3. β-actin served as loading control
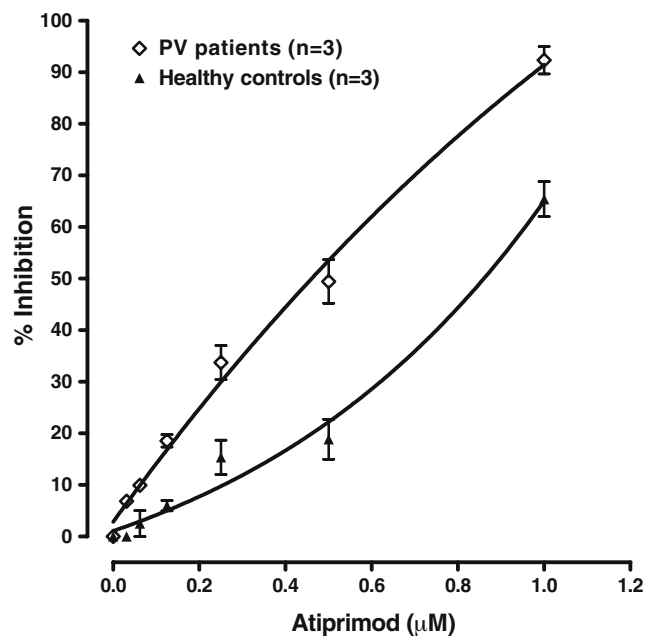
Discussion

JAK2 and JAK3 are non-receptor tyrosine kinases, predominantly expressed in hematopoietic cells. JAK2 tyrosine kinase, which is normally bound to the cytosolic domain of a type I cytokine receptor, becomes activated by transphosphorylation of a tyrosine residue in the activation loop upon cytokine stimulation. JAK3, on the other hand, is a non-receptor tyrosine kinase implicated in the signal transduction of the common gamma chain subfamily of cytokine receptors. While JAK2 is critical in hematopoiesis (mice genetically lacking the *JAK2* gene die by day 12.5 of gestation due to severe erythropoietic failure), inactivating mutations in the JAK3 gene cause immunodeficiency syndromes in both humans and mice [23]. In addition, mutations causing constitutive activation of either JAK2 or JAK3 have been implicated in human hematologic malignancies. The JAK2^{V617F} mutation is carried by a large fraction of patients with MPNs [2, 6–9]. Indeed, JAK2^{V617F} has been reported in most patients with PV and in 50% of patients with PMF or ET [6, 8]. Further, some patients with JAK2^{V617F}–negative MPNs, harbor mutations within exon 12 of *JAK2* [10]. Recently, abnormal activation of JAK3 kinase due to activating mutations in the *JAK3* gene have been reported in patients with acute megakaryoblastic leukemia and cutaneous T cell lymphoma [24, 25]. Three
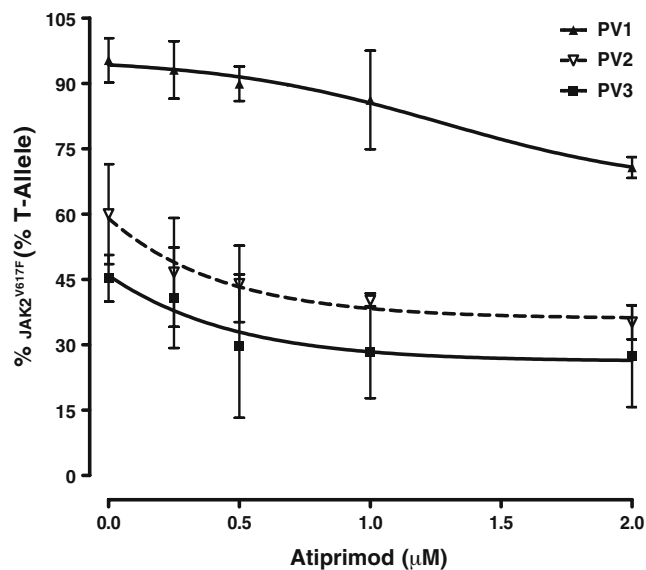

Fig. 6 Effect of atiprimod on ex vivo expanded erythroid progenitors. In vitro expanded erythroid progenitor cells from three healthy controls and three patients with PV were harvested on day 5 of phase 2 of the expansion protocol and treated with increasing concentration of atiprimod for 48 h. The MTS assay was used to evaluate cell proliferation. Data points are the mean ± SD from three independent determinations. (**b**) Non-linear regression model of the MTS results was used to estimate the inhibitory concentration (IC) values for both cell lines

Fig. 7 Quantitation of the JAK2^{V617F} mutant allele and effect of atiprimod on *JAK2^{V617F}* allele burden. Peripheral blood low-density cells from 3 patients with PV were incubated in the presence of atripimod at doses ranging from 0.25 to 2.0 μM in clonogenic assays and evaluated after 14 days. Ten colonies at each dose were microaspirated and the JAK2^{V617F} allele burden was quantitated by qRT-PCR. After DNA extraction, gDNA was used for PCR amplification with *JAK2-exon14* primers. Quantitative allele-specific suppressive PCR (ASS-PCR) was performed using the purified PCR product on a sequence detection system 7,000 platform

different JAK3 mutations, A572V, V722I, and P132T have been identified in magakaryoblastic leukemia cell lines or samples obtained from patients with megakaryoblastic leukemia. All three mutations have been shown to be capable of transforming Ba/F3 cells to factor-independent growth, and A572V recapitulates features consistent with human megakaryoblastic leukemia in a mouse model, further supporting a causal role of JAK3 mutations in leukemogenesis [25].

Small molecules capable of inhibiting the kinase activity of the above mentioned mutant oncogenic enzymes have been highly sought after as a means to develop targeted therapeutic approaches. In fact, several small molecule JAK2 inhibitors have already entered clinical trials and have shown promising results in patients with ET, PV, or PMF [14–17]. We herein described the in vitro characterization of atiprimod, a novel small molecule JAK2 inhibitor. An interesting feature of atiprimod is that not only is this molecule endowed with potent anti-JAK2 kinase inhibitory activity but also with activity against JAK3 kinase. To test these activities, we used both murine (FCDP-EpoR JAK2^{V617F} and FCDP-EpoR JAK2^{WT}) and human (SET-2 and CMK) cell lines. While FCDP-EpoR JAK2^{V617F} and SET-2 cells were used to test the activity of atiprimod against JAK2^{V617F} kinase, CMK, a megakaryoblastic leukemia cell line, was used to test the activity of the drug against mutant JAK3 as it carries the activating A572V mutation in the pseudokinase domain of JAK3 [25]. Exposure to atiprimod resulted in markedly diminished proliferation of both JAK2^{V617F}- and mutant JAK3–bearing cells. Cells bearing JAK2^{V617F} appeared to be considerably more sensitive to atiprimod as compared with those carrying wild-type JAK2. These antiproliferative effects were coupled with inhibition of JAK2 phosphorylation in FDCP-EpoR JAK2^{V617F} and SET-2 cells and dephosphorylation of mutant JAK3 in CMK cells in a dose-dependent manner.

Constitutive activation of STAT3 and AKT has been shown to be of critical importance to cell proliferation and survival. Hence, we determined the levels of total and phosphorylated STAT3 and AKT by immunoblotting. Cells carrying mutant JAK2 or JAK3 exhibited constitutive activation of STAT3/5 and PI3K/AKT but exposure to atiprimod resulted in dose- and time-dependent inhibition of phosphorylation of STAT3, STAT5, and AKT in cells harboring mutant JAK2 or JAK3 with no significant changes in total STAT3, STAT5, or AKT protein levels. This is of importance because downstream JAK2 effectors, like STAT5, are considered to mediate cell proliferation, survival, and differentiation [7]. Not surprisingly, inhibition of the JAK/STAT axis was coupled with marked cellular apoptosis. While the inhibition of phosphorylation of STAT3/5 could be accounted for by the inhibition of

JAK2 kinase, the mechanism whereby atiprimod induced decreased activation of AKT is less clear. Whether AKT inhibition is due to direct inhibition of the AKT activator PI3K or to activation of PTEN, a negative regulator of AKT phosphorylation, warrants further investigation [26].

Importantly, the results obtained with mutant JAK2- or JAK3-bearing cell lines were replicated in in vitro expansion assays in which human erythroid progenitors obtained from patients with JAK2^{V617F}–positive PV were exposed to atiprimod. Treatment with atiprimod resulted in marked anti-proliferative and proapoptotic activity of JAK2^{V617F}–positive progenitors compared with erythroid progenitors carrying JAK2^{WT} obtained from healthy volunteers, indicating a preferential effect on the malignant clones. In keeping with our findings, atiprimod has been recently shown to inhibit the clonogenic growth of AML cell lines and primary AML bone marrow cells while sparing untoward toxicity on normal hematologic precursors. This activity was also associated with inhibition of the JAK/STAT3/5 pathway and induction of apoptosis by cleavage of caspase 3 and PARP.

In conclusion, we have characterized in vitro the JAK2/JAK3 inhibitor atiprimod. This agent has activity against cells bearing mutated JAK2 or JAK3 kinase. Atiprimod inhibits the proliferation of several cell lines such as FDCP-EpoR cells engineered to express JAK2^{V617F}, other JAK2^{V617F}–positive cell lines such as SET-2, and primary cells from patients with JAK2^{V617F}–positive PV. In addition, atiprimod also inhibits the growth of CMK, an acute megakaryoblastic leukemia cell line carrying the activating mutation A572V in the JAK3 pseudokinase. This activity was coupled with inhibition of the JAK/STAT and AKT signaling pathways and resulted in apoptosis. These data support the testing of atiprimod in patients with mutant JAK2- or JAK3-positive hematologic malignancies.

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