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Inhibition of related JAK/STAT pathways with molecular targeted drugs shows strong synergy with ruxolitinib in chronic myeloproliferative neoplasm

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Summary

This study aimed to assess the antitumour effects, molecular mechanisms of action, and potential synergy of ruxolitinib with sorafenib, KNK437, dasatinib, and perifosine, in Philadelphia-negative chronic myeloproliferative neoplasms (MPN). Cytotoxic and cytostatic effects of the different compounds were determined in the *JAK2* V617F-positive cell lines, HEL and Ba/F3 $^{JAK2V617F\ EPOR}$, and in primary mononuclear and bone marrow CD34-positive cells from 19 MPN patients. Ruxolitinib [50% inhibitory concentration $(IC_{50})^{PV} = 15$ nmol/l], as well as sorafenib ($IC_{50}^{PV} = 8\mu$mol/l), KNK437 ($IC_{50}^{PV} = 100\mu$mol/l), and perifosine ($IC_{50}^{PV} = 15\mu$mol/l), were able to inhibit proliferation in cell line models and in primary cells from MPN patients. Dasatinib, KNK437, and sorafenib showed a strong synergistic effect in combination with ruxolitinib [combination index $(CI)^{PV} < 0{\cdot}3$]. Western blot confirmed that ruxolitinib blocked ERK, and consequently STAT5 activation, sorafenib inhibited ERK, P38 and STAT5, dasatinib blocked SRC and STAT5, and KNK437 decreased the stability of the JAK2 protein, reducing its expression. Inhibiting JAK2-related proliferative pathways has the potential to inhibit cell proliferation in MPNs. Furthermore, the combination of ruxolitinib with inhibitors that target these pathways has a strong synergistic effect, which may be due to decreased activation of the common effector, STAT5.

Keywords: Myeloproliferative disease, drug synergisms, stem cells, ruxolitinib, KNK437.

Introduction

JAK2 is a non-receptor tyrosine kinase that acts as an important signal transducer in cytokine signalling and promoting growth, survival, and differentiation of various cell types. A somatic point mutation in *JAK2*, giving rise to the expression of the JAK2 V617F mutant, occurs at a high frequency in myeloproliferative neoplasm (MPN) patients [>95% in polycythaemia vera (PV), and >50% in essential thrombocythaemia (ET) and primary myelofibrosis (PMF)] (Baxter *et al*, 2005; Kralovics *et al*, 2005; Ugo *et al*, 2005). The mutation confers constitutive kinase activity and results in cytokine hypersensitivity and a proliferative advantage of haematopoietic progenitor cells. Thus, inhibiting JAK2 V617F may be therapeutically beneficial. While clinical trials on JAK inhibitors in MPN patients have shown positive results, success has primarily been due to improved patient symptomatology. One clinical trial also showed an increased survival rate but a limited reduction of the tumour clone (Harrison *et al*, 2012; Verstovsek *et al*, 2012), either at the bone marrow level or in the *JAK2* V617F allele burden. Moreover, a sponsor-independent analysis of long-term patient outcomes suggests that the reduction in spleen size by ruxolitinib is modest and not always durable (Tefferi *et al*, 2012). Therefore, there is a need for advances in understanding MPN cell biology to further improve clinical outcomes and treatment optimization.

We recently reported a relationship between response to treatment with hydroxycarbamide and the expression of certain key genes in the transduction of proliferative signalling pathways, such as MAPK, AKT/PI3K, and SRC related proteins (Albizua *et al*, 2011), in addition to differential expression levels of HSP70 in ET and PV. Further recent observations suggest that two independent pathways could

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mediate STAT activation; one being dependent on JAK2 and the other independent, although related to other tyrosine kinases, such as the SRC family kinases (SFKs)(Byers *et al*, 2009; Sen *et al*, 2009). On the other hand, SFKs are reported to be independent of *JAK2* V617F-induced polycythaemia in a murine retroviral bone marrow transplantation model (Zaleskas *et al*, 2006).

Based on these previous results and the extensive literature that relates these oncogenes with MPN, we explored the applicability of these targets in treatment. To this end, we selected a series of specific inhibitors in clinical phase development for other malignancies: sorafenib (Nexavar, BAY 43-9006) is the most successful clinical inhibitor of RAF activity (Ibrahim *et al*, 2012); dasatinib (BMS354825, Sprycel) is a dual SRC-ABL1 kinase approved for the treatment of patients in all phases of chronic myeloid leukaemia (CML) (Shah *et al*, 2004); KNK437, specifically inhibits the *in vitro* synthesis of heat shock proteins, such as HSP70 (Fujii *et al*, 2012); and perifosine acts as an inhibitor of the AKT pathway and is currently used in the treatment of multiple myeloma and neuroblastoma (Jakubowiak *et al*, 2012; Sun & Modak, 2012).

We aimed to compare the *in vitro* effects of the new JAK1/2 inhibitor, ruxolitinib, with the kinase inhibitors dasatinib, sorafenib, perifosine, and the HSP70 inhibitor, KNK437, in primary cells from MPN patients. We also assessed potential synergistic effects among these compounds, which allowed us to analyse the effects of blocking the proliferative pathways related to JAK2 (MAPK, AKT and SRC) in these pathologies, and to study the underlying molecular mechanisms.

Materials and methods

Patients

A total of 19 MPN patients including 8 PV $^{JAK2\ V617F}$, 7 ET $^{JAK2\ V617F}$, and 4 ET $^{JAK2\ WT}$, as well as six healthy donors were analysed. MPN diagnosis was established on the basis of World Health Organization (WHO) 2001/2008 criteria (Tefferi, 2008), or Polycythaemia Vera Southern Study Group criteria (according to the standard criteria at the time of diagnosis). The clinical and biological variables are shown in Table I. This study was approved by the Hospital 12 de Octubre ethics committee and written informed consent was obtained from all patients, according to the Declaration of Helsinki.

Samples

Peripheral venous blood and bone marrow samples were collected in heparin and immediately processed. Leucocytes, granulocytes, and mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation, as previously described (Pharmacia, Freiburg, Germany). Erythrocytes were eliminated using a commercial red blood lysis buffer (Roche Applied Sciences, Manheim, Germany), resulting in more than 90% granulocytes. Lymphocyte contamination was assessed in five samples by flow cytometry, and was less than 2% of the total cell count.

CD34 cells from bone marrow samples were isolated by double-positive selection using a magnetic cell sorting system (miniMACS, Miltenyi Biotec, Paris, France), in accordance with the manufacturer's recommendations. The purity of the recovered cells was greater than 90%, as determined by flow cytometry. CD34 cells from two cord blood donors were used as controls.

DNA from granulocytes was extracted using a Maxwell 16 SEV automated extraction system (Promega, Manheim, Germany). Mutational screening for *JAK2* V617F was performed with real time polymerase chain reaction (PCR), using DNA from whole peripheral blood from the same study sample, as previously described (Rapado *et al*, 2009).

Cell culture

The erythroleukaemia *JAK2* V617F HEL cell line was obtained from the DSMZ collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brauncschweig, Germany) and cultured in RPMI-1640 medium. The pro B murine transformed cell lines, Ba/F3 $^{EPOR\ JAK2\ V617F}$ and BA/F3 $^{EPOR\ JAK2\ WT}$, were kindly donated by Dr Quintas-Cardama (Department of Leukemia, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA) and cultured in RPMI medium supplemented with WEHI media enriched with interleukin 3 (IL3). For the inhibition assays, $1{\cdot}5 \times 10^5$ cells were treated with ruxolitinib, dasatinib, perifosine, KNK437 or sorafenib (Selleckchem, Munich, Germany). Viability was determined by trypan blue (Sigma Aldrich, St. Louis, MO.), or the WST (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)2H- tetrazolium, monosodium salt) assay (Roche Applied Sciences, Manheim, Germany).

Colony-forming assay

To perform the colony-forming assay, 5×10^5 mononuclear cells from 16 MPN patients (9 PV, 7 ET) who were *JAK2* V617F positive at diagnosis were seeded in methylcellulose medium supplemented with recombinant human (rh) IL3 (20 ng/ml) and rhSCF (50 ng/ml). Erythropoietin (EPO)-stimulated colony growth was evaluated in the presence of rhEPO (3 μ/ml). H4230 methylcellulose semi-solid medium and cytokines were purchased from StemCell Technologies, Vancouver, BC, Canada. The same assay was also performed with 2×10^3 CD34-positive bone marrow cells per well from two PV, two ET, and two cord blood controls samples.

Cells were cultured in the presence of dasatinib (0–100 nmol/l), perifosine (0–100 μmol/l), sorafenib

Table I. Clinical and laboratory features of *JAK2* V617F-positive PV and ET patients at diagnosis and follow-up.

	PV	ET *JAK2* V617F	ET *JAK2* WT
Patients	8	7	4
Gender (M/F)	3/5	2/5	2/2
Age*,†	59·5 (35–81)	51·9 (34–94)	46·8
Splenomegaly†	2/8	3/7	0/3
Hepatomegaly†	1/8	1/7	0/3
Haemoglobin (g/l)*,†	17·35 (15·3–20·3)	14·55 (12·0–15·5)	15·9 (15·7–16·2)
Haematocrit*,†	53·6 (45·5–60·5)	44·1 (37·4–47·9)	48·3 (46·6–49·9)
Platelets ($\times 10^9$/l)*,†	545·55 (346–904)	580·5 (477–694)	762·7 (514–895)
Increased LDH†	4/8	3/7	1/3
JAK2 V617F allele burden	49·54% (8·53–100)	28·11% (11–49·8)	NA
Thrombotic/Haemorragic events†	4/8	2/5	1/3
Disease duration *,‡	37 (7–156)	29 (8–96)	53(12–144)
Treatment duration *,‡	10 (8–12)	9 (7–15)	NA
Response to hydroxycarbamide treatment	5/8	4/7	1/2

PV, polycythaemia vera; ET, essential thrombocythaemia; M, male; F, female; WBC, white blood cells; LDH, lactate dehydrogenase; NA, not applicable.
*Median value (range) is reported.
†At diagnosis.
‡In months.

(0–100 μmol/l), and ruxolitinib (0–100 nmol/l). Each assay was performed in duplicate. After 14 d of culture, the morphology and number of colonies was determined using an inverted light microscope.

Synergism analysis

This analysis used 0–500 nmol/l ruxolitinib, and constant concentrations [lower than the 50% inhibitory concentration (IC_{50})] for each of the other drugs. Results were analysed for synergistic, additive, or antagonistic effects using CalcuSyn software (Biosoft, Cambridge, UK). Synergism was indicated by a combination index, (CI) < 0·9, an additive effect was indicated by CI values between 0·9 and 1·1, and antagonism by a CI > 1·1 (Tallarida, 2001).

Flow cytometry, multiparametric analysis, and viability test

Cells from BFU-E were extracted, washed and resuspended in 10 ml of phosphate-buffered saline (PBS). A 10-μl aliquot of cells was used for the viability test with trypan blue 1:1. Cells were analysed by flow cytometry using the markers CD71-fluorescein isothiocyanate (FITC), CD45-perdinin chlorophyll (PerCP), annexin V-allophycocyanin (APC), CD41a-FITC, and CD34-APC (BD, Cockeysville, CA, USA). Cell suspensions with IgG isotype control antibodies were used as negative controls.

Protein concentrations of AKT, phospho-AKT, phospho-MEK, MAP2K1, phospho-MAP2K1, STAT1 and phospho-STAT1 were determined using the cytometric bead array (CBA) immunoassay (BD). All the analyses were performed using a FACSCalibur flow cytometer (BD).

Western blot

Cells were washed twice in PBS and proteins were extracted with the Cytobuster protein extraction reagent (Merck, Darmstadt, Germany). The protein concentration was determined using a non-interfering assay (RC-DC Protein Assay Kit; BIO-RAD, Hercules, CA, USA). Western blot was performed using rabbit actin primary antibody (protein control), anti-JAK2, anti-phospho-JAK2 (Tyr1007/1008) anti-SRC, anti-phospho-SRC (Tyr 416), anti-AKT, anti-phospho-AKT (ser473) anti-STAT5, anti-phospho-STAT5 (ser456), anti-LYN, anti-phospho-LYN (Tyr507), and rabbit secondary antibody (all from Cell Signalling, Beverly, MA, USA). The membranes were incubated with their respective secondary antibody for 1 h and signal was detected using the ECL Advance Western Blotting Detection KIT (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis

Due to the non-normal distribution of the biological results, the dichotomized data were analysed by a non-parametric Wilcoxon test. Statistical significance was considered when the *P* value was <0·05. Flow cytometry data were analysed by the Summit 4·3 program. CBA files were processed with the FCAP Array™ (BD).

Results

Effect of ruxolitinib on myeloproliferative neoplasm mononuclear cells

Our first approach was to determine the effects of ruxolitinib on mononuclear cell cultures seeded in methylcellulose, as a reference. In the colony forming assays (Fig 1A, B) there was a greater IC_{50} in MPN $^{JAK2\ V617F}$ growth in EPO-facilitated conditions compared with ET *JAK2* WT or endogenous growth (IC_{50EPO}: PV = 37 nmol/l, ET $^{JAK2\ V617F}$ = 40 nmol/l, ET $^{JAK2\ WT}$ = 12 nmol/l. $IC_{50WITHOUT\ EPO}$: PV = 15 nmol/l, ET $^{JAK2\ V617F}$ = 6 nmol/l, ET $^{JAK2\ WT}$ = 15 nmol/l) (Figure S1). Culturing the different cell lines in the presence of ruxolitinib showed an IC_{50} of 274 nmol/l in HEL cells, 158 nmol/l in BAF3 $^{JAK2\ V617F}$ cells, and 137 nmol/l in BAF3 $^{JAK2\ WT}$ cells (Fig 1C).

In CD34 cells from bone marrow aspirates of four patients diagnosed with MPN, ruxolitinib presented an IC_{50} of 50 nmol/l, while it had no effect on CD34 controls cells ($P \leq 0{\cdot}05$, Fig 1D).

Synergistic effect of sorafenib with ruxolitinib

To assess the effects of sorafenib on the different cells, we first determined the IC_{50} dose of this drug in cell lines, and found a similar response in all these series of experiments (Ba/F3 $^{EPOR\ JAK2\ WT}$: IC_{50} = 2·37 μmol/l, HEL: IC_{50} = 3·87 μmol/l, Ba/F3 $^{EPOR\ JAK2\ V617F}$: IC_{50} = 8·49 μmol/l) (Fig 2A). Comparable results were found in mononuclear and CD34 cells from patients (Fig 2C, D).

When we analysed the possible synergistic effects of sorafenib with ruxolitinib (Fig 2B) we found that a constant concentration of sorafenib (1 μmol/l) reduced the effective dose of ruxolitinib in BA/F3 cultures by 3 to 5 times. The Chow-Talalay model shows that the combined action of both drugs produced a moderate synergistic effect in BA/F3 $^{EPOR\ JAK2\ V617F}$ and BA/F3 $^{EPOR\ JAK2\ WT}$ cell lines (Table II). However, in the HEL cell line these two drugs had an additive effect.

These results were validated in mononuclear cells from PV patients, where the IC_{50} of ruxolitinib decreased from 30 nmol/l to less than 1 nmol/l in the presence of a sorafenib concentration of 1 μmol/l, which was ineffective alone (Fig 2E).

At the phenotypic level, sorafenib produced a change in the colony development. It inhibited erythroid burst-forming units (BFU-E) and erythroid colony-forming units (CFU-E) colony progression and increased the non-haemoglobinized granulocyte–macrophage CFU (CFU-GM) and megakaryocyte

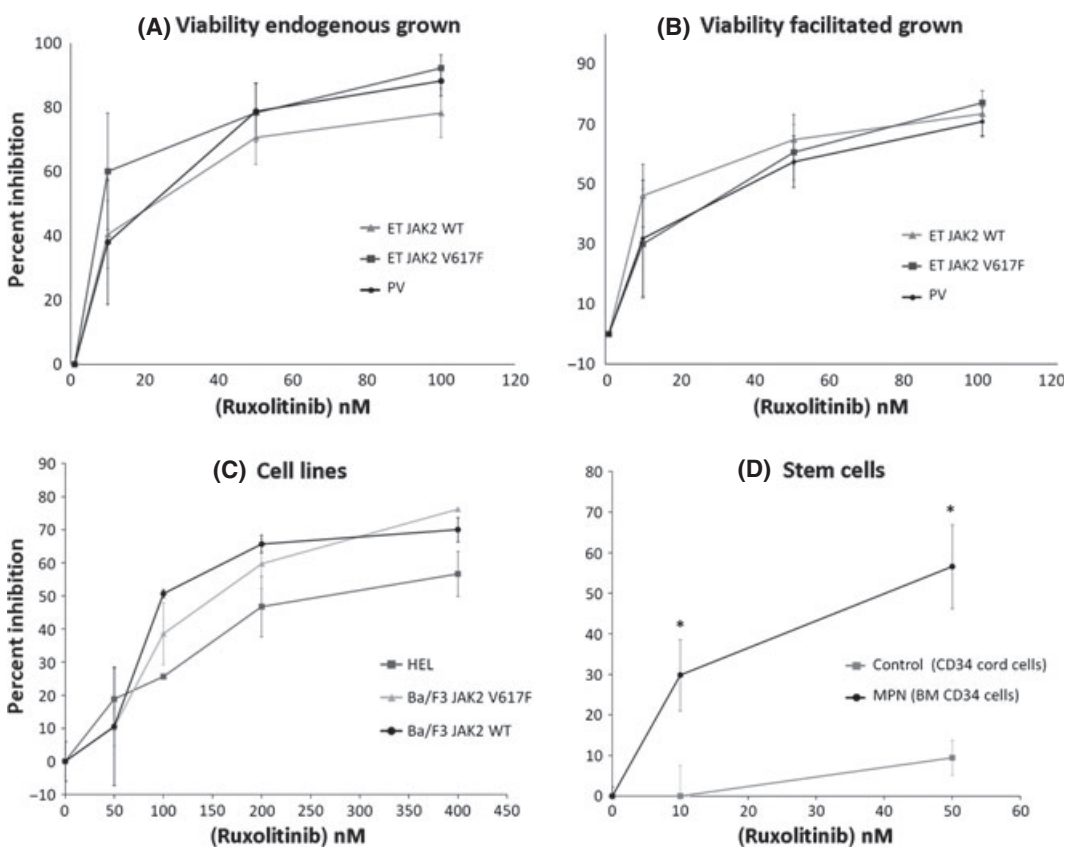

Fig 1. Effects of ruxolitinib in the different models analysed. (A) and (B): Viability test of cells from colonies assays. (A) Cultures without EPO (Supplemented with rhIL3 and rhSCF). (B) Cultures with rhEPO, rhIL3 and rhSCF (C) Viability test of the different cell lines in presence of ruxolitinib. (D) Effects of ruxolitinib in CD34 cells. * $P \leq 0{\cdot}05$.

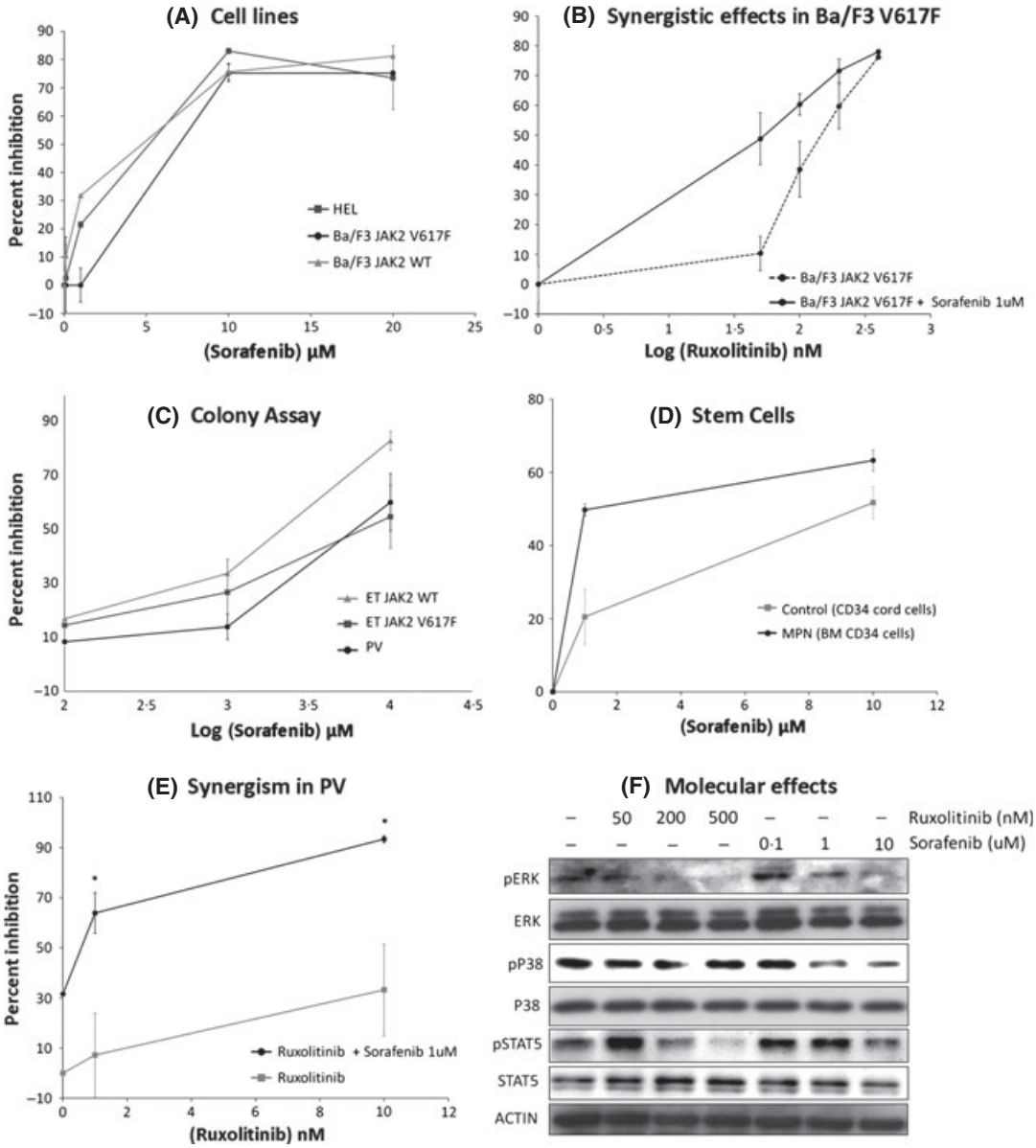

Fig 2. Effects of sorafenib in the different models analysed. (A) Viability test of cell lines. (B) Synergistic effects of ruxolitinib with sorafenib (10 nmol/l). (C) Viability test of cells from colonies assays. (D) Viability test of CD34 cells from colonies assays. (E) Synergistic effects of ruxolitinib with sorafenib in cell from polycythaemia vera patients (10 nmol/l). (F) Western blots of lysates from HEL cells. *$P \leq 0{\cdot}05$.

CFU (CFU-MK). Flow cytometry revealed that behind this phenotypic change was a decrease in CD71 erythroid cells and increased CD41 megakaryocytic and CD45 panleucocitary colonies, mainly CFU-GM (Figure S2).

Western blot analysis of the activation level within the major pathways after treatment with sorafenib showed that, while ruxolitinib blocked ERK1 activation without affecting P38, sorafenib decreased the activation of P38, in addition to ERK activation (Fig 2F). To confirm P38 inhibition, a CBA analysis was performed with proteins extracted from MPN patient cells after treatment with ruxolitinib and sorafenib. This allowed us to confirm that, besides inhibiting the canonical MAPK pathway at the RAF1 level (resulting in decreased ERK activation), sorafenib blocked the activation of P38 (Figure S2).

Effect of perifosine on MPN mononuclear cells

Perifosine was effective in reducing the number of colonies, as well as inhibiting the proliferation of mononuclear cell cultures from patients diagnosed with PV, ET $^{JAK2\ V617F}$ and ET $^{JAK2\ WT}$, with an average IC_{50} of 15 µmol/l. In control cultures, this inhibition was evident only in the absence of EPO (IC_{50} = 17 µmol/l), with no effect on erythroid cells in the EPO supplemented culture. The number of apoptotic cells doubled with 25 µmol/l perifosine [$ET_{CONTROL}$ 24% (13·8–40·5),

Table II. IC_{50} and synergic effect for the different compounds.

	Ruxolitinib	Sorafenib							KNK437							Dasatinib						
			CI							CI							CI					
	IC_{50}		[Ruxolitinib] (nmol/l)					IC_{50}		[Ruxolitinib] (nmol/l)					IC_{50}		[Ruxolitinib] (nmol/l)				
	(nmol/l)	IC_{50}^{syn}	1·0	10	50	100	200	400	(μmol/l)	IC_{50}^{syn}	1·0	10	50	100	200	400	(μmol/l)	IC_{50}^{syn}	1·0	10	50	100	200	400	IC_{50}^{syn}
HEL	274·4	249·9	–	–	1·07	0·96	1·02	1·08	3·87	6·67	–	–	0·41	0·59	0·57	0·68	17·1	111·1	–	–	0·75	0·30	0·35	0·35	–
BAF3mut	157·5	58·91	–	–	0·43	0·54	0·71	1·10	8·49	43·11	–	–	0·11	0·23	0·26	0·33	313·2	59·13	–	–	0·31	0·46	0·66	1·06	–
BAF3wt	137·4	21·11	–	–	0·79	0·71	0·79	1·30	2·37	29·43	–	–	0·43	0·39	0·50	1·45	79·8	42·08	–	–	0·31	0·47	0·67	1·20	–
PV	15·5	0·53	0·09	0·05	–	–	–	–	8·11	1·03	0·08	0·21	–	–	–	–	100	0·35	0·08	0·14	–	–	–	–	11·68

CI, combination index; PV, polycythaemia vera. (CI > 1·1 Antagonists. 1·1 < CI <0·9 Additive effect. 0·9 < CI <0·3, Synergic effect. CI < 0·3, strong synergism.The $IC50_{synergy}$ of ruxolitinib was determined in the presence of constant concentrations of aorafenib (1 μmol/l), KNK437 (1 μmol/l) y dasatinib (1 nmol/l). IC_{50}, 50% inhibitory concentration.

49·9% $ET_{25\mu mol/l}$ (38·7–78·61)]. In CD34 cells from bone marrow, perifosine presented an IC_{50} of 18 μmol/l, and 29 μmol/l in cord blood control donors. This difference was not statistically significant (perifosine data are shown in Figure S3). Synergy studies with perifosine and ruxolitinib did not show conclusive results (IC > 1) in cell lines, and did not justify the study in cells from MPN patients.

KNK437 is strongly synergistic with ruxolitinib, and is able to decrease JAK2 expression

In the HEL cell line, KNK437 showed an IC_{50} of 17 μmol/l (Fig 3A) and in combination with ruxolitinib the IC_{50} was 10 times lower (CI = 0·41, Fig 3B). A similar result was observed in the BA/F3 cell lines with a decrease from 137 nmol/l to 50 nmol/l seen in BA/F3 $^{EPOR\ JAK2\ V617F}$, and from 137 nmol/l to 29 nmol/l in BA/F3 $^{EPOR\ JAK2\ WT}$. A constant concentration of 10 μmol/l KNK437 was used and the KNK437 IC_{50} in these cell lines was 313 μmol/l and 80 μmol/l, respectively (Fig 3A).

The IC_{50} of KNK437 in cells from patients was 25 μmol/l for mononuclear cell cultures and 30 μmol/l for haematopoietic stem cell cultures. An analysis of the synergistic effects of KNK437 and ruxolitinib in mononuclear cells from PV patients showed a reduction in the ruxolitinib IC_{50} from 30 nmol/l to 1 nmol/l ($CI_{1\ nmol/l}$ = 0·082, $CI_{10\ nmol/l}$ = 0·210) in the presence of KNK437 (constant concentration of 1 μmol/l) (Fig 3C).

At the molecular level, KNK437 decreased the activation of JAK2 as well as its expression, (Fig 3D). This decrease in *JAK2* expression resulted in the inhibition of leading proliferative pathways related to JAK2.

Dasatinib reduced cell proliferation via SRC and STAT5 and acted synergistically with ruxolitinib

Dasatinib alone showed no effect in the cell lines, however, it acted synergistically with ruxolitinib (Fig 3B, Table II). Viability studies of dasatinib on methylcellulose colony cultures showed an IC_{50} of 10 nmol/l for MPN $^{JAK2\ V617F}$, and 1 nmol/l for ET $^{JAK2\ WT}$ cells. At the phenotypic level, dasatinib appeared to specifically decrease the development of erythroid colonies. This decrease was evident, not only from the number of colonies, but also from their size, highlighting growth inhibition (Figure S4).

The IC_{50} value observed in mononuclear cell colonies was maintained in CD34 positive cell cultures from MPN $^{JAK2\ V617F}$ patients and cord donor controls, and was around 20 nmol/l (Fig 4D).

The synergy studies with ruxolitinib and dasatinib in PV patient samples showed a decrease in the ruxolitinib median effective dose from 30 nmol/l to 1 nmol/l ($P < 0·05$). CI values for the drug combination were 0·084 when ruxolitinib was 1 nmol/l, and 0·137 when ruxolitinib was 10 nmol/l (Fig 3E).

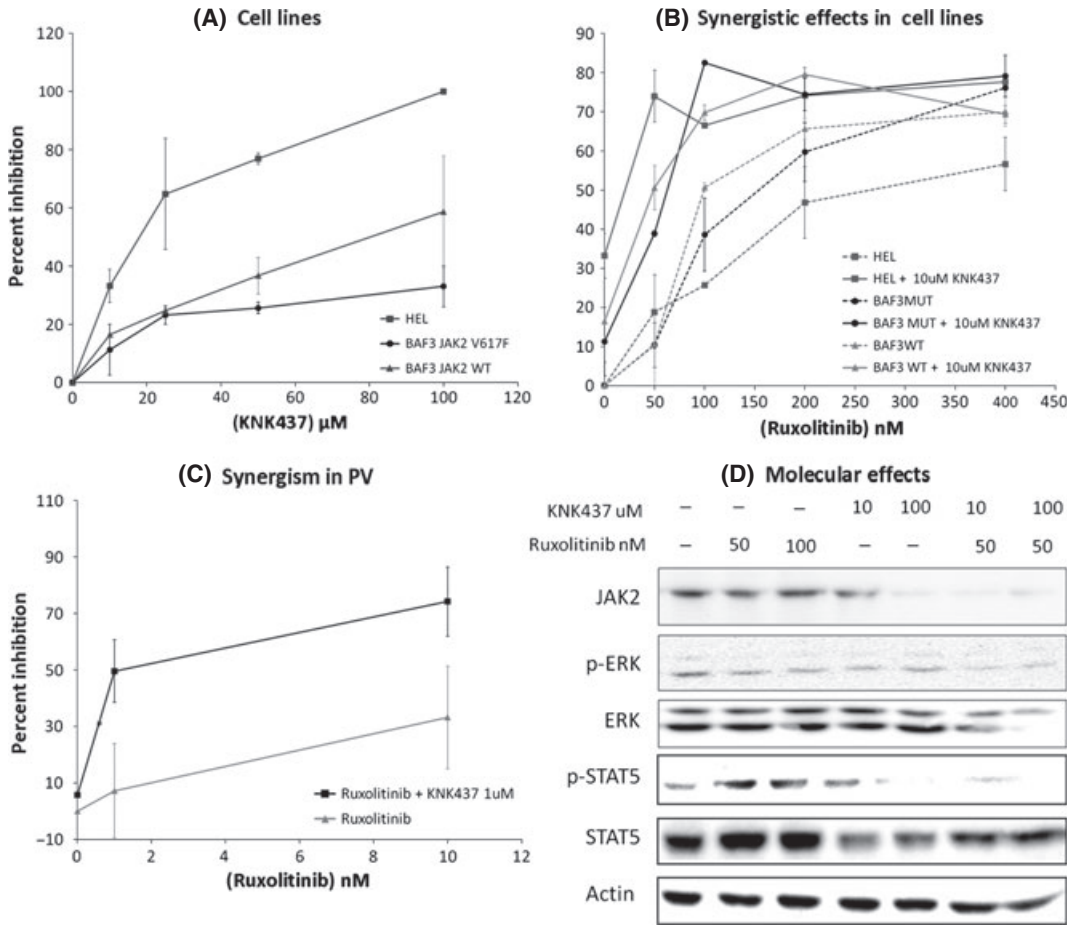

Fig 3. Effects of KNK437 in the different models analysed. (A) Viability test in cell lines. (B) Synergistic effects of ruxolitinib with KNK437 (10 µmol/l). (C) Synergistic effects of ruxolitinib with KNK437 in cell from PV patients (10 nmol/l). (D) Western blots of lysates from BaF3 *JAK2* V617F cells.

A molecular analysis of the effects of dasatinib on the BAF3 *JAK2* V617F cell line by Western blot showed that the inhibitory effect on the activation of SRC appeared to be correlated with decreased activation of STAT5. Moreover, the drug combination virtually eliminated STAT5 activation (Fig 4F).

Discussion

Although JAK inhibition is a new therapeutic avenue in MPN (Eghtedar *et al*, 2012; Mascarenhas & Hoffman, 2012), further approaches are still required as JAK inhibitors have some limitations. They are unable to eradicate the pathological clone and cannot control some symptoms, such as anaemia. A potential novel approach may be a synergist combination with the currently used JAK inhibitors. In this study we explored the synergistic effect of some molecular targeted drugs, based on our previous findings regarding molecules related to the JAK/STAT pathway in PV and ET (Albizua *et al*, 2011).

Consistent with previous reports (Quintas-Cardama *et al*, 2011) we found an inhibition of PV colony growth (IC_{50} 30 nmol/l) and a higher cytotoxicity of ruxolitinib in MPN than in controls. We also characterized ruxolitinib inhibition in ET $^{JAK2\ V617F}$ and ET $^{JAK2\ WT}$ cases. We found that the inhibition of CD34-positive cell proliferation in MPN samples was significantly higher than that in CD34 cells from control cord donors (IC_{50MPN} = 35 nmol/l, $IC_{50CONTROL}$ > 400nmol/l). These results indicate that ruxolitinib was not only very effective at inhibiting the MPN cell proliferation, but also acted selectively against pathological stem cells.

Having characterized the effects of ruxolitinib on our cell lines and cultured cells, we assessed the effects of the other selected drugs, both individually and in combination with ruxolitinib.

The therapeutic index of sorafenib was similar to that previously described for other malignancies (Wilhelm *et al*, 2004; Morgillo *et al*, 2011; Fecteau *et al*, 2012). Its synergistic effect appears to be related to the blockage of STAT5 activation. We observed a decrease in P38 protein activation by Western blot analysis of *JAK2* V617F cell lines treated with sorafenib. This decrease was confirmed in MPN patients by CBA analyses. Furthermore, we observed a marked decrease in the CD71 erythroid lineage, but there was no effect on,

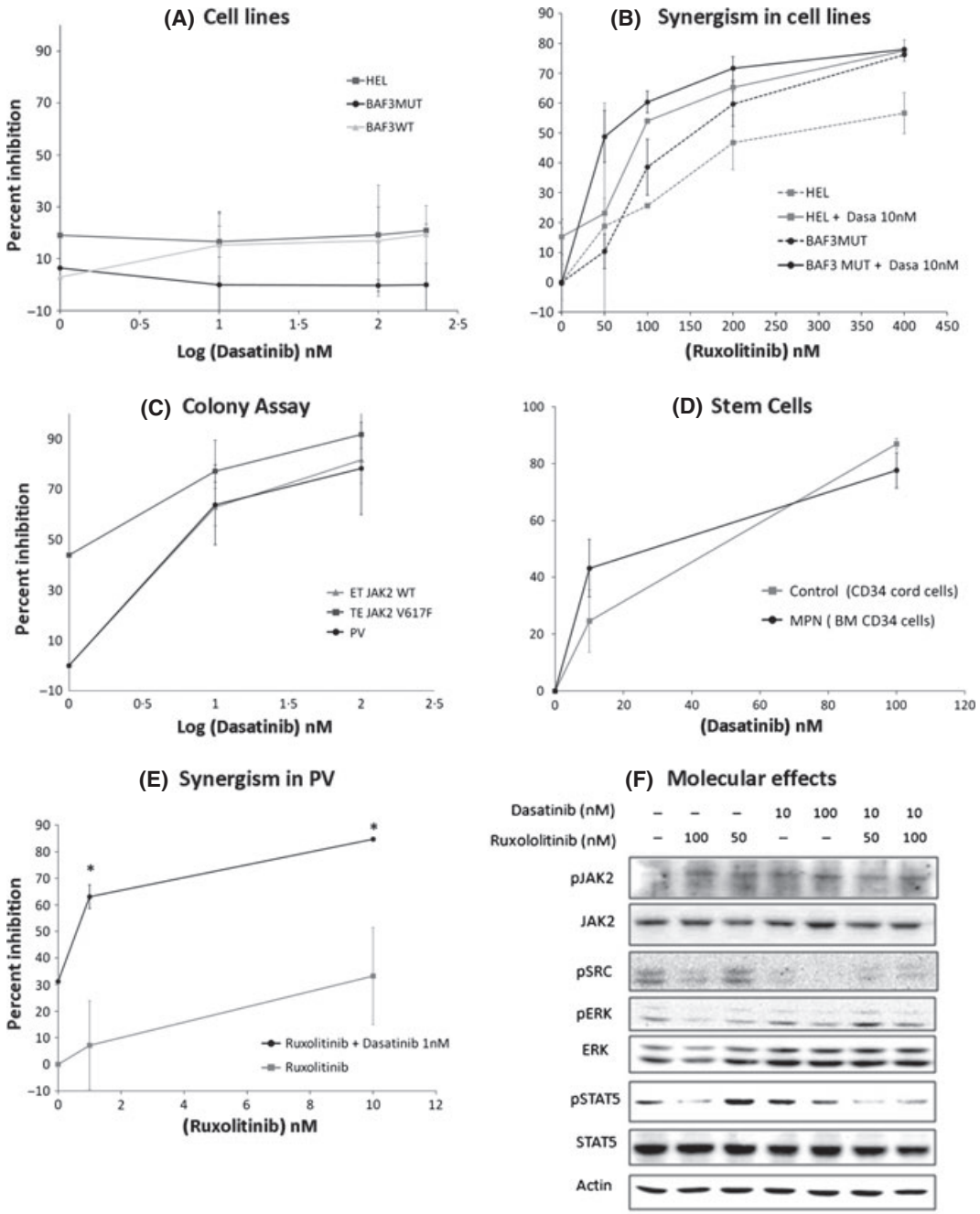

Fig 4. Effects of Dasatinib in the different models analysed. (A) Dasatinib did not affect the cell lines (BAF3 MUT: BA/F3 *JAK2* V617F EPOR. BAF3 WT: BA/F3 *JAK2* WT EPOR). (B) Synergistic effects of ruxolitinib with dasatinib (10 nmol/l). (C) Viability test of cells from colonies assays. (D) Viability test of CD34 cells from colonies assays. (E) Synergistic effects of ruxolitinib with dasatinib in cell from PV patients (10 nmol/l). (F) Western blots of lysates from BaF3 *JAK2* V617F cells. $*P \leq 0{\cdot}05$.

and in some cases we even observed an increase in, the CD41 megakaryocytic and CD45 pan-leucocyte lineages. Desterke *et al* (2011)reported that P38 activation was associated with increased apoptosis of megakaryocytes. Together these findings suggest that sorafenib has a dual effect on haematopoietic cells. On the one hand it reduces the proliferation of erythroid cells by inactivation of the classical MAPK pathway. On the other hand, at high concentrations it promotes the survival of non-erythroid cells by blocking apoptosis via P38.

The heat shock protein HSP70 is a chaperone molecule responsible for ensuring the correct folding of a large number of targets in response to stress signals (Song *et al*, 2001). Its inhibition increases polyubiquitination and proteasome degradation (Yang *et al*, 2008). HSP family members are associated with numerous proliferative pathways involved in

myeloid proliferation, such as JAK/STAT, AKT and MAPK (Madamanchi *et al*, 2001; Dame *et al*, 2002; Lurie *et al*, 2008).

The HSP70 inhibitor KNK437 acted synergistically with ruxolitinib in both the *JAK2* V617F cell lines tested and in culture colonies of PV. As shown in Table II, these effects were more evident in patient cells, reaching an IC_{50} less than 0·1 (very strong synergistic effect). A molecular analysis of KNK437 indicated that it affects the stability of JAK2, as its inhibition decreased the expression of this protein. The inhibition of another heat shock protein, HSP90, has been shown to act synergistically with JAK2 inhibitors, in the same way HSP70 inhibition acts synergistically with increasing JAK2 degradation and increased STAT5 inhibition. These data suggest that HSPs may be key in the pathogenesis of MPN, and with the inhibition of JAK2 expression, they may be excellent therapeutic targets.

We also showed that dasatinib, although not effective in inhibiting cell line proliferation (consistent with previous observations in the HEL cell line (Snead *et al*, 2009)), presented a significant inhibitory effect in the MPN mononuclear cell cultures. A preliminary study showed a similar degree of inhibition in patients with PV (Wappl *et al*, 2008). This effect observed in PV is similar to that presented by the ET $^{JAK2\ V617F}$ cases here, but the ET $^{JAK2\ WT}$ cases were up to 10 times more sensitive to this compound. This may indicate that the SRC protein family members are important in the pathogenesis of MPN, but their effects are masked by the presence of *JAK2* mutations.

Okutani *et al* (2001) demonstrated that SRC is involved in signal transduction mechanisms mediated by erythropoietin, linking this protein with the activation of STAT5. Fig 4E shows that, at least in the BA/F3 *JAK2* V617F cell line, inhibition with dasatinib completely eliminated the activation of SRC and in turn decreased the activation of STAT5, at concentrations close to 100 μmol/l. Ruxolitinib, for its part at 100 nmol/l, also removed the activation of STAT5. This effect was not observed at concentrations less than 50 nmol/l. These results suggest that the molecular phenomenon that determines the synergy between these two drugs is the inhibition of the common effector, STAT5. In fact, all the pathways inhibited by the compounds studied here converge on the effector protein STAT5. Moreover, all the combinations of ruxolitinib with the different drugs used resulted in a critical decrease of STAT5 activation. These results suggest that the synergistic effects observed with the different compounds are, at the molecular level, due to a stronger inhibition of STAT5.

Furthermore, synergistic effects have been found between different JAK2 inhibitors and histone deacetylase inhibitors, such as panabinostat (Wang *et al*, 2009) or AUY922 (Fiskus *et al*, 2011) and other HSP90 inhibitors (Weigert *et al*, 2012), in a similar manner to that described here. Thus, this synergistic effect may present a recurrent mechanism that may help to reduce the ruxolitinib dose and consequently minimize its side effects, which cause intolerance in 5–10% of cases in clinical trials, and molecular resistance in 20%. We believe that the results presented here justify the design of clinical trials to study the benefits of ruxolitinib in combination with these different molecular targeted drugs.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Ruxolitinib. Supplementary data.
Fig S2. Sorafenib. Supplementary data.
Fig S3. Perifosine. Supplementary data.
Fig S4. Dasatinib. Supplementary data.

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