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SERENA MARTINELLI

Study of intracellular
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in Chronic
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Chapter 1

Introduction

1.1 Background

Chronic myeloproliferative neoplasms (MPN) are a group of blood malignancies that originate from the transformation of a pluripotent stem cell, and are characterized by clonal proliferation of one or more hematopoietic progenitors in the bone marrow and extramedullary sites. The biological definition of such diseases is historically represented by the absence of the Philadelphia chromosome (crPh-), and the fusion gene BCR / ABL known by the t (9; 22), which characterizes chronic myeloid leukemia (CML) (crPh +) (Barosi G et al. 2008; Vannucchi AM et al., 2008).

The three main myeloproliferative disorders (crPh-) are the Polycythemia Vera, Essential Thrombocythemia and Primary Myelofibrosis. However, the World Health Organization (WHO) includes among the myeloproliferative neoplasms also less frequent diseases: systemic mastocytosis, chronic eosinophilic leukemia, chronic neutrophilic leukemia, and chronic myelomonocytic leukemia. The annual incidence of MPN is 1 to 3 cases per 100,000 / inhabitants for PV and ET, while the IMF is more rare.

Primary Myelofibrosis (PMF) is a chronic and malignant neoplasm, characterized by splenomegaly, presence of circulating hematopoietic progenitors and anisopoichilocytosis with dacryocytes, a different stages of marrow fibrosis and extramedullary hematopoiesis. It is a rare disease, with an incidence of 0.5-1.5 cases per 100,000 inhabitants per year and affects both sexes equally; the average age at diagnosis is 60 years. Approximately 20% of patients are completely asymptomatic at diagnosis, but when symptoms are present they are related to the stages of anemia or splenomegaly (Cervantes F et al., 1997). The terms primary and idiopathic mean that in these cases the disease is of unknown or spontaneous origin. This is in contrast with myelofibrosis that develops secondary to polycythemia vera or essential thrombocythaemia (secondary myelofibrosis).

The reticulinic fibrosis is an exaggeration of normal pattern of fibers in the medulla, and is comprised of collagen type I and III and fibronectin. The fibrous collagen, however, is responsible for the disruption of the bone marrow architecture and is present in both primary and secondary myelofibrosis. In Idiopathic Myelofibrosis has been observed a steady increase in the deposition of collagen, especially in patients with long-term illness. The increase of these proteins is observed also in the serum, especially of patients with active disease. The marrow fibrosis is a potentially reversi-

ble event, as demonstrated by its regression after allogeneic bone marrow transplantation or after prolonged administration of chemotherapy or interferon. (Mc Carthy DM, 1985). PMF is the traditionally more problematic of the MPN because of the risk of leukemic transformation (ie, MPN blast phase), and decreasing in life expectancy (Gangat N et al., 2011).

The Essential Thrombocythemia (ET) is a chronic myeloproliferative disorder characterized by abnormal proliferation of megakaryocytes that induce an increasing number of circulating platelets. The true incidence is unknown. It affects mainly individuals with a mean age at diagnosis of 50-60 years and does not like any sex. The platelets survival is normal, the thrombocythemia is due to an increase in platelets production in the bone marrow associated with an increase in the mean megakaryocytic volume, in the number of nuclear lobuli and in the nuclear polyploidy (Buss DH et al., 1985). The main clinical manifestations of ET are thrombotic and hemorrhagic complications that still remain the leading cause of morbidity and mortality in these patients; rarely there are evolving forms in myelofibrosis post-ET and transformation into acute leukemia. ET shows a typical bone marrow biopsy that shows mature megakaryocytes distributed in clusters with no dysplasia (typical of myelofibrosis).

Polycythemia Vera (PV) is one of the most common chronic myeloproliferative disease characterized by clonal expansion of the erythroid lineage, variably associated with thrombocytosis and leukocytosis. It has a rate of about 2 cases / 100,000 per year; there was a slight predominance in males. The average age at diagnosis is 60 years. It is also documented that the 5% of patients with PV has less than 40 years, and only 0.1% have less than 20 (Berlin NI, 1975). PV is characterized by bone marrow hyperplasia with hyperproliferation of erythroid lineage also in absence of erythropoietin (EPO). This aspect is demonstrated in vitro with the spontaneous growth of erithroid endogenous colonies (EEC) without the addition of exogenous EPO (Prchal JF et al., 1974). The main clinical manifestations of PV are consequences of excessive proliferation of red cells. Erythrocytosis, creating an increase in blood viscosity, causes alteration of the blood circulation. Characteristic is the generalized pruritus, reported in half of the cases, frequently caused by water. The main complications are thrombotic events that may affect any district, also uncommon locations as the large abdominal vessels. In PV patients the hematocrit is often high, not just because of the expansion of red cell mass, but also because of the increase in erythrocyte number. Leukocytosis and thrombocytosis are present in more than 50% of cases. During evolution, the disease changes, and patients may evolve in myelofibrosis and more rarely in acute myeloid leukemia. The most frequent cause of mortality in patients with PV is the cardiovascular event (41% of deaths).

The first clinician to provide a definition of the Polycythemia Vera was Vaquez (Vaquez H et al., 1892) in 1892 when he described it as a condition of "persistent and excessive hypercellularity accompanied by cyanosis." While was Epstein (Epstein E et al., 1934) in 1930 who described thrombocythemia.

Nevertheless, the protagonist is Dameshek (Dameshek W, 1951) who in 1951 grouped under the term of myeloproliferative neoplasms four disorders pathophysiologically and clinically correlated: the Polycythemia Vera (PV), the Primary Myelofibrosis (PMF), the Essential Thrombocythemia (ET) and Chronic Myelogenous Leukemia (CML) just to emphasize the spectrum of clinical conditions interconnected

between different disease entities. The '70s were fertile for scientific discoveries regarding MPNs: in fact it was found the spontaneous growth in vitro of endogenous erythroid colonies (EEC) by plating the bone marrow of polycythaemic patients in the absence of growth factors (Zanjani ED et al., 1977). Through studies based on X chromosome inactivation in blood of women with polycythemia and thrombocythemia, was documented the clonal nature of these pathologies (Fialkow PJ et al., 1981). These findings suggested that the Myeloproliferative crPh- could originate from the clonal proliferation of a hematopoietic stem cell.

In the following years a number of studies showed that the Myeloproliferative Diseases and other related blood disorders derived from the proliferation of the pluripotent stem cell and that clinical and phenotypic diversity is linked to different mutations in genes coding for proteins with tyrosine-kinase function (Kozbor D et al., 1986). In this regard the discovery of the Philadelphia crPh translocation and BCR-ABL1 as molecular marker of Chronic Myeloid Leukemia has certainly represented a turning point (Bartram CR et al., 1983).

In 2001 the World Health Organization formed the Myeloproliferative Diseases part of Chronic Myeloproliferative Diseases with also Chronic Neutrophilic Leukemia, Chronic Eosinophilic Leukemia / Hypereosinophilic Syndrome and Chronic Myeloproliferative Diseases unclassifiable (Jaffe ES et al., 2001). The criteria proposed by the WHO was based on the integration of clinical, laboratory and histopathological data for diagnosis and for the first time was given relief to the bone marrow biopsy as necessary for a correct diagnosis of ET and PMF and a complementary tool in the diagnosis of PV.

However, the differential diagnosis between MPN Ph-negative was often difficult due to the lack of specific cytogenetic abnormalities and the frequent overlap of the clinical features between the different entities with both myelodysplastic syndromes / myeloproliferative diseases (eg. The atypical chronic myeloid leukemia and leukemia chronic myelomonocytic) and so-called reactive forms. The WHO criteria in 2001 were valid until have been described genetic mutations which were able to induce phenotypes similar to those of MPN in experimental animal models (Levine RL et al., 2005; James C et al., 2005). These recurrent anomalies strengthened the original vision of Dameshek, according to which these hematologic disorders are supported by a common pathogenic mechanism (Dameshek W, 1951).

In the light of this recent findings, in 2008 the WHO has revised the classification of hematological pathologies (Swerdlow SH et al., 2008) (Table 1). The Chronic Myeloproliferative Diseases were renamed in Myeloproliferative Neoplasms because of the clonal nature of the disorders and the common trend to evolve in Acute Myeloid Leukemia (Vardiman JW et al., 2009). The review made by the new WHO classification has also been influenced by two main factors: 1) the identification of specific genetic abnormalities used as diagnostic markers for the MPNs Ph-negative, with particular reference to the JAK2V617F mutation, and 2) the acquisition of new histopathological informations. This factor lead to define eight types of MPNs (Polycythemia Vera, Essential Thrombocythemia, Primary Myelofibrosis, Chronic Myeloid Leukemia, Chronic Eosinophilic Leukemia, Chronic Neutrophilic Leukemia, Systemic Mastocytosis, and Chronic Myelomonocytic Leukemia, Myeloproliferative Neoplasms unclassifiable).

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Among all the diseases included under WHO 2008 classification, classical MPN Ph- (PV, PMF and ET) are the most interesting entities from a scientific point of view because of the numerous new discoveries regarding molecular genetics. A further revision of this classification is expected in the future due to the newly discovered calreticulin (CALR) mutations (Tefferi A et al., 2014).

All the MPN are clonal disorders that originate at the level of a pluripotent hematopoietic stem/progenitor cell that, while retaining a relatively normal differentiation program unlike that in acute leukemia, has lost some of the regulatory mechanisms that control proliferation; indeed, one of the best characterized abnormalities of hematopoietic cells in these neoplasms is their substantial independence from regulatory cytokines, in particular erythropoietin (EPO) (Prchal JF et al., 1974) or thrombopoietin (TPO) (Taksin AL et al., 1999), the two main cytokines for the erythroid and megakaryocytic (MK) lineage, respectively.

Later on were found evidences about the possible molecular mechanisms of Myeloproliferative Disorders, including the association between various chronic myeloid neoplasms and constitutive activation of specific proteins with tyrosine kinase function (Campbell PJ et al., 2006). The frequent presence of chromosomal abnormalities in the short arm of chromosome 9 (Kralovics R et al., 2002) and the evidence of the increased activity of some signal transduction pathways (Röder S et al., 2001, Ugo V et al., 2004) pointed the scientific research towards specific molecular targets. In April 2005 five independent research groups identified a single point mutation in exon 14 of the somatic gene Janus Kinase 2 (JAK2), present in most patients with MPNs Ph-negative (Levine RL et al., 2005; James C et al., 2005; Baxter EJ et al., 2005; Kralovics R et al., 2005; Zhao R et al., 2005). The JAK2V617F mutation consists of the substitution of a thymine with a guanine at nucleotide 1849 and results in the substitution of a valine residue with a phenylalanine residue in position 617 of the protein JAK2. This residue is located at the pseudokinase domain of the protein, which regulates negatively the kinase domain of JAK2. The JAK proteins are a family members of the Janus kinase and act as intermediary between membrane receptors and intracellular messengers. When a particular cytokine or a growth factor binds to the specific receptor on the cell surface, the JAK proteins associated with the cytoplasmic region of the receptor is phosphorylate and facilitate the phosphorylation of intracellular second messengers, in particular those which belong to the family of STAT (Figure 1).

Once activated STAT dimerization, it enters the nucleus and acts as a transcription factor. JAK2 appears to be activated in particular when the receptors bind hematopoietic growth factors such as EPO, IL3, IL5, G-CSF, GM-CSF and TPO. Indeed JAK2 is functionally associated with homodimeric receptors for growth factors type I, that have no intrinsic kinase activity. In addition to its role in signaling, JAK2 functions as transport molecule and contributes to the stabilization of MPL expressed in the membrane (this last feature may account for abnormalities of MPL expression) (Baker SJ et al., 2007). Each JAK2 protein is composed of 7 homologous domains; the C-terminal domain has kinase activity (JH1), the pseudokinase domain catalytically inactive (JH2) plays important regulatory functions. The V617F mutation enhances the kinase activity of JH1, presumably by preventing the inhibition exerted by JH2 on JH1. The SH2 domain consists of the regions JH3 and JH4 with binding capacity

specific for the phosphorylation. Finally the domain band 4.1, consists from the regions JH4-JH7 and it has activities of protein-protein interaction (Figure 2). The presence of two similar domains JH1 and JH2 in protein, one active and the other inactive, recalls the Roman god Janus who had the ability to look in two opposite directions.

Biochemical studies have clarified that the JAK2V617F mutation causes the activation cytokines-independent of the downstream signaling pathway mediated by a variety of second messengers including regulatory proteins of the STAT family (Signal Transducers and Activators of Transcription) (Levine RL et al., 2005). The JAK2V617F mutation is present in 95% of patients with PV, in 50-60% of patients with ET and in 40-50% of patients with PMF (Tefferi A et al., 2010; Vainchenker W et al., 2011). It was rarely identified in other myeloid neoplasms but is absent in all non-myeloid malignancies, this is why JAK2V617F it is the first genetic marker directly related to the pathogenesis of myeloproliferative disorders and in particular to MPNs Ph-negative.

Approximately 25-30% of patients with PV or PMF, and only 2-4% of patients with ET, have the V617F mutation of JAK2 in a state of homozygosity. This condition is characterized by a JAK2 mutated allele higher than 51% in granulocytes. By FISH and PCR Taqman was observed that the presence of homozygosity is secondary to a mechanism of mitotic recombination at the level of chromosome 9p with duplication of the mutated allele, and not to an allelic deletion.

Kralovics reported a significant association between the presence of homozygosity and a longer duration of disease in different forms of MPN; this observation was confirmed by others (Levine RL et al., 2005) raising the possibility that the homozygous status is an evolutionary event that occurs over time. Kralovics (Kralovics R et al., 2005) has therefore proposed a two-step model for the role of JAK2 in the evolution of MPN.

1st step: the somatic mutation is acquired in hematopoietic progenitor or stem cell. This subsequently leads to the expansion of an heterozygous clone that replaces the normal hematopoiesis.

2nd step: the mitotic recombination in heterozygous stem cell from which originates a cell homozygous. This expands and having a proliferative advantage can replace heterozygous hematopoiesis.

The same mutation has also been observed less frequently in myelodysplastic syndromes, in systemic mastocytosis, in neutrophilic leukemia, chronic ipereosinophilic syndromes and other atypical myeloproliferative disorders (Jelinek J et al., 2005; Steensma DP et al., 2005; Jones AV et al., 2005), particularly in refractory anemia with ringed sideroblasts and thrombocytosis. Instead it is uncommon in chronic myelomonocytic leukemia and acute leukemia, and is absent in the forms of acute and chronic lymphoproliferative disorders (Levine RL et al., 2005). In familial forms of MPN, for patients affected by PV or ET, the presence of the mutation in homozygous form is associated with an increased risk of complications such as more frequent evolution in AML and PMF (Bellane-Chantelot C et al., 2006).

In 2006, the Gary Gilliland research team identified in some patients with PMF-JAK2 wild type a new somatic point mutation in the gene Myeloproliferative Leukemia Virus Oncogene (MPL), coding for the thrombopoietin receptor (Pikman Y et al.,

2006). The MPLW515L mutation consists of the substitution of a guanine with a thymine at position 1544 resulting in replacement of a tryptophan residue with a residue of leucine at codon 515 in the juxtamembrana region, essential for the maintenance of the receptor in an inactive state in the absence of ligand. Subsequently, other point mutations were described always at the level of tryptophan in position 515 (MPLW515K, MPLW515R, MPLW515A) (Pardanani AD et al., 2006). The most common mutations are the MPLW515L and MPLW515K, with an overall incidence estimated at around 3 to 4% in ET and 8-10% in PMF, but quite exceptional in PV (Tefferi A, 2010; Vainchenker W et al., 2011).

In 2007 were described other mutations in exon 12 of the JAK2 gene in patients with PV JAK2V617F-wild type (Scott LM et al., 2007), and their frequency has been estimated at around 3% in PV, but are absent in ET and PMF (Tefferi A, 2010). The expression of MPLW515L / K gives to the cell cytokines independent growth and hypersensitivity to thrombopoietin (Figure 3). This is due to the constitutive phosphorylation of JAK2, STAT3, STAT5, AKT, ERK. The functional consequences of the MPL mutation are very similar to those induced by JAK2V617F.

Despite major advances in understanding the molecular mechanisms of the MPNs Ph-negative, for several years the 30-40% of cases of ET and PMF was orphaned of a genetic marker of the disease. The discovery of MPL mutations in 2006 (Pikman Y et al., 2006) only partially addressed the problem because of its low mutational frequency, estimated at 10–20% in JAK2-unmutated ET or PMF (Rotunno G et al., 2013; Tefferi A et al., 2014; Pardanani AD et al., 2006). In December 2013, two groups reported the occurrence of novel calreticulin (CALR) mutations in JAK2/MPL - unmutated PMF or ET (Nangalia J et al., 2013; Klampfl T et al., 2013). Both groups found mutual exclusivity between CALR, JAK2 and MPL mutations. CALR is a multi-functional Ca^{2+} binding protein chaperone mostly localized in the endoplasmic reticulum (ER). Located on chromosome 19p13.2, CALR contains nine exons and its protein three domains: N-domain (residues 1–180), P-domain (residues 181–290) and C-domain (residues 291–400). Knocking out CALR in mice is lethal and causes impaired cardiac development (Mesaeli N et al., 1999). In the study by Klampfl et al., 15 CALR mutations were not seen in 382 cases of PV but were detected in 25% of patients with ET (n=311) and 35% of those with PMF (n=203). The authors subsequently enriched their JAK2/MPL - unmutated patient population by adding 211 cases with ET or PMF and reported CALR mutational frequencies of 67% and 88% in JAK2/MPL unmutated ET (n=289) and PMF (n=120), respectively. In regards to clinical and laboratory correlative studies, CALR mutations, compared with JAK2, were associated with lower hemoglobin level, lower leukocyte count, higher platelet count, lower risk of thrombosis and better survival in ET and in PMF. In the same period Nangalia et al. conducted an exome sequencing on 258 patients with MPNs. CALR mutational frequencies were 71%, 56% and 86% in JAK2/MPL-unmutated ET (n=112), PMF (n=32) or post-ET MF (n=14), respectively. Similar to the observations by Klampfl et al., Nangalia et al. also found an association between CALR mutations and higher platelet count and lower hemoglobin level in ET; in addition, their study suggested an increased incidence of fibrotic transformation in CALR-mutated ET without apparent survival difference. All CALR mutations seen in the above two stud-

ies were exon 9 frame-shift mutations with somatic insertions or deletions. Two variants constituted more than 80% of the CALR mutations seen: type 1 variant resulted from 52 bp deletion and was more frequent in PMF, and type 2 variant resulted from 5 bp TTGTC insertion. Only three cases of homozygous CALR mutations were reported. All CALR mutations resulted in one base pair reading frame shift and an altered C-terminal that is missing the KDEL (lysine, aspartic acid, glutamic acid and leucine) endoplasmic reticulum retention motif and is positively rather than negatively charged. Recently were reported two cases of ET and PMF with the coexistence of mutations in JAK2 and CALR. However, the actual frequency, the pathogenetic and clinical significance of the double mutation has not yet been clarified (McGaffin G et al., 2014).

So the PV is almost invariably associated with mutations of JAK2 (JAK2V617F or mutations of exon 12), but cases of ET and PMF without a specific molecular marker amount to about 10% and for this reason are defined "triple negative" (Tefferi A et al., 2014). These studies suggest that the molecular mutational profiles of MPN subtypes, in determining prognosis, and also in making therapeutic decisions. Figure 4 shows the distribution of mutations of JAK2, MPL and CALR in MPNs classic Ph-negative.

There are significant differences in phenotype among the JAK2, CALR, and MPL mutational categories; JAK2 mutations are generally associated with older age, higher hemoglobin level, leukocytosis, lower platelet count, and increased risk of thrombosis; a higher JAK2 mutant allele burden with pruritus and fibrotic transformation in PV; mutant CALR in ET with younger age, male sex, higher platelet count, lower hemoglobin level, lower leukocyte count, and lower incidence of thrombotic events; and mutant CALR in PMF with younger age, higher platelet count, and lower frequencies of anemia, leukocytosis, and spliceosome mutations (Tefferi A et al., 2014). Furthermore, more than 80% of patients with mutant CALR harbor 1 of 2 mutation variants: in ET, type 2 CALR mutation was associated with significantly higher platelet count (Tefferi A, Guglielmelli P et al., 2014) and, in PMF, with higher Dynamic International Prognostic Scoring System (DIPSS)-plus score, circulating blast percentage, and leukocyte count and inferior survival (Verstovsek S et al., 2010). Triple-negative PMF patients appear to have a less favorable prognosis than patients with mutations in JAK2, CALR, or MPL (Tefferi A, Lasho TL, 2014), whereas patients with CALR mutations tend to have a better prognosis than patients with JAK2 or MPL mutations (Klampfl T, 2013; Rumi E, 2014). Non-type 1 or type 2 CALR mutations are operationally classified into "type 1-like" and "type 2-like" variants on the basis of their structural similarities to type 1 and type 2 CALR variants, respectively, which is in turn based on α -helix content of the mutant C-terminus (Vannucchi A et al., 2014).

In addition to the driver mutations explained, numerous studies have identified other somatic mutations in 25-30% of patients with MPNs. The identified genes encode for factors involved in epigenetic regulation (EZH2, ASXL1, TET2, DNMT3A, IDH1, IDH2), the spliceosome complex (SF3B1, SRSF2, U2AF1) and rarely interfering with the JAK- STAT signaling pathway (SH2B3 / LNK) (Vainchenker W et al., 2011). Mutations in TP53, TET2, SH2B3, and IDH1 are more frequently observed in

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leukemic blasts from transformed MPN patients, suggesting a role for these gene mutations in leukemic transformation (Abdel-Wahab O, 2010). In addition, novel recurrent mutations occurring at low frequencies have been also found in CHEK2, SCRIB, MIR662, BARD1, TCF12, FAT4, DAP3, and POLG (Tenedini E et al., 2013). The common feature of these gene mutations is that they alone do not cause a MPN phenotype. They are typically also found in other hematologic malignancies, such as myelodysplastic syndrome (MDS) and acute leukemias. We therefore propose to call them “non phenotypic driver mutations” or perhaps “important passenger mutations”. Many of these mutations are sequentially acquired in cells that already carry a mutation in one of the phenotypic driver genes, most frequently JAK2V617F, and are thought to be involved in disease progression (Vainchenker W et al., 2011). Surprisingly, the same genes in some patients can be mutated before JAK2V617F, and in other patients, after JAK2V617F (Schaub FX et al., 2010). Thus, the order of events is not uniquely linked to the gene function. Two recent studies examined the broad mutational landscape of patients with PV, ET, and PMF using exome sequencing or capture-based next-generation sequencing of a set of 104 genes (Figure 5) (Lundberg P et al., 2014). Both studies found that after JAK2V617F and CALR, the most frequent somatic mutations in MPN occur in genes involved in epigenetic regulation (TET2, DNMT3A, ASXL1, and EZH2). Mutations in other genes were found at lower frequencies, many of them in single individual patients, which will make it difficult to assess their functional and prognostic relevance. Poor survival and increased risk of leukemic transformation correlated with the increasing number of somatic mutations in individual patients (Guglielmelli P et al., 2014). Among the rather rare mutation events, the TP53 mutations stand out, because, when present, they are associated with poor prognosis and high risk of progression to acute myeloid leukemia (AML) (Harutyunyan A et al., 2011). Overall, only about 10% of MPN patients are in the category of “triple negative,” in which the driver mutation is still unknown.

Ortmann and colleagues (Ortmann CA, 2015) provide a comprehensive investigation of the biologic consequences of the order of driver events in relation to clinically relevant outcome measures in MPNs. The authors investigate the combination and order of the driver mutations JAK2 and TET2 in myeloproliferative disorders with JAK2V617F mutations (Polycythemia Vera, Essential Thrombocythemia, and Myelofibrosis), and they provide compelling evidence that mutation order influences the biology and outcome of the disease. TET2 presents a 2-oxoglutarate/Fe²⁺ oxygenase activity that converts 5 methylcytosine (5mC) in 5-hydroxymethylcytosine (5-hmC) and participates in active DNA demethylation (Tahiliani M et al., 2009). TET2 mutations are the most prevalent epigenetic regulators found in MPN (15%). More specifically, the TET2 mutation was found in approximately 4% of ET, 10% to 16 % of PV, and 8% to 15% of PMF (Delhommeau F et al., 2009). They are loss of function mutations, more frequently on one allele, but mutations on a second allele can be observed during disease progression (Saint-Martin C et al., 2009). TET2 plays a role in clonal dominance because in human primary cells and in mouse models its deficiency leads to the amplification of hematopoietic stem cells (HSC) and hematopoietic progenitors (Moran-Crusio K et al., 2011; Qivoron C et al., 2011). Mutations in TET2 and a clonal hematopoiesis have been identified in aged people presenting no sign of hemopathy (Busque L. et al., 2012). The authors (Ortmann CA, 2015) found that when

a TET2 mutation is acquired first, expansion of hematopoietic stem and progenitor cells is favored, but expansion of lineage-committed erythroid progenitor cells is blocked until a JAK2 mutation is acquired (Swanton C, 2015). Conversely, in patients in whom a JAK2 mutation occurs first, there is an increase in the number of megakaryocyte and erythroid cells but no expansion of hematopoietic stem and progenitor cells until a TET2 mutation occurs. In cells with a prior mutation in TET2, upregulation of genes involved in DNA replication and mitosis, which is normally stimulated by the JAK2V617F mutation alone, is prevented. This explains why single hematopoietic stem and progenitor cells with a JAK2 mutation in TET2- non mutated cells have increased proliferation relative to cells in which JAK2 is mutated as second event and TET2 mutation occurs first (Swanton C, 2015). The influence of mutation order is mirrored by the effect on clinical outcomes. Patients in whom the JAK2 mutation occurs first, as compared with those in whom the TET2 mutation occurs first, are younger at the onset of disease, are more likely to present with PV than ET, and are more likely to have thrombosis.

In addition to JAK/STAT signaling pathway, constitutive activation of other pathways through the phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK) has been documented in MPN (Kubota Y et al., 2004). Activation and autophosphorylation of Tyrosine Kinase Receptors (RTK) by the intracytoplasmatic tyrosine portions facilitate their interaction with p85 (PI3K regulatory subunit). PI3K transfers the γ -phosphate from ATP to phosphatidylinositol 4,5-bisphosphate (PIP2) generating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and ADP. RTK can activate PI3K also through RAS protein, that bounds and activate p110 subunit (PI3K catalytic subunit). PI3K is negatively regulated by phosphatases like PTEN; reduced expression of PTEN contributes to oncogenesis activating PI3K. PIP3 recruits the protein kinase B/Akt through a link of the plekstrin homology domain (PH) next to the plasmatic membrane where Akt is phosphorylated and activated by the kinases PIP3-dependent 1 and 2 (PDK1- PDK2). Once activated, Akt phosphorylates several substrates involved in the regulation of many cellular processes including cell survival, proliferation, differentiation, apoptosis inhibition, autophagy as well as angiogenesis. All of these processes are being considered as crucial features for the establishment and the maintenance of the tumorigenic phenotype (Guertin DA et al., 2007). Although Akt resulted constitutively activated in JAK2V617F mutated cells in vitro (James C et al., 2005; Bumm TG et al., 2006) and in V617F transgenic (Shide K et al., 2008) or knock-in mice (Akada H et al., 2010). Akt is activated via PI3K in response to ligand engagement of the erythropoietin (EPO) receptor and has a role in normal erythroid differentiation. Akt support erythroid differentiation in JAK2-deficient fetal liver progenitor cells through a mechanism downstream of EPO receptor (Ghaffari S et al., 2006).

The main downstream target of Akt is the mTOR complex. It can integrate signals coming from outside and inside the cell, like signals correlated to the cellular energetic status, or to the presence of nutritional substances and growth factors. Akt can directly phosphorylate and activate mTOR (mammalian Target of Rapamycin). The beginning of proteins transcription is regulated by phosphorylation of 4EBP1 protein: when 4EBP1 is not phosphorylated it binds eIF4E leading to the inhibition of transcription. After proliferating stimuli, 4EBP1 is phosphorylated by mTOR and other kinases: this

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induces the release of eIF4E that can form the transcription complex (Mendez R et al., 1996; Rousseau D et al., 1996). mTOR is the direct responsible of the 4EBP1 phosphorylation: it has been demonstrated that mTOR inhibition blocks 4EBP1 phosphorylation (Berretta L et al., 1996; Dilling BD, 2002). mTOR proteins have pleiotropic functions and they are involved in mRNA transcription regulation, protein translation and degradation and ribosomal biogenesis. mTOR exists in two complexes, mTORC1 and mTORC2. mTORC1 is formed by mTOR and RAPTOR, is sensible to Rapamycin and phosphorylates S6 kinase and 4EBP1, TORC1 is mainly involved in protein translation regulation and is found constitutively hyperactivated in a wide range of cancers and hematologic disorders (Foster JG et al., 2012); mTORC2 is formed by mTOR and RICTOR: it affects cytoskeleton changes and phosphorylates Akt leading to a positive feedback (Figure 6). The most recent evidence indicates a role also of mTORC2 in cancer development: many gliomas overexpress the mTORC2 subunit RICTOR, with a consequent enhanced mTORC2 activity that confers to cells increased proliferation and invasion potential (Hietakangas V et al., 2009). Prostate cancer development induced by the loss of PTEN in mice, requires mTORC2 function (Guertin DA et al., 2009). The downstream effector p70S6K plays a critical role in cell growth; the p70S6K gene is found amplified in approximately 9% of primary breast cancer and elevated levels of its mRNAs are found in approximately 41% of tumors (Lluis F et al., 2009; Wu GJ et al., 2010). Several lines of evidence support the involvement of the PI3K/Akt/mTOR pathway in hematologic disorders: the pathway results as constitutively activated in lymphoma (Lenz G, 2010), myeloma (Peterson TR et al., 2009) and acts as principal mediator of FMS-like tyrosine kinase 3 signaling in acute myeloid leukemia (Brandts CH et al., 2005), and BCR/ABL signaling (Kharas MG et al., 2005). It has also been shown that transplantation of cells with a hyperactivated PI3K/mTOR pathway induces leukemia in mice (Feng Z et al., 2005; Dowling RJ et al., 2010). PI3K signaling is of key importance in normal erythropoietin-induced erythroid differentiation and in spontaneous PV erythroid differentiation (Ugo V et al., 2004); conceivably, several kinases which are part of this pathway such as Akt and mTOR have been reported constitutively phosphorylated in bone marrow (Grimwade LF et al., 2009), in megakaryocytes of MPN patients (Vicari L et al., 2012) and in JAK2 V617F mutated cell lines (James C et al., 2005).

Regarding the treatment, for decades the armamentarium to treat Philadelphia-negative MPNs was reduced to a small handful of cytotoxic drugs like hydroxyurea (HU); busulfan; and, in some countries, pipobroman (Barbui T et al., 2011). Since the discovery of the JAK2V617F mutation 10 years ago followed by the discovery of many other genetic alterations (Vainchenker W et al., 2011), our knowledge of the pathophysiology of these disorders has dramatically changed. With conventional therapies, the treatment of MPNs mainly aims at reducing the risk of vascular events (including thrombosis and hemorrhage), which are the main causes of mortality and morbidity over short and medium time periods (Landolfi R et al., 2004; Harrison CN et al., 2006). However, the outcome of patients with these chronic malignancies is different over the long-term evaluations (i.e., after 15 to 20 years of evolution), when transformation to myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) becomes a major concern, as demonstrated by the long-term analyses of a

randomized study in patients with PV after more than 16 years of median follow-up time (Kiladjian JJ et al., 2008). The development of new therapies raises the hope of new objectives, including reduction of the long-term risk of transformation to MDS or AML; achievement of molecular or histopathologic complete remissions. Recognizing that the life expectancy in ET and PV may not be diminished in select populations, the primary goals of therapy for both PV and ET include prevention of thrombotic and bleeding complications in addition to minimizing the risk of progression to MF or acute leukemia. In contrast, MF treatment goals are based on assessment of both disease burden (symptoms, cytopenias, splenomegaly) and impact of disease on survival. However, to date, only allogeneic hematopoietic stem-cell transplantation (ASCT) can cure selected high-risk patients with MF.

Ruxolitinib (INCB018424), is a JAK2/JAK1 inhibitor and the only one approved by the U.S. Food and Drug Administration in 2011 and the European Commission in 2012, based on the results of 2 pivotal phase III trials, COMFORT (CONtrolled MyeloFibrosis study with ORal JAK inhibitor Treatment) -I and -II. Whatever the JAK2 status, ruxolitinib therapy led to a rapid and sustained reduction of the spleen size (average of 30%), a sustained reduction of myelofibrosis-related symptoms, and an improved quality of life in most patients (Harrison C et al., 2012; Verstovsek S et al., 2012). However, JAK2 is indispensable for hematopoiesis, particularly for red blood cell and platelet production. Therefore, it is not possible to strongly inhibit wild type JAK2 without inducing an anemia or a thrombocytopenia and presently no inhibitor is specific for JAK2V617F. Because of these limits, other attempts have been done either to indirectly target JAK2V617F or other downstream molecules of the pathway to obtain some “synthetic lethality” or to target effectors, which might be important for clonal development. In addition, interferon alpha (IFN α) is one of the rare molecules that targets the JAK2V617F clone, but the precise mechanism remains unknown. Since MPN disorders are the result of constitutive activation of the JAK-STAT signaling pathway, with subsequent propagation of downstream mediators (STAT3/5, PI3K, MAPK), combination treatments targeting multiple levels of the signaling cascade became a rational intervention. A variety of small-molecule novel therapies have been under parallel investigation with JAK2 inhibitors to emphasize synergistic biological activity and to compensate for treatment-related adverse events such as anemia. The PI3K pathway is of particular interest given its pleiotropic effects on cellular proliferation, metabolism, and mediation of cellular drug resistance (Burchert A et al., 2005). Several pan or more specific PI3K inhibitors have been developed and tested in vitro and in clinical trials. Choong et al. have recently shown that pan-class I, but not gamma- or delta-specific PI3K inhibitors, synergized with JAK2 inhibitors. As a PI3K target, mTOR was also targeted by allosteric (RAD001) or ATP competitive (PP242) inhibitors. The investigation led to a good efficacy in cell lines and in MPN primary cells (Bartalucci N. et al., 2013). PI3K inhibitor used in combination with ruxolitinib has demonstrated significant antiproliferative activity, suggesting that inhibition of JAK2 signaling plays an essential role in increasing sensitization of cells to PI3K inhibition (Choong ML et al., 2013). mTOR similarly functions as a regulatory serine/threonine kinase important to cellular metabolism, apoptosis, and proliferation (Loewith R et al., 2002). Use of mTOR inhibitors in singularity within MPN disorders has demonstrated modest results without affecting

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JAK2V617F mutational burden, likely reflecting the existence of alternative regulatory pathways (Guglielmelli P et al., 2011).

1.2 Drugs used in this study

Ruxolitinib: it binds and inhibits JAK1 and JAK2 (and with much lower affinity, JAK3) both the WT and JAK2V617F forms: this should lead to the inhibition of cell signaling mediated by growth factors and proliferation of cancer cells (Quintas-Cardama A et al., 2010).

AZD1480: It is a potent inhibitor of JAK2. Inhibition of JAK2 is associated with the inability of STAT3 to translocate to the nucleus and generate tumor growth. AZD1480 in mice prevents the growth of human solid tumors which show persistent activation of STAT3 (Hedvat M et al., 2009).

Everolimus (RAD001): It is an inhibitor of the serine-threonine kinase mTOR derived from Rapamycin. Is associated with high affinity to the molecule FKBP12, to generate an immunosuppressive complex that binds and inhibits mTOR: thus exerts the inhibition of cell proliferation and growth blocking the transition from the G1 phase to the S phase of cell cycle. The antineoplastic effects of RAD001 involves other several mechanisms, including induction of apoptosis, expression inhibition of genes that control cell adhesion, migration, and angiogenesis. (Yee KW et al., 2006).

PP242: Is a potent inhibitor of mTORC1 and mTORC2 and also shows a minimal activity against a small range of tyrosine, when used at high concentrations (Apsel B et al., 2008). PP242 unlike Rapamycin and its cytostatic derivatives, as RAD001, induces death of human and murine leukemic cells. PP242 in vivo induces the effects of treatment with tyrosine kinase inhibitors more effectively than Rapamycin.

BEZ235: It is a dual inhibitor of both the PI3K class I and of the complexes 1 and 2 of the mTOR kinase. The PI3K signaling pathway is the most frequently mutated and constitutively activated in human cancers. A dual inhibition should be more effective than the single inhibition of mTOR in controlling tumor growth, if the overgrowth is not controlled effectively by inhibition of the mTORC1 only (Maira SM et al., 2008; Serra V et al., 2008).

BKM120: specifically inhibits class I PI3K in the PI3K/AKT kinase (or protein kinase B) signaling pathway in an ATP-competitive manner, thereby inhibiting the production of the secondary messenger phosphatidylinositol-3,4,5-trisphosphate and activation of the PI3K signaling pathway. This may result in inhibition of tumor cell growth and survival in susceptible tumor cell populations. Activation of the PI3K signaling pathway is frequently associated with tumorigenesis.

1.3 Aim of the study

The chronic Myeloproliferative Disorders Philadelphia (Ph) -negative (MPN; also known as myeloproliferative neoplasms according to WHO 2008) include the Polycythemia Vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) (Campbell PJ et al., 2006). Their clinical course is mostly characterized by

cardiovascular events, but all MPN have an inherent potential of clonal evolution and progression to acute leukemia. They have common features, such as the origin from pluripotent hematopoietic cells, the presence of a neoplastic clone that coexists with normal hematopoiesis, and the altered production of one or more blood cells; the presence of hematopoietic progenitors capable of in vitro growth regardless of the presence of specific cytokines ("endogenous colonies"). The MPN are characterized by mutations in Val617Phe exon 14 of JAK2 (James C et al., 2005; Levine RL et al., 2005; Kralovics R et al., 2005; Baxter EJ et al., 2005), by Calreticulin mutations (Nangalia J et al., 2013; Klampfl T et al., 2013), by mutation in exon 12 of JAK2 (Scott LM et al., 2007) and the mutation codon 515 MPL (Pikman Y et al., 2006). However, while the majority of patients with PV presents the mutation Val617Phe (Vannucchi AM, Antonioli E et al., 2007;), or less frequently mutations of exon 12 of the JAK2 (Scott LM et al., 2007), approximately 40% of those with ET or PMF is wild-type (Barosi G et al., 2007), and only 5-10% of MPL mutations (Guglielmelli P et al., 2007). In addition to the altered JAK2/STAT5 signaling pathway, cells of patients with PV or PMF, show signs of self-activation of the PI3K / AKT (Levine RL et al., 2005; Kralovics R et al., 2005; Baxter EJ et al., 2005; Pikman Y et al., 2006), which could represent an additional / separate therapeutic target. The PI3K / AKT pathway acts as a regulator of cell survival, stimulating cell proliferation and inhibiting apoptosis. It is known that in normal cells the signals coming from EPOR and TPOR activate the PI3K / AKT and are involved in the regulation of erythroid and megakaryocytic cells differentiation (Ghaffari S et al.; 2006; Zhao W et al., 2006). A key element of these pathways is represented by mTOR ("mammalian Target of Rapamycin"), a member of the family of the phosphatidylinositol kinase-related kinase (PIKK) that has kinase activity, and is activated via the PI3K. The activation of PI3K / AKT and the subsequent phosphorylation of mTOR leads to the phosphorylation of important downstream targets, inducing an increased transcription of targets mRNAs. Then, downstream of mTOR, different signaling pathways may be influenced. The identification of the deregulation of the signaling of these molecules in various types of cancer has attracted the interest on the possible use of mTOR and its effectors as therapeutic targets. Indeed, inhibition of these molecules might affect multiple signaling pathways as indicated by preliminary data in solid and hematological tumors (Bjelogrić SK et al., 2006).

The aim of the study was to analyze the involvement of JAK / STAT and PI3K / AKT signaling pathways in the deregulation of functional hematopoietic progenitors of patients with polycythemia vera and primary myelofibrosis. In this study we set out to evaluate the effect of some drugs acting on specific target of the JAK / STAT and PI3K / AKT signaling pathways, either individually or in combination, using several cellular models, CD34+ cells from patients with MPN and two different murine models. My aim was therefore to provide the conditions for the combined use of these drugs in the treatment of chronic myeloproliferative neoplasms.

POLYCYTEMIA VERA

Major criteria	<ol style="list-style-type: none">1. Hemoglobin > 18.5 g/dl (♂), > 16.5 g/dl (♀) or other evidences of increase in red cell mass.2. Presence of JAK2V617F mutation or other analogue mutations (like exon 12 mutation of JAK2).
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Minor criteria	<ol style="list-style-type: none">1. Bone marrow hypercellularity and hyperplasia of red cells, granulocyte and megakaryocytes.
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2. Erythropoietin serum levels less to normal range.

3. Spontaneous growth in vitro of endogenous erythroid colonies (EEC).

ESSENTIAL THROMBOCYTEMIA

Major criteria	<ol style="list-style-type: none">1. Platelets count $\geq 450 \times 10^9/L$.2. Bone marrow megakaryocytic hyperplasia and presence of mature megakaryocytes.3. Absence of WHO criteria for diagnosis of PV, PMF, CML, MDS or other myeloid neoplasms4. Demonstration of JAK2V617F or other clonal marker or absence of thrombocytosis.
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PRIMARY MYELOFIBROSIS	
Major criteria	<p>1. Bone marrow megakaryocytic hyperplasia, megakaryocytes proliferation and atypia accompanied by either reticulin and/or collagen fibrosis, or, in the absence of reticulin fibrosis, the megakaryocytes changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis. (pre-fibrotic PMF).</p> <p>2. Not meeting WHO criteria for CML, PV, MDS or other myeloid neoplasm.</p> <p>3. Demonstration of JAK2V617F or other clonal marker or no evidence of reactive marrow fibrosis.</p>
Minor criteria	<p>1. Leukoerythroblastosis</p> <p>2. Increased serum LDH</p> <p>3. Anemia</p> <p>4. Palpable splenomegaly</p>

Table 1. Criteria for diagnosis of Polycitemia Vera, Essential Thrombocytemia and Primary Myelofibrosis: For PV diagnosis is required the presence of both major criteria plus one minor criteria or the presence of the first major criteria and two minor criteria. For ET diagnosis is required the presence of all major criteria. For PMF diagnosis is required the presence of all the major criteria and two minor criteria.

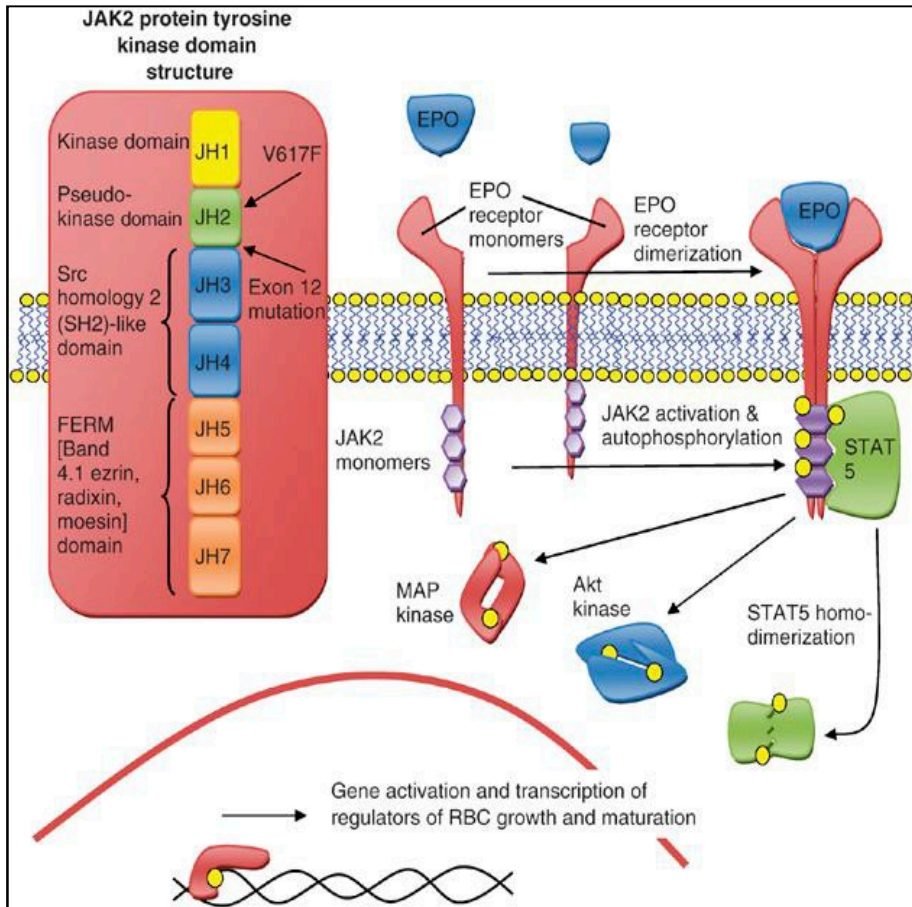


Figure 1. JAK2 protein structure and signal transduction pathway of JAK2-STAT. (Patnaik M M and Tefferi A, The complete evaluation of erythrocytosis: congenital and acquired, Leukemia, 2009).

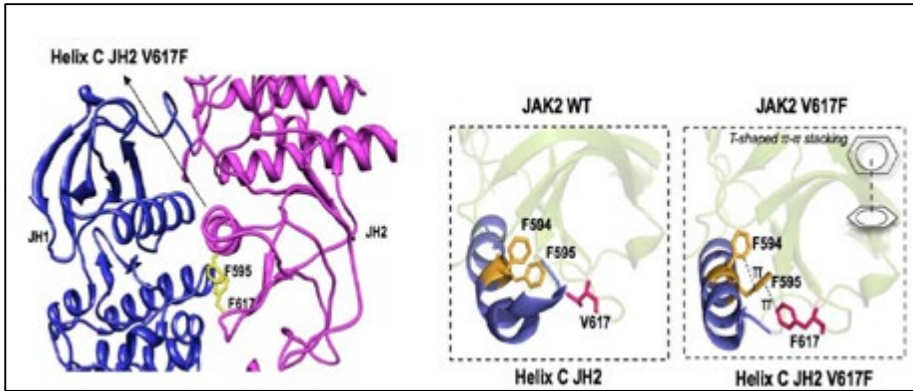


Figure 2. X-ray crystal structures of JAK2 protein and of JH2 and JH2 V617F. (Institut de Duve web site).

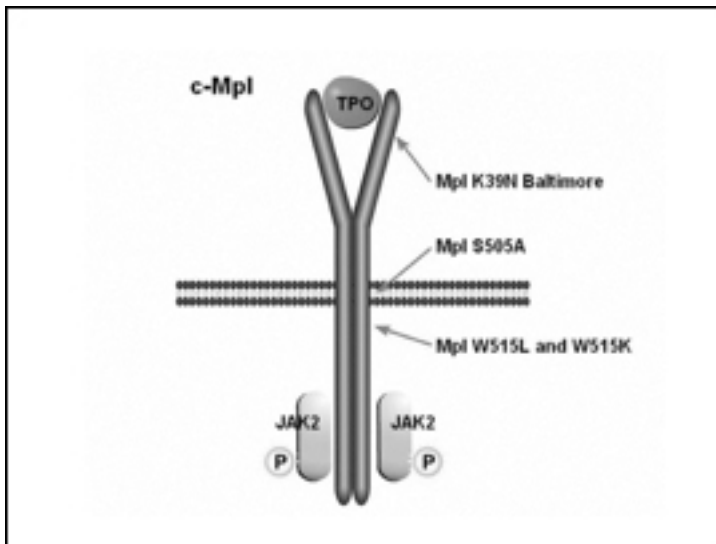


Figure 3. Mutations on TPO receptor MPL.

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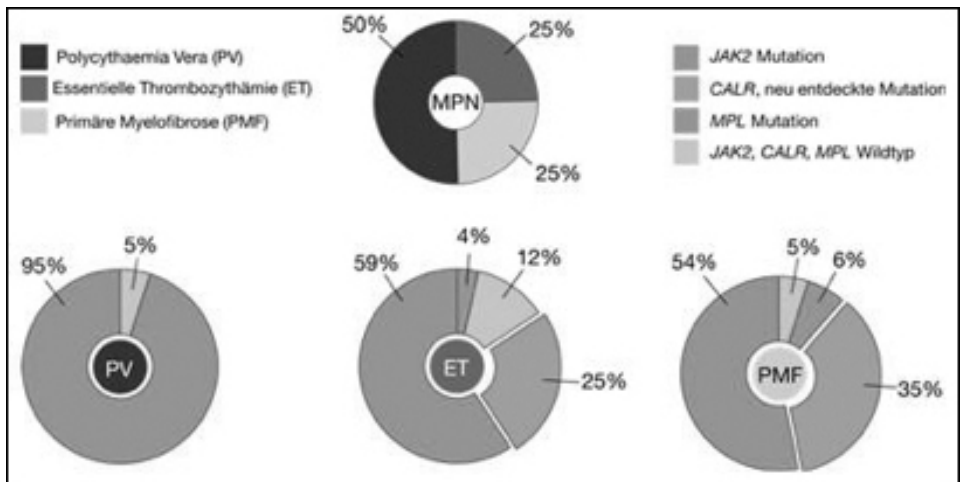


Figure 4. Distribution of mutations of JAK2, MPL and CALR in Myeloproliferative Neoplasms Philadelphia-negative.

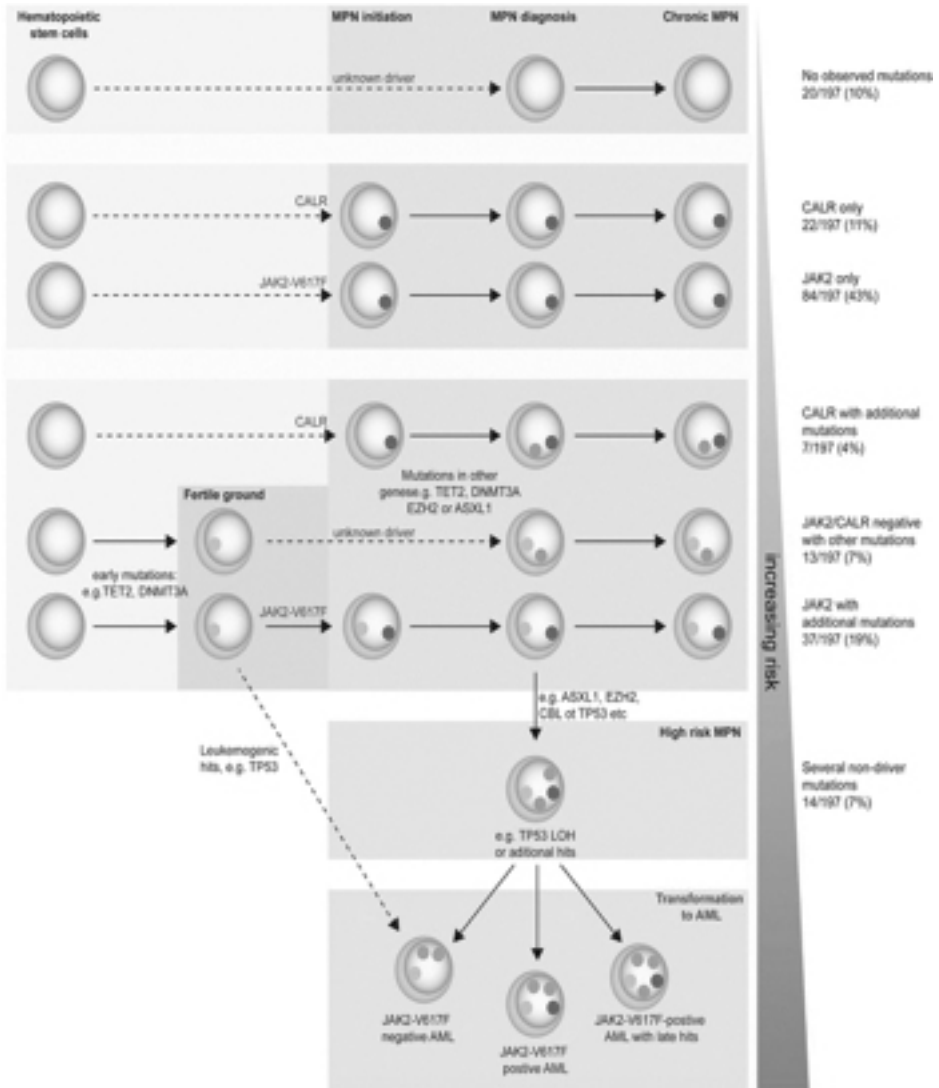


Figure 5. Model of MPN disease evolution and risk stratification in correlation to mutational events (Lundberg P et al., 2014).

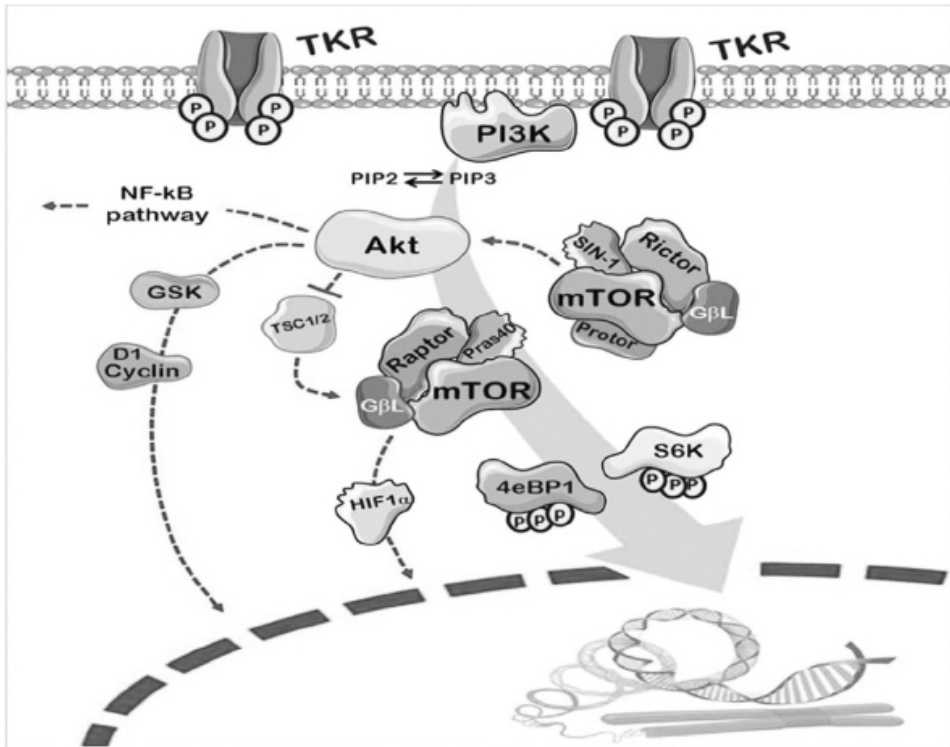


Figure 6. The PI3K/Akt/mTOR Signaling Pathway. The 2 TORC Complexes are Represented in Their Main Components (Bartalucci N et al., Rationale for Targeting the PI3K/Akt/mTOR Pathway in Myeloproliferative Neoplasms. Clinical Lymphoma, Myeloma & Leukemia 2013).

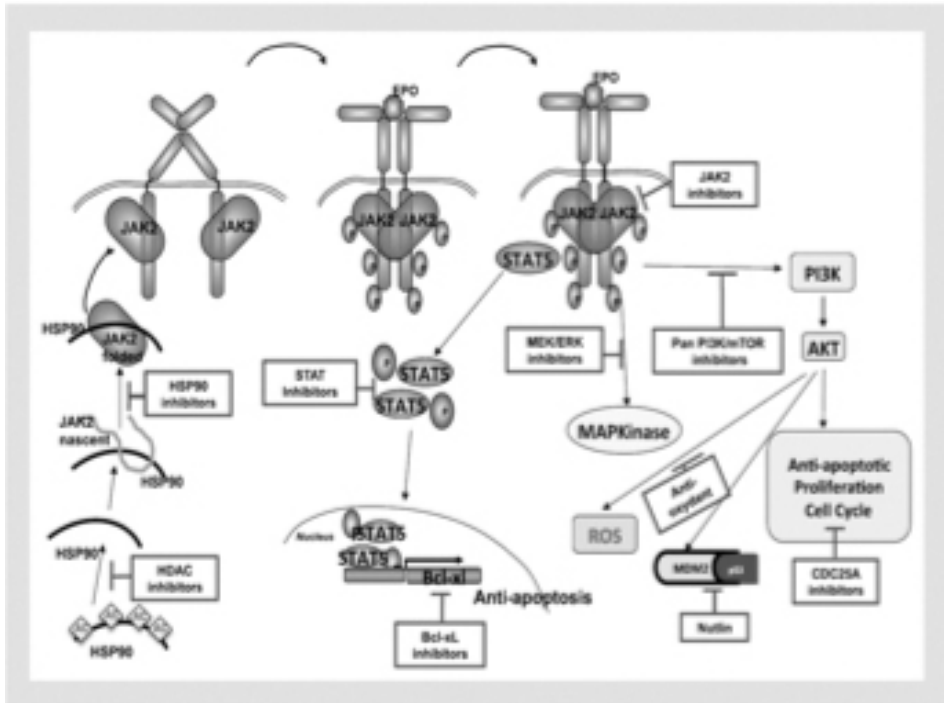


Figure 7. Potential druggable targets in MPN. (Pasquier F et al., Clin Lymph Myelom Leuk, 2015).

Chapter 2

Methods and materials

2.1 Reagents

RAD001 (an mTOR specific allosteric inhibitor with activity against TORC1) was provided by Novartis (Basel, CH); PP242 (an ATP domain inhibitor of mTOR, with activity against TORC1 and TORC2) was obtained from Sigma-Aldrich (St. Louis, Germany). The JAK1/JAK2 kinase ATP-competitive inhibitors AZD1480 and ruxolitinib were provided by D. Huszar (AstraZeneca, Waltham, MA, USA) and T. Radimeski (Novartis, Basel, CH), respectively. BEZ235, a dual PI3K/mTOR inhibitor and BKM120 an inhibitor of PI3K were provided by Novartis. The compounds were dissolved in 100% DMSO (Sigma-Aldrich, St Louis, MO, USA) to a final stock concentration of 10 mM. Each stock was used only once by diluting in culture medium. Antibodies against phospho(p)-STAT5 (Tyr694), STAT5, p- JAK2 (Tyr1007/1008), JAK2, p-4EBP1 (Thr70), 4EBP1, p-mTOR (Ser2448), mTOR, p-Akt (Ser473), Akt and Tubulin were from Cell Signalling Technology (Danvers, MA, USA).

2.2 Cellular lines and primary cells cultures

The cell lines used were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, www.dsmz.de). Murine Ba/F3 and Ba/F3-EPOR cells expressing JAK2 wt or JAK2V617F (VF) were donated by R. Skoda (Basel, Switzerland). JAK2V617F-Ba/F3-EPOR luc⁺ cells (clone 8) were kindly provided by T. Radimerski (Novartis, Basel). The cells were maintained in RPMI medium supplemented with 10% FBS, 1% L-glutamine and 1% Penicillin-Streptomycin. WEHI-conditioned medium (10%) was used for propagation of IL-3-dependent Ba/F3 and Ba/F3-EPOR wt cell lines; at the time of experiments with drugs, Ba/F3-EPOR cells were switched to rhEPO (1 U/ml).

HEL: this cell line was derived from the peripheral blood of a 30 year old man with acute erythroblastic leukemia (AML-M6) arose after treatment for Hodgkin's lymphoma. The cells show a rounded morphology, appear great, and occasionally giant multinucleated; show capacity for induced and spontaneous synthesis of globin. The HEL cell line presents in its genome multiple copies of the mutation JAK2V617F (Quentmeier H et al., 2006).

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K562: this cell line was derived from the pleural effusion of a Caucasian woman of 53 years old affected by chronic myelogenous leukemia; the cells have a lymphoblastic morphology. They have a human diploid karyotype with 46 chromosomes in which there is the terminal deletion of the long arm of chromosome 22 (del (22)(q12)), Philadelphia chromosome; there is also a reciprocal translocation of an unbalanced chromosome 17 on the long arm of one of chromosome 15. The majority of the mononuclear cells are undifferentiated and do not produce immunoglobulin, show no alkaline phosphatase activity and myeloperoxidase and are not able to phagocytose particles (Baker EJ et al., 2002). The cell line K562 is WT for the mutation JAK2V617F.

SET2: This cell line was derived from peripheral blood of a 71 year old woman affected by essential thrombocythemia evolved into megakaryoblastic leukemia. The cells are found mostly individually suspended and some grow losing grip. Note the rare presence of giant cells. The line is positive for the mutation JAK2V617F heterozygous.

Ba/F3: This cell line was derived from the peripheral blood of BALB / c mice affected by pro-B leukemia. They are IL-3 dependent and show round morphology, some appear polymorphic, meet individually suspended or occasionally in groups. Show a diploid karyotype mouse with 33% of polyploidy (40 (36-42) 2n).

WEHI-3B: This cell line was derived from a murine myelomonocytic leukemia of BALB / c mice. The cells are similar to macrophages, small and round. They grow both in adherence and in suspension and produce IL-3.

PRIMARY CELLS: For the evaluation of molecular targets and for cellular studies 30 ml of peripheral blood were taken to collect mononuclear cells and other cellular populations from MPN patients diagnosed according to the 2008 WHO criteria after obtaining an informed consent. The peripheral blood was layered on Lymphoprep (Lympholyte, Cedarlane, Canada) and centrifuged at 1600 rpm for 30 min at 20° C. It has been recovered the ring of mononuclear cells, which were washed in sterile PBS at 4°C, and centrifuged at 1200 rpm for 10 minutes at 8 ° C for two times. The cells were counted using a Burkner chamber with a dilution factor of 1: 300.

CD34+ CELL ISOLATION: The purification of CD34 + cells from peripheral blood or cord blood was performed using immunomagnetic separation according to the Miltenyi procedure: the layer of mononuclear cells was washed with buffer 1 (Ca-Mg free PBS, 2 mM EDTA), centrifuged at 1000 rpm for 10 minutes and then resuspended in buffer 2 (Ca-Mg free PBS, 0.5% BSA, 2 mM EDTA) to a final volume of 300 µl containing up to 10⁸ mononuclear cells. To this suspension were then added 100 µL of FcR blocking reagent and 100 µL of CD34 microbeads (FcR blocking reagent. Human IgG. MACS CD34 microbeads: microbeads super-paramagnetic conjugated to murine monoclonal antibodies anti-human CD34 (isotype: mouse IgG1) per 10⁸ total cells; the whole was incubated for 30 minutes at 4°C and agitated every 10 minutes. After incubation buffer 2 was added up to a volume of 10 ml and centrifuged at 1000 rpm for 10 minutes at 8°C; very carefully removed the supernatant, the pellet was resuspended in a final volume of 500 µl buffer 2 per 10⁸ total cells. For separating CD34 positive was taken a column MACS MS (2x10⁸cells total 1x10⁷ positive cells). After washing with 500 µl of buffer 2 the column MACS MS separation inserted in the magnetic support, it was applied to the column the cell suspension

which was then allowed to drain by gravity the fraction CD34 negative; then 3 washes of the column with 500 μ l of buffer 2 were performed. Were then added further 500 μ l of buffer 2 in the column, which was quickly detached from the magnetic media to recover the fraction CD34 positive by pressure with a piston-column. In some cases it was necessary to perform additional steps of purification of CD34 positive fraction recovered in a new column. CD34+ were plated at the concentration of 5×10^5 / mL in IMDM medium (Iscove's Modified Dulbecco's Medium, Lonza, Belgium) with the addition of 20% human serum, penicillin / streptomycin 1% L-glutamine 1%; plus a cytokines cocktail allowing CD34+ proliferation: SCF 50ng/mL; FLT3L 50ng/mL; TPO 20ng/mL; human IL-3 10ng/mL; human IL-6 10ng/mL. After 6 days of culture the cells were washed in PBS, counted and apoptosis and proliferation have been evaluated after addition of various drugs.

2.3 Cell proliferation test

The evaluation of cell proliferation in presence of various amount of the drugs was performed using the colorimetric assay WST-1 (Cell Proliferation Reagent WST-1). The principle of this method is the increase in absorbance due to increased cell proliferation detectable by spectrophotometer. The tetrazolium salt WST-1 is turned in the Formazan molecule by the activity of mitochondrial enzymes. An expansion in the number of cells causes an increase in the activity of cellular mitochondrial dehydrogenases which leads to an increase in the Formazan formation, this increase is directly related to the number of metabolically active cells in the culture. 10 μ L of WST-1 were added to 100 μ L of cell suspension of HEL, K562, SET2, BaF3, CD34+ plated at a concentration of 2×10^5 / ml (4×10^5 for CD34+) on 96 multiwells (Falcon, USA) with escalating doses of the various drugs. The identification of the IC50 dose was detected using the software Origin 7.5. Each individual test was carried out in triplicate and the final datum was obtained as media of three tests. The spectrophotometer used is EL808 ELISA (Biotek) and data were analyzed with the Gen5 (Biotek).

2.4 Agar clonogenic test (semisolid medium)

To evaluate the decrease in clonogenic potential of some drugs HEL, K562 and SET2 cell lines were plated on agar. It was used the DMEM medium (Dulbecco's Modified Eagle Medium, GIBCO BRL Life Technologies) to which was added 10% FBS, P / S and L-Glut 1%. To this medium were added $0,8-1 \times 10^4$ cells / mL. Bactoagar powder 0.5% previously boiled was supplemented to the suspension rebalanced at 37 ° C. After quickly agitated were dispensed 300 μ L of suspension in 24 multiwells. The appropriate concentrations of the drugs were supplemented directly in the wells.

2.5 Clonogenic test of human progenitors (semisolid medium)

Two semisolid media were used: a preparation of methylcellulose containing an optimal blend of cytokines (Methocult GF H4434, StemCell Technologies Inc, USA) to induce the growth of myeloid colonies (with rh SCF, rh GM-CSF and rh IL-3) and erythroid colonies (with rh IL-3 and rh EPO); a preparation of methylcellulose "base" (MethoCult H4531, StemCell Technologies Inc, USA), (Table 1) without any cytokine, allowing the growth of the erythroid endogenous colonies (EEC). Were added 1.0×10^5 / mL of mononuclear cells for the methylcellulose complete medium and 2.5×10^5 / mL mononuclear cells for the "base" methylcellulose medium to vials of 2.5 mL of semisolid (methylcellulose) medium, in IMDM culture medium (Lonza, Belgium), Penicillin / Streptomycin (Lonza, Belgium) and L-glutamine (Lonza, Belgium) 2 mM; the cells were subsequently plated in Petri dishes (Tissue Culture Dish, 35x10 mm, Falcon, USA). After 14 days of culture in an incubator at 37°C and 5% CO₂, colonies were counted and recognized as belonging to the myeloid or erythroid lineage according to conventional morphological criteria. The presence or absence of hemoglobin pigment were done by examination under an inverted microscope (Figure 1).

For the evaluation of the megakaryocytic colonies was used MegaCult-C serum-free medium (Stem Cell Technologies Inc., USA) (Table 2) to which were added CD34 positive cells purified by immunomagnetic separation at a concentration of 5×10^3 / ml in a final volume of 3.3ml (consists of 0.4 ml of cell suspension, 1.7 ml of MegaCult non-C Medium supplemented with cytokines and 1.2ml of Collagen Solution). They were then added to the medium 50 ng / ml of Thrombopoietin, 10ng / ml of human IL-6 and 10 ng / ml of human IL-3. After 12 days of culture in an incubator at 37°C and 5% CO₂, megakaryocytic colonies were recognized and counted (CFU-MK) (Figure 8).

2.6 Cell cycle analysis

1×10^6 cells were recovered after 18 hours of drugs treatment and washed in PBS by centrifugation at 1200 rpm for 5 minutes; subsequently the supernatant was aspirated and the cell pellet resuspended in 500 µL of a 95% ethanol cold solution and then incubated on ice for 20 minutes. After washing with PBS, the cell pellet was resuspended in 500 µL of PBS with the addition of 5 µg of RNase (Roche) and incubated at 37°C for 20 minutes. The suspension was then transferred into a FACS tube and has been carried out a wash in PBS before addition of 500 µl of a propidium iodide (PI) solution at a concentration of 10 µg / ml and subsequent incubation on ice for 10 minutes. The addition of propidium iodide and subsequent incubation were performed protected from light. PI is an intercalating agent and a fluorescent molecule and when is bound to nucleic acids, the fluorescence excitation emission is detected. After incubation, we proceeded to the cytofluorimetric evaluation with FACS Scan (Becton Dickinson) using the Cell Quest Pro software (Becton Dickinson) and a minimum of 30,000 events.

2.7 Apoptosis evaluation

1x10⁶ cells were removed from the wells after 24 hours of incubation with the various compounds and washed in PBS by centrifugation at 1200 rpm for 5 minutes. The pellet was resuspended in 100 µl of Incubation Buffer with the addition of 2 µl of Annexin-V-FLUOS labeling solution (Roche) and 2 µl of Propidium iodide and incubated at room temperature for 15 minutes. Then 500 µl of Incubation Buffer were added and we proceeded to the evaluation using flow cytometer FACS scan (Becton Dickinson) using the Cell Quest Pro software.

2.8 Protein extraction and SDS page western blotting

Harvested cells were resuspended in RIPA lysis buffer (50 mM pH 7.4 Tris-HCl, 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing a protease inhibitor cocktail (Halt Protease Inhibitor Cocktail Kit, Pierce, Rockford, IL, USA). After freezing at -80 ° C and thawed at room temperature the samples for 3 times, the cell suspension was centrifuged at 7000 rpm for 15 minutes at 4°C and supernatant containing the extracted proteins was recovered. An aliquot of cell lysate was used for protein quantification using a BCA kit (Sigma-Aldrich). The proteins were resuspended in 4x Sample Buffer (Tris-HCl pH=6,8 250mM, glycerol 10%, SDS 8%, Bromophenol blue) and after having been heated at 95 ° C for 10 minutes, the samples were applied on a polyacrylamide gel 3-8% for the detection of proteins p-JAK2 JAK2 , mTOR, p-mTOR or 4-12% for the detection of the protein p-4EBP1, 4EBP1, p-STAT5, STAT5, tubulin (Resolving Gel 10%: 3.3ml BisAcrylamide, 2.5ml Buffer 1.5 M Tris-HClpH 8.8, 0.1ml SDS, 4.1ml H₂O milli-Q; Stacking Gel 4%: 1.3ml BisAcrylamide, 2.5ml Buffer 0.5 M Tris-HClpH 6.8, 0.1ml SDS, 6.1ml H₂O milli-Q). After an electrophoretic separation with running buffer (Tris 15 g/L, glycine 72 g/L, SDS 5 g/L, H₂O milli-Q) the proteins were blot on PVDF using blotting buffer (Tris 25 mM, glycine 192 mM, Methanol 20%, SDS 0,25%, H₂O milli-Q) membranes and subsequent locking of the membranes for 1 hour at room temperature with a solution of TBS-Tween (Tris aminomethane 20 mM, NaCl 500 mM, pH 7,6) 0.1% and 1% BSA. The next incubation with the primary antibody took place overnight at 4°C with a 1: 1000 dilution of the antibody in a solution of TBS-Tween 0.1% and 1% BSA. After incubation, three washes of the membranes were carried out to remove the excess of unbound antibody with a solution of TBS-Tween 0.5%. The incubation with the secondary antibody was performed with a dilution of 1: 7000 in a solution of TBS-Tween 0.1% and 1% BSA for one hour under stirring at room temperature and then three washes with a solution TBS-Tween 0.5% was accomplished. The membranes were incubated for 1 minute with ECL reagent and detected by the acquisition system Image Quant 350 (GE Health Care). The stripping of the membranes (stripping solution: 62,5 mM Tris-HCl pH 6,7, 2% SDS, 100 mM β-mercaptoethanol) was performed at room temperature for one hour with the stripping solution with subsequent washings of the membranes with a TBS-Tween 0.5% solution; after stripping was performed a new saturation of the membrane with a solution of TBS-Tween 0.1% and 1% BSA for 1 hour at room temperature which was followed by incubations with primary antibody

Study of intracellular signaling pathways in Chronic Myeloproliferative Neoplasms and the secondary before the new revelation with the ECL reagent. In each experiment, we proceeded to the assessment on the same membrane of the phosphorylated form of the protein, the non-phosphorylated form and has conducted an audit of the normalization evaluating the expression of the protein α -tubulin.

2.9 Mouse models

All animal procedures were performed according to Italian laws in an animal facility (Di.V.A.L., University of Florence) under humanized conditions.

2.9.1 SCID Ba/F3 *JAK2*^{V617F} –Luc+ mouse model

This mouse model is generated by intra venous injection in a SCID immunodeficient mouse (4–6 weeks; Harlan, Indianapolis, IN, USA) of 3×10^6 murine leukemic proB Ba/F3 cells expressing *JAK2*V617F mutation and Luciferase enzyme. At specified time points thereafter, mice were injected with Xeno Light D-luciferin (Caliper, Waltham, MA, USA) to generate a measurable bioluminescence signal that is proportional to leukemic burden (luc+ cells); measurement was performed 15 min after luciferin injection using the Photon Imager apparatus (Biospace Lab, Paris, France). Baseline measurement performed on day 6 after luc+ cell injection was used to establish individual bioluminescence level, then mice were randomly divided into four treatment cohorts of six mice each having comparable baseline disease burden. Drugs were administered daily by gavage and imaging was performed at weekly intervals after the first drug dose; mice were followed daily for survival and euthanized when they developed hind limb paralysis or became moribund. This model was used to evaluate effectiveness doses and selectivity of drugs treatment.

2.9.2 C57Bl6/J *JAK2*^{V617F} KI mouse model

This mouse model is generated with the insertion in mouse genome of inverted *JAK2*V617F mutated sequence that is activated by the mating with a VavCre transgenic mouse expressing Cre recombinase under the control of Vav promoter in Hematopoietic and Endothelial tissue. Progeny is heterozygous *JAK2*V617F and develop a Chronic Myeloproliferative Neoplasms from birth with characteristic symptoms like high hematocrit values, high platelets and white blood cells count, big spleen and bone marrow fibrosis.

Three months-aged KI mice received the drugs for indicated periods and were euthanized by CO₂ inhalation. Blood samples were collected by retro-orbital plexus puncture; blood parameters were measured using the Sysmex XE5000 (Sysmex, Hyogo, Japan) cell counter, while reticulocytes (number per high-power field, HPF) were counted in methylene blue-stained blood smears. The spleen was collected and weighted; to accomplish for variations in body weight at baseline, a spleen index (i.e., spleen weight/body weight x100) was calculated. Cuts of the spleen were fixed in PBS-buffered formalin (4%), paraffin embedded, sectioned and haematoxylin and eosin stained.

For colony assay: bone marrow cells were collected from JAK2V617F KI and JAK2 wt mice and plated at 1.5×10^5 /mL in methylcellulose (MethoCult GF M3434, Stem Cell Technologies) supplemented with recombinant cytokines (SCF, mIL-3, hIL-6, hEPO). In experiments testing drug combination a 1:1 ratio of JAK2V617F KI and JAK2 wt bone marrow mice cells (at a 1×10^4 /ml) was used. Colonies were enumerated on day 4 using standard criteria.

2.10 Statistical methods

The Mann–Whitney U or Fisher test was used for comparison (SPSS software; StatSoft Inc., Tulsa, OK, USA). The level of significance from two-sided tests was $P < 0.05$. The analysis of drug synergism was performed by calculation of the combination index (CI), that is a measure of the interaction between two drugs. The CI was calculated according to the median- effect principle of the Chou and Talalay method using the CalcuSyn software 2.1 (BioSoft, Cambridge, UK) (Chou T-C, 2010). According to this formula, when CI is less than 0.9 the interaction of two drugs is considered synergistic, when CI is 0.9–1.1 the interaction is additive, and when CI is greater than 1.1 the interaction of two drugs results in an antagonist effect.

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METHOCULT GF H4434	METHOCULT H4531
1.0 % methylcellulose in IMDM	1.0 % methylcellulose in IMDM
30 % Fetal Bovin Serum (FBS)	30 % Fetal Bovin Serum (FBS)
1 % Bovine Serum Albumin (BSA)	1 % Bovine Serum Albumin (BSA)
10 ⁻⁴ M 2- mercaptoethanol	10 ⁻⁴ M 2- mercaptoethanol
2mM L- glutamine	2mM L- glutamine
50ng/mL rh Stem Cell Factor	10 % Agar Leukocyte Conditioned Me- dium
10ng/mL rh GM-CSF	
10ng/mL rh IL-3	
3 unità /mL rh Eritropoietina (EPO)	

Table 1. Description of semisolid media.

COMPONENTS	FINAL CONCENTRATION
Collagen	1,1 mg/ml
Bovine serum albumin	1%
Recombinant human insulin	10 µg/ml
Human transferrin	200 µg/ml
L-glutamin	2 mM
2-Mercaptoethanol	10 ⁻⁴ M
Iscov's MDM	

Table 2. Description of MegaCult-C serum-free medium.

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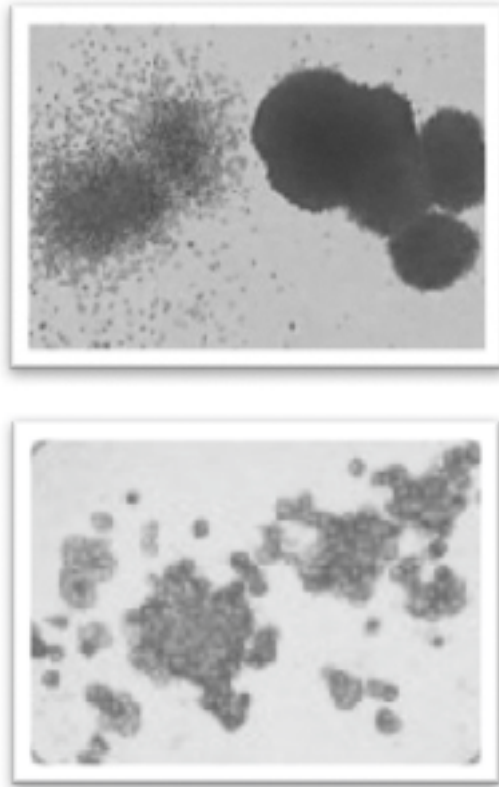


Figure 1. Human CFU-GM, BFUE and CFU-MK.

Chapter 3

Results

3.1 Impairment of cell viability

To ascertain the effects of mTOR inhibition on the growth of cell lines harboring the JAK2V617F mutation we employed the selective allosteric mTOR inhibitor RAD001 and the ATP competitive inhibitor of the active site of mTOR, PP242. Concurrently, we used the two ATP-competitive JAK2 inhibitors ruxolitinib and AZD1480, the PI3K and mTOR inhibitor BEZ235 and the PI3K inhibitor BKM120. Ba/F3 JAK2 wt and JAK2 Ba/F3-EPOR wt cells engineered to express the erythropoietin receptor and the cytokine-independent counterpart with ectopic expression of JAK2V617F (Ba/F3 VF) and Ba/F3 EPOR-VF were exposed to increasing drug concentrations in proliferation assay. We found that Ba/F3 VF and Ba/F3-EPOR VF cells were definitely more sensitive to inhibition of the mTOR pathway than the wt counterpart: the IC₅₀ (mean±SD) was respectively 10±4 nM and 651±50 nM versus 2,600±1,200 nM and 11,000±1,000 nM for RAD001. In case of PP242 the IC₅₀ were 800±200 nM and 500±100 nM for Ba/F3 VF and Ba/F3 EPOR-VF versus 3,400±300 nM and 5,931±1,000 nM, respectively for Ba/F3 JAK2 wt and JAK2 Ba/F3-EPOR wt (P<0.001 for both drugs). Similarly, JAK2 inhibitors effectively prevented the growth of Ba/F3 VF and Ba/F3 EPOR-VF cells with greater sensitivity compared to their wt counterpart; the IC₅₀ was respectively 300±80 nM and 313±23 nM versus 500±100 nM and 752±30 nM in case of AZD1480 and 34±2 nM and 220±20 nM versus 1,600±500 nM and 457±15 nM in case of Ruxolitinib (P<0.001 for both). We found that also BEZ235 and BKM120 inhibited the proliferation of Ba/F3 VF and Ba/F3-EPOR VF cells at concentrations significantly lower than the wt counterparts: the IC₅₀ value was 87±50 nM in Ba/F3-EPOR VF cells compared with 676±200 nM in the wt counterpart and 64 ±10 nM in the Ba/F3 VF cells compared with >1000 nM in the wt cells in case of BEZ235. The IC₅₀ for Ba/F3 VF and Ba/F3 EPOR-VF were respectively 364±200 nM and 1,100±207 versus 5,300±300 nM and 3,122±100 nM for Ba/F3 JAK2 wt and JAK2 Ba/F3-EPOR wt in case of BKM120 (P<0.05). We later evaluated the effect of the drugs on human cell lines JAK3V617F mutated HEL and SET2 compared to the K562 JAK2 WT cell line. The IC₅₀ was 1,400 nM for HEL, 200 nM for SET2, while the K562 cell line showed a value of 16,000 nM under RAD001 treatment. Also in case of PP242 treatment the JAK2V617F mutated cell lines were significantly more sensitive compared to control line; in fact the values of IC₅₀ were for HEL and SET2, respectively 1,500 nM and 285 nM, while for the K562 was 9,300 nM. Using the JAK2 inhibitors AZD1480 and Ruxolitinib on human cell

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lines, we found that SET2 and HEL cell lines were very sensitive to AZD1480 and Ruxolitinib (90 nM and 160 nM respectively for the SET2 and 860 nM and 790 nM for HEL), while the K562 line JAK2 WT was much more resistant showing IC₅₀ 5,300 nM and > 20,000 nM respectively for AZD1480 and Ruxolitinib. Using PI3K and mTOR inhibitors the viability of human HEL and SET2 cells was affected by BEZ235 and BKM120 with IC₅₀ values respectively of 387±90 nM (HEL) and 334±40 nM (SET2), and 2,000±500 nM (HEL) and 1,000±300 nM (SET2). Conversely, the BCR/ABL mutated K562 cell line was inhibited at BEZ235 concentrations about 15-fold higher (IC₅₀ value = 5,000±1000 nM; P<0.01 versus SET2 and HEL) and BKM120 about 4,5-fold higher (IC₅₀ value = 4,500±800 nM; P<0.01). (Table 1).

3.2. Effect of the inhibitors on the clonogenic growth potential in agar

The clonogenic potential of human JAK2V617F mutated HEL and SET2 cells, evaluated in semisolid cultures, was potently inhibited by mTOR and JAK2 inhibitors at very low nanomolar concentrations, as shown in Table 5. We found that colony formation by HEL and SET2 cells was efficaciously prevented at nanomolar concentrations of RAD001 (mean value, 0.93 nM and 0.044 nM for HEL and SET2 cells, respectively) and PP242 (0.172 nM and 0.062 nM), and similar for JAK2 inhibitor AZD1480 (0.46 nM and 0.035 nM) and Ruxolitinib (0.37 nM and 0.027 nM) (Table 2). We are not able to provide obvious explanations for such contrasting findings concerning the efficacy of RAD001 in clonogenic versus proliferation assay, that could not be actually ascribed to different culture conditions, variable times of drug exposure, types of plastics used as well as a number of other variables we deliberately introduced in the system. One possibility might be that optimal inhibition of HEL and SET2 cell proliferation by RAD001 requires some kind of cell-to-cell interactions that are favoured in the semisolid media as compared with liquid cultures. These experiments also indicated that HEL cells, that display multiple copies of JAK2V617F, resulted significantly less sensitive than the JAK2V617F heterozygous SET2 cells.

3.3. Effect of the inhibitors on cell cycle in set2 cell line

We then evaluated the effect of increasing drug amounts on the rate of cell division through the analysis of changes in cell cycle of SET2 cells that could contribute to slowed cell division. After 24 hours, mTOR, JAK2, and PI3K and mTOR inhibitors induced a block in the G₀/G₁ phase with subsequent decrease in the S-phase. Whereas treatment with the PI3K inhibitor BKM120 led to a significant increase in the G₂-M population and the increase in G₂-M occurred in a dose-dependent manner (Figure 1).

3.4. Effect of the inhibitors on apoptosis in set2 cell line

The results obtained from the study of apoptosis, using as a model SET2 cell line, have shown that treatment with RAD001 was little effective in inducing cell death, while PP242 caused in dose-dependent manner, a modest apoptosis at high concentrations. These data suggest that RAD001 and PP242 have a more potent cytostatic effects rather than cytotoxic. Conversely treatment with JAK1 / JAK2 inhibitors induced apoptosis proportionally with increasing dose of the compounds: both drugs AZD1480 and Ruxolitinib caused an increase in the proportion of apoptotic cells up to 40% (with 10xIC₅₀) compared to control, while the proportion of necrotic cells did not increase in a dose dependent manner. The SET2 cells were also sensitive to BEZ235 and BKM120 showing an increase in the number of apoptotic cells up to 40% compared to control, with only 5xIC₅₀. These data indicate that the effect of inhibitors of JAK1 / JAK2, PI3K inhibitor and dual inhibitor of PI3K and mTOR in leukemia cell lines JAK2V617F is predominantly mediated by apoptosis (Figure 2).

3.5. Effect of the inhibitors on protein phosphorylation

Inhibition of the mTOR- and JAK2-dependent signaling pathways in Ba/F3-EPOR (Figure 3) and SET2 (Figure 4) cells exposed to the relevant drugs was supported by results of western blot experiments showing reduced levels of phosphorylated 4eBP1, in case of mTOR inhibitors and the double inhibitor of PI3K and mTOR, and phosphorylated JAK2 and STAT5 in case of JAK2 inhibitors in a dose dependent manner (Figure 4). Unexpectedly also the mTOR inhibitors RAD001 and PP242 induced a reduction in STAT5 phosphorylation.

To confirm that the decrease in STAT5 phosphorylation was directly mediated by inhibition of mTOR and not the result of a direct effect of RAD001 on STAT5 phosphorylation, mTOR silencing was made using specific siRNA anti-mTOR, or control siRNA without target (scramble) using HEL cell line. Despite treatment with anti mTOR siRNA induced a decrease in the levels of mTOR of only 50-60% after 24 hours, the level of 4EBP1 phosphorylation decreased drastically compared to cells that were treated with scramble, while the content of total protein 4EBP1 did not change at all. After 48 hours both phospho-mTOR and phospho-4EBP1 were barely detectable. In a similar way the levels of phosphorylated STAT5 appeared markedly reduced at 24 and 48 hours in cells that were transfected with siRNA specific anti-mTOR compared to the control while the total concentration of STAT5 not change (Figure 5).

3.6. Effect of the inhibitors on the clonogenic potential of human primary cells

The inhibition effect of various compounds was then evaluated on the clonogenic potential of hematopoietic progenitors from patients with MPN. For this purpose were used mononuclear cells and CD34+ cells isolated from patients affected by PV with JAK2V617F mutation (n = 6), PMF with JAK2V617F mutation (n = 6) and healthy

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controls (n = 6). Mononuclear cells were isolated and plated in semisolid culture medium at the concentration of 1×10^5 / mL in the presence of cytokines favoring the growth of erythroid colonies (BFU-E), granulocyte-macrophage (CFU-GM) or 2.5×10^5 / mL in the absence of EPO for the growth of erythroid colonies independent from erythropoietin (EEC). In addition, CD34 + cells from patients with PV, PMF or from healthy controls were purified and plated in a collagen medium for the growth of megakaryocyte colonies (CFU-MK). Experiments were carried out using RAD001, AZD1480, Ruxolitinib, PP242, BEZ235 and BKM120. It was found that the growth of CD34 + cells from patients with MPN was inhibited at concentrations of RAD001 significantly lower compared to controls. IC₅₀ values for BFU-E, CFU-GM and CFU-MK controls were respectively 432 nM, 335 nM and 572 nM, in cases of PV were respectively 91 nM, 75 nM and 50 nM and PMF were respectively 42 nM, 46 nM and 44 nM. These differences were statistically significant (P<0.01). The inhibition of the growth of progenitors cells following treatment with PP242 was found for BFU-E, CFU-GM and CFU-MK in controls, respectively, 233 nM, 148 nM and 13 nM, respectively in the cases of PV 58 nM, 31 nM and 3, in the PMF respectively 38 nM, 46 nM and 5 nM. Also using inhibitors of JAK1 / JAK2 AZD1480 and Ruxolitinib was observed preferential inhibition of progenitors colony from patients with MPN: the formation of BFU-E for AZD1480 was inhibited at IC₅₀ values of 226 nM, 135 nM and 27 nM respectively for healthy subjects, PV patient and PMF patients (P<0.01). For Ruxolitinib the formation of BFU-E was inhibited at IC₅₀ of 167 nM, 27 nM and 46 nM respectively in healthy subjects, PV patients and PMF patients; IC₅₀ of CFU-GM were respectively in healthy subjects, PV patients and PMF patients 193 nM, 46 nM and 9 nM (P<0.01) for AZD1480 and 209 nM, 39 nM and 43 nM for Ruxolitinib. IC₅₀ values for CFU-MK were for AZD1480: 400 nM for healthy controls, 44 nM for patients with PV, 59 nM for patients with PMF (P<0.01), for INC424 IC₅₀ values were: 12 nM for healthy controls, 4 nM for patients with PV and 4 nM for patients with PMF. Treatment with BEZ235 induced a decrease in the growth of CD34 + cells for BFU-E, CFU-GM and CFU-MK in controls respectively 177 nM, 143 nM and 11 nM, in cases of PV respectively 98 nM, 44 nM and 2 nM, and PMF respectively 99 nM, 108 nM and 0.7 nM. Using BKM120 the inhibition of clonogenic potential of BFU-E, CFU-GM and CFU-MK was in controls respectively >1000 nM, 258 nM and 40 nM; in cases of PV respectively 204 nM, 177 nM and 14 nM; and in PMF respectively 162 nM, 225 nM and 12 nM (Table 3). The drugs were added only once at the beginning of the cultivation, the colonies enumerated after 14 days, and the IC₅₀ expressed as a percentage of the clonogenic growth compared to control plates containing only DMSO.

As regards the growth of EPO-independent erythroid colonies (EEC), mononuclear cells derived from peripheral blood of patients suffering from PV were plated in a methylcellulose semisolid medium in the absence of EPO. The EEC developed in the presence of increasing concentrations of the various drugs were enumerated after 10-12 days and expressed as a percentage of the clonogenic growth compared to control plates containing DMSO only. The IC₅₀ obtained for RAD001, PP242, AZD1480, Ruxolitinib, BEZ235 and BKM120 were respectively 15 nM, 1 nM, 19 nM, 1,8 nM, 20 nM and 8 nM. Only for RAD001 we evaluated both PV JAK2V617F heterozygous and homozygous. The EEC of JAK2 in PV with homozygous showed

an IC₅₀ of 3nm, while those in heterozygous form 30nm: this shows a selectivity of the drug against the mutated clone (Table 3).

3.7. Effect of the inhibitors on the clonogenic potential of primary cells from murine bone marrow

We next used progressively increasing amounts of the double inhibitor of PI3K and mTOR BEZ235 and the JAK1/JAK2 inhibitor Ruxolitinib in clonogenic assay to estimate the relative selectivity of the drugs against haematopoietic progenitors obtained from bone marrow of JAK2V617F KI mice and the JAK2 wild-type counterpart. As shown in Figure 6 a 50% inhibition of colony formation was observed for wild-type mice at 83.2 nM BEZ235 and > 200 nM Ruxolitinib while for the JAK2V617F KI mice at 9.4 nM BEZ235 and 22.5 Ruxolitinib to indicate a selectivity of the drugs in the inhibition of the mutated clone.

3.8. Effect of drugs combinations on proliferation of cell lines

SET2 and Ba/F3 EPOR JAK2V617F cell lines were simultaneously treated with combinations of mTOR inhibitors, JAK1 / JAK2 inhibitor, double inhibitor of PI3K and mTOR and PI3K inhibitor, and was evaluated the effect of inhibition of proliferation. SET2 cells were incubated with different concentrations of RAD001 or PP242, AZD1480 or Ruxolitinib, BEZ235 and BKM120; using combinations of these drugs was given a Combination Index (CI) that oscillates between 0.37 and 0.77, this showed a strong synergistic activity of the two drugs used. Also in Ba/F3 cells EPOR JAK2V617F combinations of RAD001 and AZD1480 showed CI of less than 1.0 (0.41-0.98) (Table 4). Triple combination with BKM120, RAD001 and Ruxolitinib on SET2 cell line resulted in a very low Combination Index, indicating a strong synergism between the three compounds, allowing to use low doses of every single drug.

3.9. Effect of drugs combinations on clonogenic growth

We then evaluated the effects of concurrent inhibition of mTOR and JAK2 signaling in SET2 cells. Cells were put in culture using progressively increasing concentrations of the drugs and the number of colony formed at day 7 was measured; the combination index (CI), as a measure of the interaction between two drugs, was calculated as detailed in the Methods section. Results presented in Table 5 indicate that combination of a mTOR and JAK2 inhibitor resulted in markedly lower concentrations of the individual drugs necessary to produce a 50% inhibition in the assay. For example, the clonogenic growth of SET2 cells could be efficaciously inhibited with RAD001 amounts of 8.4 to 7.9-fold lower, 3.8 to 2.1-fold in case of PP242, 8.5 to 35-fold in case of AZD1480 and 7.2 to 2.1-fold in case of Ruxolitinib, depending on the drug combinations. The calculated CI values was always lower than 1, ranging from

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0.23 to 0.9, to indicate synergism of the mTOR and JAK2 inhibitors in the two cell lines and experimental settings.

3.10. Effect of drugs combinations on protein phosphorylation

We analyzed by western blot the level of phosphorylated mTOR, 4EBP1 and STAT5 in Ba/F3-EPOR VF cells exposed to BEZ235 and Ruxolitinib alone and in combination (Figure 7). We found that drugs combination produced greater inhibition of phosphorylated mTOR, 4EBP1 and particularly STAT5 compared with single drugs; of note, no significant changes were observed in Ba/F3-EPOR wt cells. The treatment conditions were 6 hours with doses of 30 nM for BEZ235 and 80 nM for Ruxolitinib.

3.11. Effect of drugs combinations on erithroid endogenous colonies growth inhibition

We next determined the effects of co-treatment of the mTOR inhibitors, JAK1 / JAK2 inhibitors and the double inhibitor of PI3K and mTOR in an EEC assay (n = 4 PV patients). A 50% inhibition of EEC colony formation was observed for RAD001 plus AZD1480 at 2.3 nM and 3.1 nM respectively, compared with 15 nM and 19 nM for the two drugs alone, with a CI of 0.61. For RAD001 plus Ruxolitinib the IC50 were respectively 1.9 nM and 0.2 nM compared with 15 nM and 1.8 nM for the drugs alone, with a CI of 0.26. Co-treatment of PP242 and AZD1480 resulted in IC50 of 0.04 nM and 0.7 nM respectively compared with 1 nM and 19 nM for the drugs alone, with a CI of 0.13, while Co-treatment of PP242 and Ruxolitinib resulted in IC50 of 0.05 nM and 0.1 nM with a CI of 0.2. A 50% inhibition of colony formation was observed for BEZ235 and AZD1480 at 3.4 nM and 3.2 nM compared with 20 nM and 19 nM, respectively, for the two drugs alone with a CI of 0.33. For BEZ235 and Ruxolitinib IC50 was found at 4.4 nM for BEZ235 and 0.4 nM for Ruxolitinib, with a CI of 0.2 (Table 6).

3.12. Combination of BEZ235 and Ruxolitinib synergistically inhibits cd34+-derived colonies from PMF patients

Efficacy of BEZ235 and Ruxolitinib combination was also analyzed by measuring the inhibition of GFU-GM and BFU-E colony formation by CD34+ cells of three PMF individuals. As shown in Figure 15, the combination of BEZ235 and Ruxolitinib resulted in synergistic inhibition of colony formation compared with single agents. Erythroid and myeloid colonies were counted together to calculate the total cell fraction affected (Fa. Only Fa50 and Fa80 values are shown). The CI of #MF1 at Fa50 and Fa80 were respectively 0.78 and 0.43; regarding #MF2 the CI at Fa50 and Fa80 were respectively 0.78 and 0.82. Then, the CI of #MF3 at Fa50 and Fa80 were respectively 0.47 and 0.66 (Figure 8).

3.13. Combination treatment of BEZ235 with Ruxolitinib improves survival in mice injected with Ba/F3 cells

To corroborate the findings of synergism shown in-vitro by the combination of BEZ235 and Ruxolitinib we used two mouse models. The first is based on the rapid, uncontrolled proliferation of Ba/F3-EPOR VF cells, stably transfected with luciferase, after systemic injection in immunodeficient mice; the progression of disease is monitored by bioluminescence at predefined time points. This represents an acute, aggressive model due to the fast growth rate and dissemination of leukemic cells with death of untreated animals occurring 10–15 days after injection. Mice were randomized to treatment groups 6 days after injection based on the bioluminescence signals; this point constitutes the baseline lecture before starting mice treatment; Mice were then treated with BEZ235 and Ruxolitinib alone and in combination, and were followed by bioluminescence analysis at weekly intervals. In preliminary dose-finding experiments (Figure 9) we determined that 50% of the animals were still alive after 15 days if receiving 60 mpk Ruxolitinib and 60 mpk BEZ235 single-agent.

Therefore, for combination treatments, we used the closest lower dose of BEZ235 (45 mpk) and the highest dose of Ruxolitinib (60 mpk). Mice from the vehicle group started to die by day 7, and by day 14 less than 20% were still alive, compared with 30% of the Ruxolitinib and 83% of the BEZ235 group; on the other hand, 50% of animals in the BEZ235 plus Ruxolitinib combination group were still alive by day 26 when all of the mice in the other groups (vehicle, BEZ235 and Ruxolitinib alone) had died. Bioluminescence analysis was performed at weekly intervals and quantitative bioluminescence measurement was performed at day 7, when at least 50% of the animals in the vehicle group were alive (Figure 10 A). At this time point, mice treated with combined BEZ235 and Ruxolitinib showed significantly lower level of whole body bioluminescence readings ($0.46 \pm 0.23 \times 10^6$ counts per minute, cpm) compared with animals receiving vehicle ($7.2 \pm 2.4 \times 10^6$ cpm; $P < 0.01$), Ruxolitinib ($4.7 \pm 1.9 \times 10^6$ cpm; $P < 0.01$) and BEZ235 ($2.11 \pm 0.7 \times 10^6$ cpm; $P = 0.04$) single agent. Of note, BEZ235 as single agent was still significantly more efficacious in comparison with vehicle ($P < 0.05$; Figure 10 B). Kaplan–Meyer analysis showed that survival of mice receiving the combination of BEZ235 and Ruxolitinib (median survival post-injection, 30.0 days, range 26–35) was significantly longer than mice receiving vehicle (15.0 days, range 8–18; $P < 0.01$), Ruxolitinib (18.5 days, range 15–28; $P < 0.01$) and BEZ235 (24.0 days, range 20–28; $P = 0.04$) alone (Figure 10 C). Treatment started on day 7 after injection.

3.14. Combination treatment of BEZ235 with Ruxolitinib improves survival in knock-in mice

The second in-vivo model was a conditional Knock In (KI) mouse. KI mice develop a progressive myeloproliferative disease starting from the first months after birth, characterized by marked erythrocytosis with thrombocytosis and leukocytosis, and splenomegaly, that mimics PV in early phase and evolves into myelofibrosis at later stages. In a first series of experiments, KI mice ($n = 8$ /group; two experiments)

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received BEZ235 45 mpk, Ruxolitinib 60 mpk single and in combination, or an equivalent volume of vehicle for 7 days. We used this short lapse of time based on the observation that first effects of Ruxolitinib on symptoms and splenomegaly in patients with myelofibrosis can be appreciated as early as at 2–4 weeks of treatment. We documented a prompt, dramatic reduction of spleen weight in mice receiving the two drug concurrently (Figure 11 A): the mean spleen index (i.e., the spleen weight normalized by the animal weight) in mice of the combination group was 1.4 compared with 6.5, 3.5 and 3.4 in mice receiving vehicle, BEZ235 and Ruxolitinib alone, respectively ($P < 0.05$ for all). The mean reticulocyte count decreased from 48, 50 and 44 per HPF in the vehicle, BEZ235 and Ruxolitinib group, respectively, to 3/HPF in the combination group ($P < 0.01$; Figure 11 B). Western blot analysis of whole spleen extracts documented a more pronounced decrease of pSTAT5 and p4eBP1 levels in mice receiving both drugs as compared with single agent (Figure 11 C).

In a second set of experiments, animals were treated for a longer period (16 days) with the combination of BEZ235 and Ruxolitinib. We confirmed the impressive reduction of splenomegaly (mean spleen index decreased from 9.0 in the vehicle to 2.3 in treated mice; $P < 0.01$, Figure 12 A), the decrease of reticulocyte count (from 60 to 29/HPF; $P < 0.05$, Figure 12 B) and the down-regulation of phosphorylated STAT5 and 4EBP1 (Figure 12 C) as seen in the short treatment set.

Although not statistically significant, there was a trend towards reduced leucocyte and platelet counts in treated mice compared with vehicle group (data not shown). Finally, histology showed a marked reduction of megakaryocytes and myeloid cells infiltrating the spleen in mice treated with drug combination (Figure 13). Arrows point to single/clustered megakaryocytes, greatly diminished in mice treated with combination of BEZ235 and Ruxolitinib. There was also a re-expression of the lymphoid component of the spleen, although the normal follicular architecture was not fully re-established. Treatment was well-tolerated and the body weight loss was $<10\%$ of baseline in all treatment groups.

Cell line	IC ₅₀ (nM)					
	RAD001	PP242	AZD1480	Ruxolitinib	BEZ235	BKM120
Ba1F3 JAK2 wt	2,600 ± 1,200	3,400 ± 300	500 ± 100	1,600 ± 500	10,000 ± 500	5,300 ± 800
Ba1F3 JAK2 V167F	10 ± 4**	800 ± 200**	300 ± 80**	34 ± 2**	64 ± 10**	364 ± 200*
Ba1F3 JAK2 EPOR-wt	11,000 ± 1,000	5,931 ± 1,000	752 ± 30	457 ± 15	676 ± 200	3,122 ± 1000
Ba1F3 JAK2 EPOR-V167F	651 ± 50**	500 ± 100**	313 ± 23**	220 ± 20**	87 ± 50**	1,100 ± 207*
K562	16,000 ± 2,500	8,300 ± 1,000	>30,000	>20,000	5,000 ± 1,000	4,500 ± 800
HEL	14,000 ± 2,800	1,500 ± 113**	860 ± 20**	790 ± 150**	387 ± 90**	2,000 ± 500
SET2	17,000 ± 3,000	285 ± 11**	90 ± 5**	160 ± 24**	334 ± 40**	1,000 ± 300

Table 1. Effects of mTOR (RAD001 and PP242), JAK2 (Ruxolitinib and AZD1480), PI3K (BKM120) and double inhibitor of PI3K and mTOR (BEZ235) inhibitors on the proliferation rate of murine and human JAK2V617F mutated cell lines (*P<0.05 **P<0.01).

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	IC50 (nM)			
	RAD001	PP242	AZD1480	Ruxolitinib
HEL	930±0.01**	172±0.059**	460±0.03**	370±0.177**
SET2	44±0.015**	62±0.019**	35±0.01**	27±0.009**

Table 2. Activity of mTOR (RAD001 and PP242) and JAK2 (Ruxolitinib and AZD1480) inhibitors on the clonogenic potential of HEL and SET2 cell lines.

BFU-E	IC50 (nM)					
	RAD001	PP242	AZD1480	Ruxolitinib	BEZ235	BKM120
Ctrl	432±22	233±50	226±17	167±70	177±50	> 1,000
PV	91±15*	58 ±18*	135±19*	27±7*	98±20*	204±10**
MF	42±10*	38±8*	27±8*	46 ±10*	99 ± 8**	162±45**

CFU-GM	IC50 (nM)					
	RAD001	PP242	AZD1480	Ruxolitinib	BEZ235	BKM120
Ctrl	335±16	148±40	193±13	209±40	143±20	258±100
PV	75±4*	31±15*	46±5*	39±6*	44±10*	177±50
MF	46±13*	46±20*	9±3*	43±4*	108±6	225±70

CFU-MK	IC50 (nM)					
	RAD001	PP242	AZD1480	Ruxolitinib	BEZ235	BKM120
Ctrl	572±13	13± 2	400±12	12±5	11±3	40±20
PV	50±5*	3± 1*	44±2*	4± 2*	2±1**	14±1*
MF	44±5*	5± 2*	59±3*	4±3*	0.7±0.1**	12±7*

EEC	IC50 (nM)					
	RAD001	PP242	AZD1480	Ruxolitinib	BEZ235	BKM120
PV	15±10	1±0.7	19±2	1.8±1	20±10	8±3

Table 3. Inhibition of clonogenic potential of hematopoietic progenitors of MPN patients by mTOR inhibitors, inhibitors of JAK1 / JAK2, dual inhibitor of PI3K and mTOR and PI3K inhibitor.

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Cell line	IC ₅₀ (nM)		Drug combination		C Index
	IC ₅₀ (nM)	IC ₅₀ (nM)	IC ₅₀ (nM)	IC ₅₀ (nM)	
Ba/F3 EPOR JAK2V617F	RAD001 651 ± 50	AZD1480 313 ± 23	RAD001 149	AZD1480 72	0.41
	RAD001 651 ± 50	Ruxolitinib 220 ± 20	RAD001 363	Ruxolitinib 125	0.44
	PP242 500 ± 100	AZD1480 313 ± 23	PP242 225	AZD1480 141	0.68
	PP242 500 ± 100	Ruxolitinib 220 ± 20	PP242 400	Ruxolitinib 121	0.98
	BEZ235 87 ± 50	Ruxolitinib 220 ± 20	BEZ235 31	Ruxolitinib 78	0.57
	BEZ235 87 ± 50	AZD1480 313 ± 23	BEZ235 22	AZD1480 81	0.54
	BKM120 418 ± 200	Ruxo 220 ± 20	BKM120 212	Ruxo 111	0.46

Cell line	IC ₅₀ (nM)		Drug combination		C Index
	IC ₅₀ (nM)	IC ₅₀ (nM)	IC ₅₀ (nM)	IC ₅₀ (nM)	
SET2	RAD001 17,000±3,000	AZD1480 90±5	RAD001 1,737	AZD1480 9.2	0.45
	RAD001 17,000±3,000	Ruxo 160±24	RAD001 3,098	Ruxo 29.1	0.70
	PP242 285±11	AZD1480 90±5	PP242 138	AZD1480 55	0.66
	PP242 285±11	Ruxo 160±24	PP242 66	Ruxo 47	0.43
	BEZ235 334 ± 40	AZD1480 90 ± 5	BEZ235 196.8	AZD1480 53.2	0.77
	BEZ235 334 ± 40	Ruxo 160 ± 24	BEZ235 55.1	Ruxo 26.4	0,37
	BKM120 1,000 ± 300	Ruxo 160 ± 24	BKM120 321	Ruxo 51	0.71

Cell line	IC50 (nM)			IC50 (nM) combination			C Index
	BKM120	RAD001	INC424	BKM120	RAD001	INC424	
SET2	1,100 ± 207	651 ± 50	220 ± 20	224 ± 150	143 ± 100	48 ± 34	0.27

Table 4. Ba/F3 EPOR JAK2V617F and SET2 cells treated with combinations of mTOR inhibitors, JAK1 / JAK2 inhibitor, dual mTOR and PI3K inhibitor and PI3K inhibitor.

Cell line	IC ₅₀ (nM)		Drug combination		C Index	
	IC ₅₀ (nM)	IC ₅₀ (nM)	IC ₅₀ (nM)	IC ₅₀ (nM)		
SET2	RAD001	AZD1480	RAD001	AZD1480	0,23±0,1	
	44±15	35±10	5,2±4	4,1±3		
	RAD001	Ruxo	RAD001	Ruxo		0.32±0,04
	44±15	27±9	6,2±1	3,7±0,4		
PP242	AZD1480	PP242	AZD1480	0,4±0,05		
62±19	35±10	16±4	1±0,5			
PP242	Ruxo	PP242	Ruxo	0,9±0,13		
62±19	27±9	29±16	12,8±7			

Table 5. Combining mTOR and JAK2 inhibitor resulted in synergistic inhibition of clonogenic potential of SET2 cells.

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Pts (PV)	IC ₅₀ (nM)		Drug combination		CI index
			IC ₅₀ (nM)		
	RAD001 15±10	AZD1480 19±2	RAD001 2.3	AZD1480 3.1	0.61
	RAD001 15±10	Ruxolitinib 1.8±1	RAD001 1.9	Ruxolitinib 0.2	0.26
	PP242 1±0.7	AZD1480 19±2	PP242 0.04	AZD1480 0.7	0.13
	PP242 1±0.7	Ruxolitinib 1.8±1	PP242 0.05	Ruxolitinib 0.1	0.2
	BEZ235 20±10	AZD1480 19±2	BEZ235 3.4	AZD1480 3.2	0.33
	BEZ235 20±10	Ruxolitinib 1.8±1	BEZ235 4.4	Ruxolitinib 0.4	0.2

Table 6. Combination of BEZ235 and Ruxolitinib synergistically inhibits the growth of EEC from PV patients.

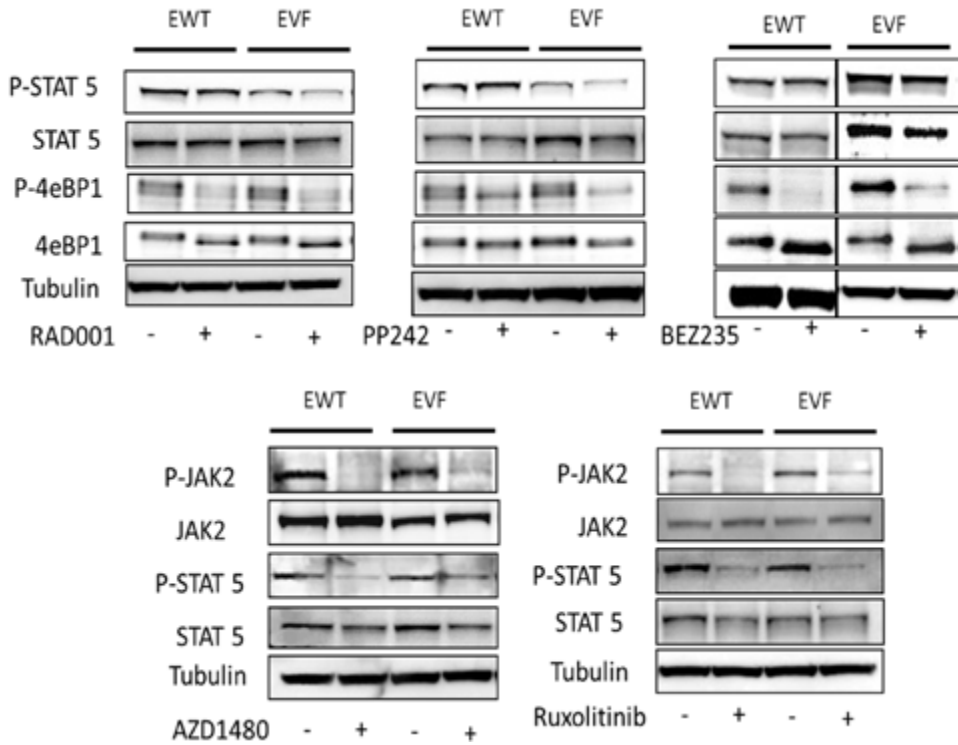


Figure 3. Effect of mTOR inhibitors, JAK1/JAK2 inhibitors, the double inhibitor of PI3K and mTOR and the the PI3Kinhibitor on mTOR and JAK/STAT signaling in Ba/F3 EPOR cells. JAK2 WT and mutated Ba/F3 cells were incubated for 6 hours with RAD001 650 nM, AZD1480 313nM, PP242 500 nM, Ruxolitinib 220 nM and BEZ235 87 nM. Tubulin was used for loading normalization. One representative of two to four similar experiments for the various drugs.

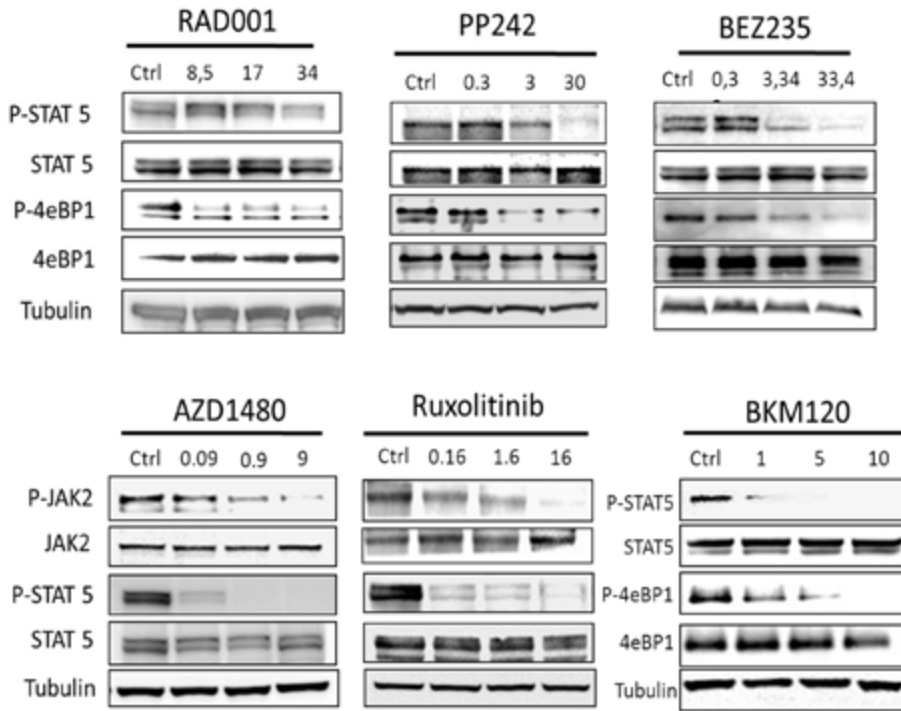


Figure 4. Effect of mTOR inhibitors, JAK1/JAK2 inhibitors, the double inhibitor of PI3K and mTOR and the PI3Kinhibitor on mTOR and JAK/STAT signaling in SET2 cells. SET2 cells were incubated for 24 hours with increasing concentrations of the drugs expressed in μ M. Tubulin was used for loading normalization. One representative of two to four similar experiments for the various drugs.

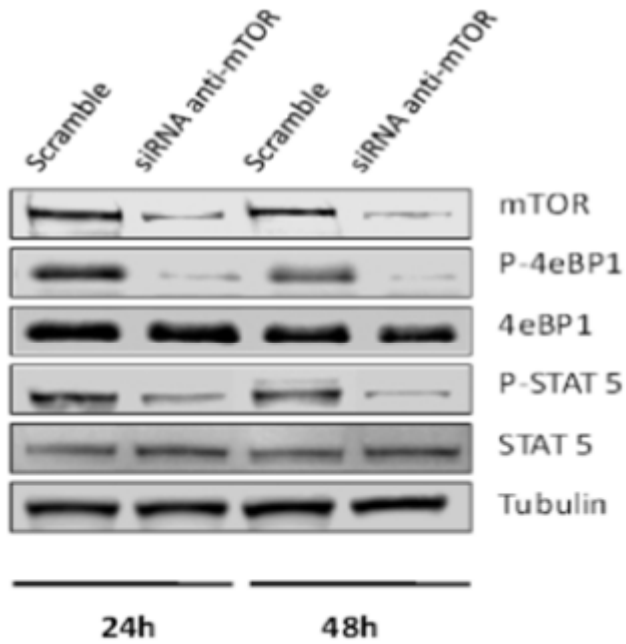


Figure 5. Inhibition of STAT5 and 4EBP1 phosphorylation by anti-mTOR siRNA on HEL cells.

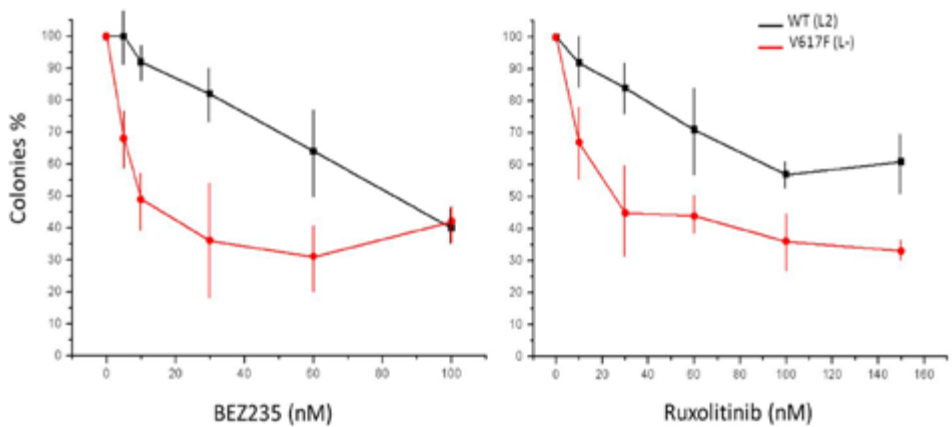


Figure 6. Inhibition of clonogenic potential of hematopoietic progenitors obtained from JAK2V617F KI mice and the JAK2 wild-type counterpart using dual inhibitor of PI3K and mTOR and JAK1 / JAK2 inhibitor.

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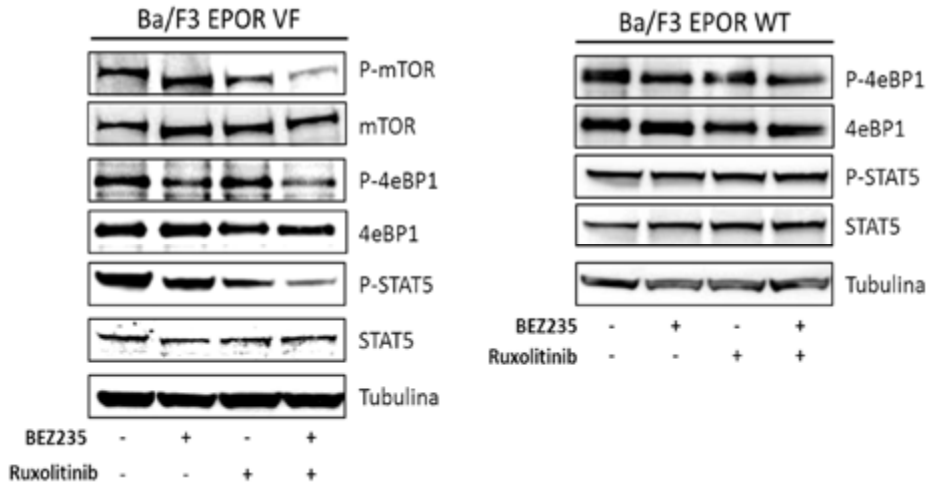


Figure 7. Ba/F3-EPOR VF and wt cells were exposed for 6 hours to 30 nM BEZ235 and 80 nM ruxolitinib, individually or in combination.

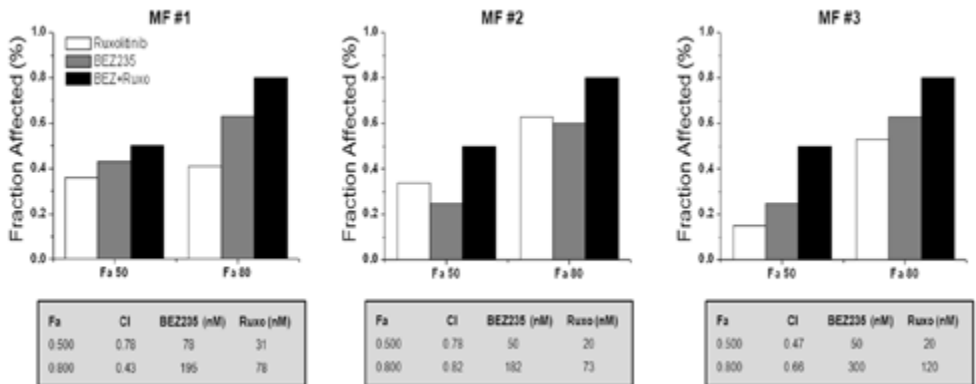


Figure 8. Combination of BEZ235 and Ruxolitinib synergistically inhibits the growth of BFU-E and CFU-GM from PMF patients.

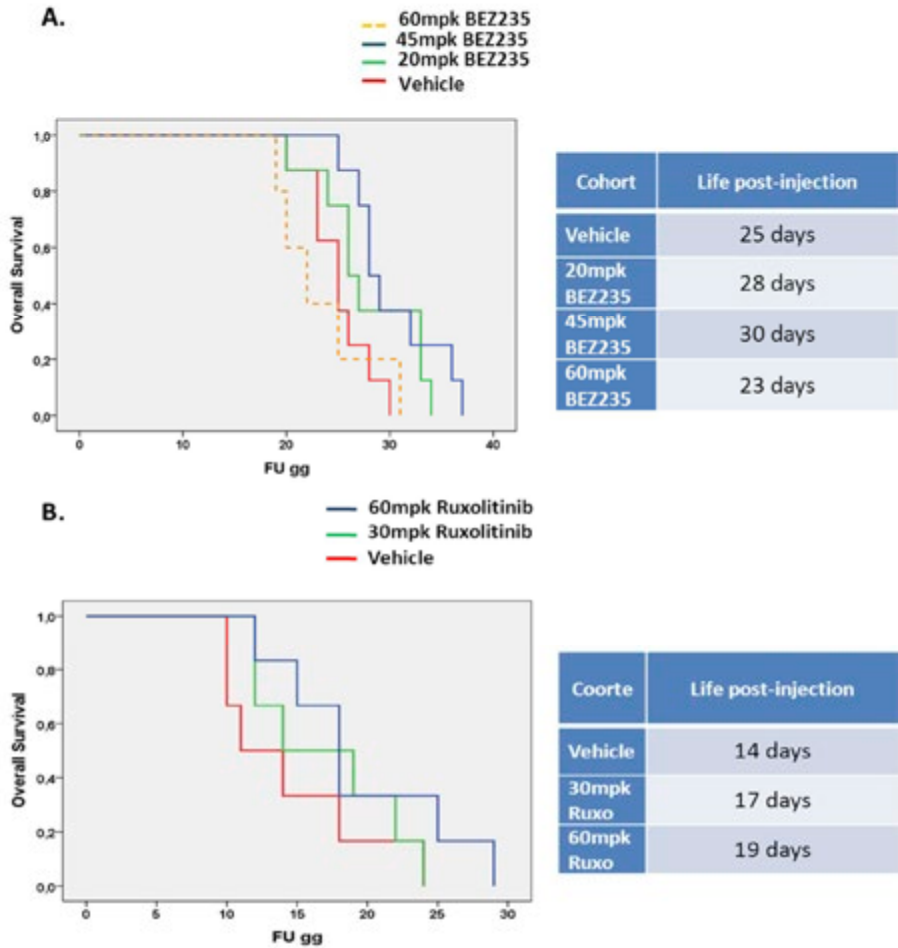


Figure 9. Single treatment of BEZ235 (A) and Ruxolitinib (B) on immunodeficient mice.

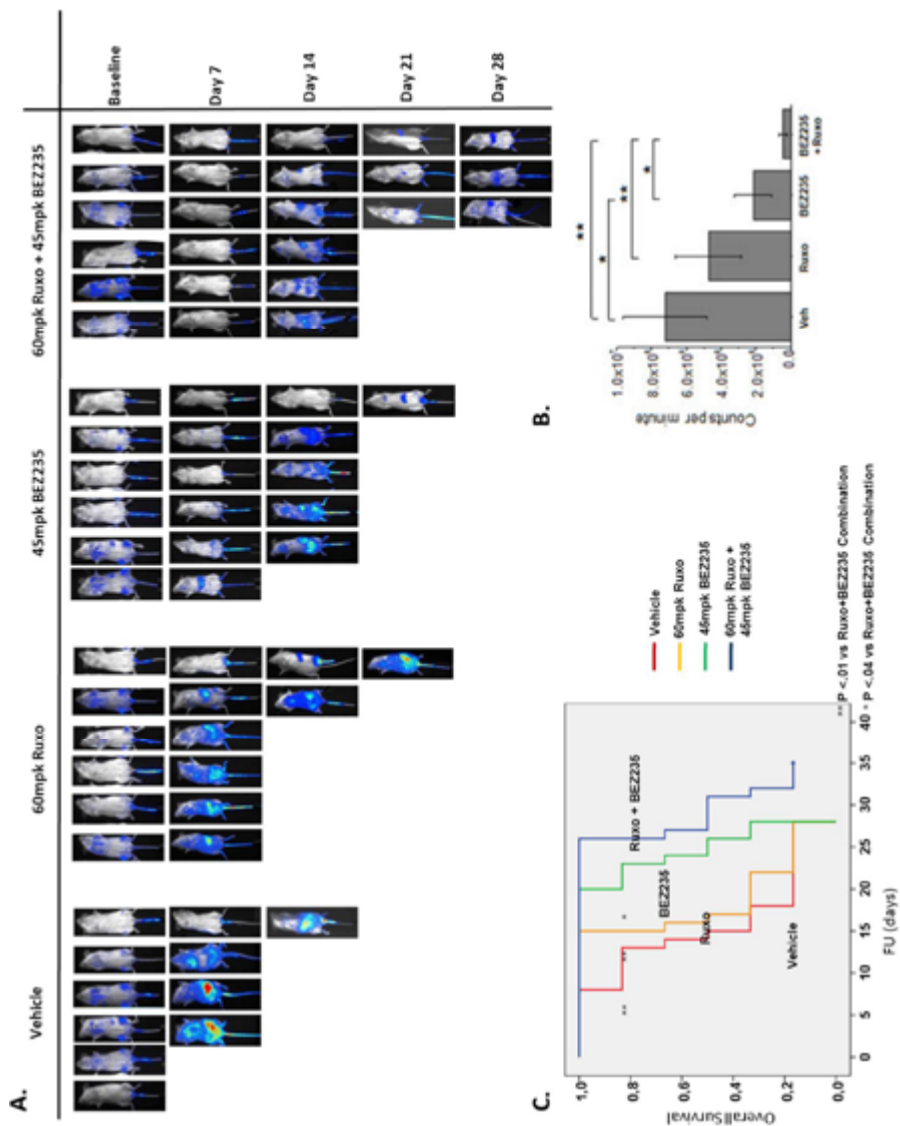


Figure 10. Combined treatment with BEZ235 and Ruxolitinib reduces dissemination of leukemic cells and improves survival in a JAK2V617F-driven mouse model. (A) SCID mice injected with 3x10⁶ JAK2V617F Ba/F3-EPOR luc⁺ cells were randomized into four treatment groups (vehicle, Ruxolitinib BEZ235 and combination). Images are from a representative experiment of three performed. (B) Bioluminescence activity (expressed as counts per minute, cpm; Mean \pm SD of four to six mice/group in each experiment, n = 3) was measured on day 7 of treatment (day 13 after cells injection). (C) Kaplan–Meyer estimate of survival in mice injected with JAK2V617F Ba/F3-EPOR luc⁺ cells

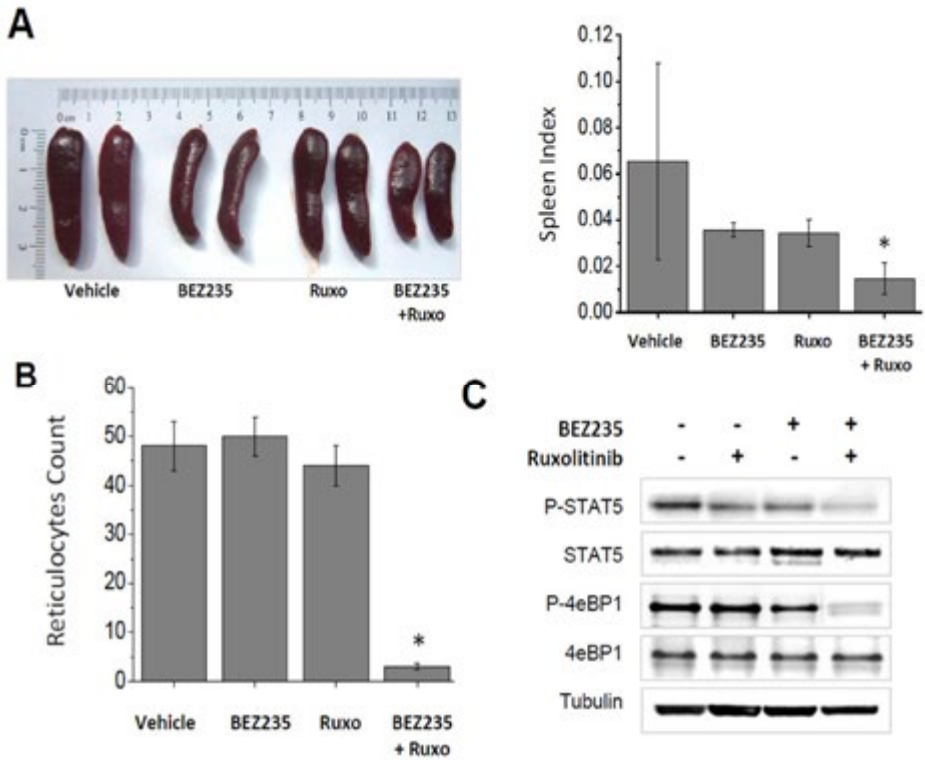


Figure 11. JAK2V617F KI mice treated with BEZ235 45 mpk, Ruxolitinib 60 mpk, single and in combination. In (A), on the left, representative images of the spleen at the end of treatment period, on the right the spleen index expressed as mean \pm SD. In (B) the effects of treatment on reticulocyte count. (C) Western blot analysis of whole spleen extracts. One representative experiment of two performed with similar results.

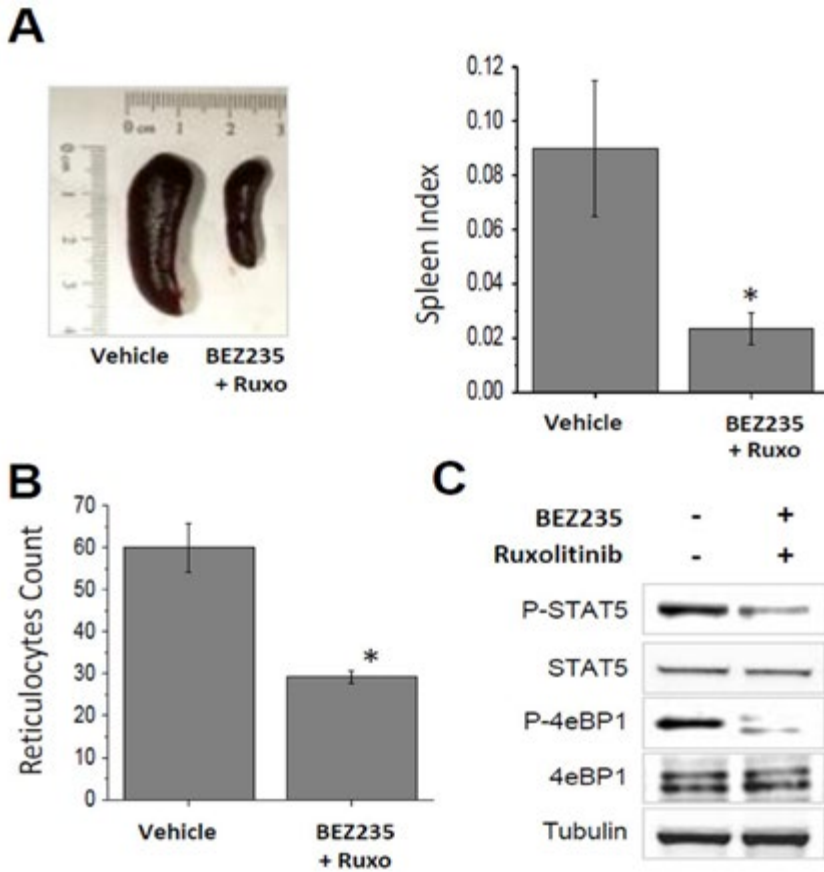


Figure 12. (A) On the left, representative images of the spleen at the end of treatment period, on the right the spleen index (mean \pm SD). In (B) the effects of treatment on reticulocyte count and in (C) western blot analysis of spleen extracts; one representative experiment of two performed with similar results.

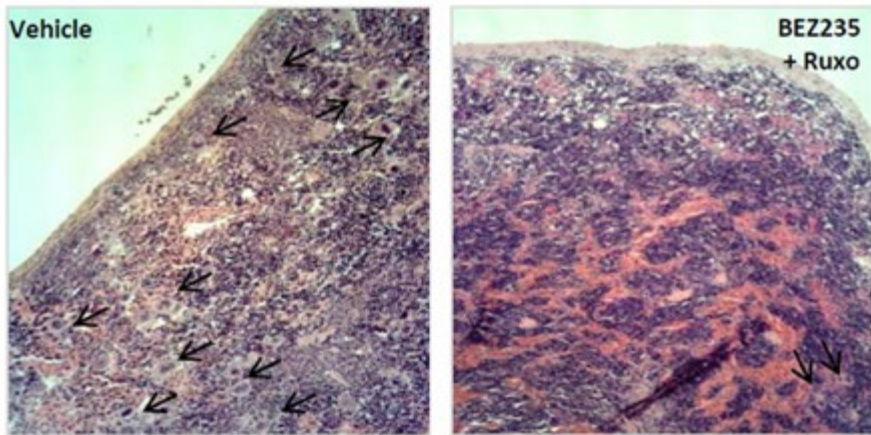


Figure 13. Haematoxylin-eosin staining of the spleen of vehicle and combination treatment. Images were acquired using a LEICA DM LS2 microscope with a Leica N Plan 10x/0.25 na objective and saved using Adobe Photoshop.

Chapter 4

Discussion

Deregulation of the JAK2/STAT pathway represents a central mechanism in the pathogenesis of MPNs: in fact, the JAK2V617F gain-of-function mutation occurs in the majority of patients with PV and 60% of PMF and essential thrombocythemia (Vainchenker W et al., 2011; Levine RL et al., 2007), other mutations (MPL, LNK, CBL) found in 5–10% can similarly activate the JAK/STAT pathway (Pikman Y et al., 2006; Pardanani A et al., 2011). Mouse models indicate that those mutations are able to induce a myeloproliferative disorder (Li J et al., 2010; Akada H et al., 2010). The JAK/STAT pathway is involved in the deregulated cytokines expression that accompanies MPNs and underlies some tracts of the clinical phenotype (Tefferi A et al., 2011) and finally targeting activated JAK2 with ATP-competitive JAK2 inhibitors resulted in measurable clinical improvements in patients with myelofibrosis (Verstovsek S et al., 2012; Harrison C et al., 2012). The clinical efficacy of JAK2 inhibitors has been ascribed to a variable degree of myelosuppression and a general down-regulation of inflammatory cytokine signaling (at least in part mediated by the concomitant anti-JAK1 properties of some JAK2 inhibitors such as ruxolitinib) (Tefferi A, 2012) well keeping in mind that none of the available molecules is specific to mutant as opposed to wild-type JAK2. Conceivably, in contrast to the capacity of JAK2 inhibitors to reduce the enlarged spleen and improve disease symptomatic manifestations, changes in the burden of mutated cells have been variable (with SAR302503 being reported as the most effective until now (Pardanani A et al., 2008)) but usually modest, at least in the short-term follow-up. Also, while generally well tolerated, JAK2 inhibitors caused some degree of unwanted myelosuppression, particularly anemia and thrombocytopenia. Therefore, current efforts are directed towards additional targets involved in the deregulated proliferation of MPN with the hope to maximize anticancer efficacy and/or improve the tolerability profile of available JAK2 inhibitors. In this regard, two preclinical studies with the heat shock protein-90 (HSP90) inhibitors PU-H71 (Marubayashi S et al., 2010) and AUY-922 (Fiskus W et al., 2011) showed marked degradation of JAK2 in MPN cellular models, including JAK2 inhibitor-persistent cells (Koppikar P et al., 2012) consistent with JAK2 being a client protein of the HSP90 chaperone complex; HSP90 inhibition normalized blood cell count, reduced allelic burden and improved survival in mice. Acetylation of HSP90 is probably involved also in the activity of histone deacetylase inhibitors, such as givinostat (Guerini V et al., 2008) and panobinostat (Wang Y et al., 2009). On the other hand, we have provided evidence that the PI3K/Akt/mTOR pathway may represent an additional suitable target for therapy; in fact, the mTOR inhibitor RAD001 (everolimus)

resulted active against JAK2V617F mutated cells *in vitro*, and produced clinical responses in MF patients in a phase 1/2 trial (Guglielmelli P et al., 2011). The serine/threonine protein kinase Akt is downstream of PI3K (Engelman JA et al., 2006); its main target is the serine/threonine kinase mTOR that exists in two multi-protein complexes, mTORC1 (with RAPTOR) and mTORC2 (with RICTOR). mTORC1, that is inhibited by rapalogs, phosphorylates the eukaryotic initiation factor 4E-binding protein 1 (4EBP1) and S6 kinase 1 controlling the level of cap-dependent mRNA translation. On the other hand, mTORC2 is largely rapamycin insensitive, and regulates the activity and stability of Akt by phosphorylating a conserved regulatory residue (Ser473). Substrates of mTORC2 include the FOXO1 and FOXO3a transcription factors (Su B et al., 2011). Therefore, the signaling network controlled by Akt and mTOR has a central role in a variety of cellular processes that include cell growth, metabolism and proliferation; the activity of this network is elevated in most human cancers, including MPN, as supported by findings that: erythroblasts of JAK2V617F conditional KI mice showed strong Akt activation, particularly in animals homozygous for the JAK2 mutation (Akada H et al., 2010), elevated levels of phosphorylated STAT5, Akt and mTOR were found in the bone marrow of MPN patients (Grimwade LF et al., 2009) and a strong inhibition of EEC formation and EPO-induced erythroid differentiation in PV progenitor cells was produced by a PI3K/Akt inhibitor (Ugo V et al., 2004). Therefore, the PI3K/Akt/mTOR pathway represents an attractive target for cancer therapy (Engelman JA et al., 2009). Nevertheless, outside patients with renal cell carcinoma, refractory mantle cell lymphoma and ER-positive/HER2-negative breast cancer when combined with hormone therapy, the antitumour activity of the first generation of mTOR inhibitors (rapalogs) has fallen short of expectations. Reasons for this may be that these allosteric inhibitors produce incomplete inhibition of mTORC1, do not target mTORC2, and favour a rebound activation of Akt. A new generation of ATP-analog inhibitors has been developed, that include molecules targeting preferentially both mTORC1 and 2 or inhibiting also PI3K, due to similarities in the kinase domains; this double activity may be of added value for more profound inhibition of abnormal signaling in cancer cells.

In this study we have focused on the PI3K/Akt pathway, with the objective to characterize the efficacy of mTOR and PI3K inhibitors in different cellular models of MPNs, including primary cells, and in two different mouse models. This data shown that JAK2V617F mutated human and murine leukemia cell lines are sensitive to mTOR inhibitors showing a dose-dependent inhibition of cell proliferation and clonogenic potential that mainly reflected a cytostatic rather than an apoptotic effect. However, the ATP mimetic inhibitor PP242 resulted a more potent and dose-dependent inducer of apoptosis than the allosteric inhibitor RAD001, leading to speculate that such a difference may be attributable to the activity of PP242 against both mTORC complexes as opposed to the inhibition of mTORC1 only exerted by RAD001. Moreover mouse and human cells and cell lines expressing JAK2V617F are sensitive also to BEZ235 and BKM120 as single agent showing proliferation arrest, induction of apoptosis at slightly higher concentrations and, in case of BEZ235 cell cycle blockade, while BKM120 caused an increased G2/M phase of cell cycle as already reported in other cell models. We also showed that mTOR inhibitors, but also PI3K inhibitors, inhibited the proliferation of CD34 + cells and hematopoietic colony formation from

MPN patients at doses significantly lower than healthy subjects and potently reduced the generation of erythroid independent colonies (EEC) that are considered to closely represent the MPN clone since they are mostly JAK2V617F mutated. BEZ235 and BKM120 with particular efficacy inhibited megakaryocytic colonies formation: this is probably due to the important role played in this population by PI3K pathway (Vicari L et al., 2012). Furthermore they potently inhibited formation of EEC that are mostly derived from JAK2V617F mutated progenitors (Dupont S et al 2007). These results, indicating sensitivity of MPN cells to mTOR inhibitors, provided mechanistic explanation for the findings of a phase I/II trial that showed efficacy of RAD001 (Everolimus) against splenomegaly and symptomatic burden in patients with myelofibrosis (Guglielmelli P et al., 2011) and reinforce the rationale for designing clinical trials with novel, and possibly more effective, drugs targeting the activated PI3K/Akt/mTOR pathway. In fact, the abnormal activation of mTOR in cancer cells and its role in many critical cellular processes, together with the availability of a growing number of molecules entering the clinical scenario, makes it an attractive target for therapy in neoplasia where involvement of the mTOR pathway contributes to disease pathogenesis; activation of this pathway has been demonstrated in MPN cells (Bumm TG et al., 2006) JAK2V617F-expressing mice (Akada H et al., 2006) and primary samples (Ugo V et al., 2004). However, in most trials performed in different clinical settings (Meric-Bernstam F, Gonzalez-Angulo AM 2009) RAD001 and other rapalogs resulted in disease stabilization rather than tumor regression, an effect largely attributable to the predominantly cytostatic effect of these agents. Indeed, in a mouse myeloproliferative disease model characterized by constitutively active STAT5, treatment with rapamycin effectively reduced myeloid cell proliferation in transplanted mice and significantly prolonged survival; however, the myeloproliferative disease recurred once the treatment was stopped (Li G et al., 2009). Such prevalent antiproliferative rather than proapoptotic effect of rapamycin derivatives including RAD001 prompted on one side studies of combination with agents that preferentially induce apoptosis or are directed against other disease-associated targets on the other side the development of novel ATP-competitive mTOR inhibitors or dual PI3K/mTOR inhibitors. Bearing this in mind, we explored the effects of combining mTOR, Pi3K and JAK1/JAK2 inhibitors in vitro. We first determined that, in the same experimental settings, JAK2 inhibitors (AZD1480 (Hedvat M et al., 2009) and Ruxolitinib (Quintas-Cardama A et al., 2010) efficaciously impaired the growth of JAK2V617F mutated mouse and human cell lines and primary cells by slowing the progression to S-phase of the cell cycle and exerting a more definite apoptotic effect, at least in part mediated by down regulation of BclXL and PIM. Accordingly, we determined that combination of mTOR and JAK2 inhibitor resulted in significant synergism concerning the inhibition of proliferation and colony formation of mouse and human JAK2V617 mutated leukemia cell lines and prevented at very low nanomolar concentration the formation of EPO independent erythroid colonies (Bogani C et al., 2013). Combinations of mTOR inhibitor with chemotherapeutics, targeted drugs or antibodies are being explored in preclinical models and have been preliminary reported in clinical trials. At this regard, combination of rapamycin with ABT-737, an inhibitor of Bcl-XL, has been shown to provoke synergistic effects in mice with a myeloproliferative disorder due to constitutively active STAT5 and persistent activation of

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Akt/mTOR signalling (Li G et al., 2010). Moreover, the activity of BEZ235 and BKM120 against MPN cell lines and primary cells was synergistically enhanced by combination with the JAK1/JAK2 inhibitor ruxolitinib. Furthermore, we showed that combination of BEZ235 with ruxolitinib improved survival in an acute JAK2V617F-driven myeloproliferative disease in mice, and reduced splenomegaly, inhibited red cell production and improved spleen histopathology in a JAK2V617F KI mouse model. The dual PI3K and mTOR inhibitory activity of BEZ235 may indeed be advantageous (Bartalucci N et al., 2013); recent *in vitro* data from Fiskus et al. in different MPN cell models, including cell lines that had been selected for resistance against JAK2 inhibitors, support the involvement of activated PI3K/Akt in MPN cell proliferation and survival and the effectiveness of its inhibition (Fiskus W et al., 2013). The novel findings of a significant synergism exerted *in vivo* by combination of BEZ235 and ruxolitinib, that we report herein by using both a leukaemia model in immunodeficient mice injected with Ba/F3 cells harbouring JAK2V617F mutation and a JAK2V617F KI mouse model closely mimicking human MPN, give further strong support to the potential therapeutic relevance of dual JAK2 and PI3K/mTOR inhibition. Of relevance is also the fact that we observed strong synergistic activity in these models by using doses of the drugs that were lower than those showing activity when used as single agents. Since inhibition of normal haematopoiesis exerted by JAK2 inhibitors represents their main dose-limiting toxicity, we believe that our observations are important in the clinical setting by suggesting that enhanced activity could be obtained with lower dose of JAK2 inhibitor when used in combination with a PI3K/mTOR inhibitor, such as BEZ235, minimizing toxicity at the same time.

Overall, the data generated in this framework indicate that drug-mediated inhibition of PI3K/Akt/mTOR signalling is efficacious against MPN cells and can enhance the effects of JAK2 inhibition, describing synergistic activity of mTOR- and JAK/STAT-directed therapies in cellular models of MPN. Therefore, concurrent targeting of the PI3K and JAK/STAT pathways may foresee the opportunity of testing this association in clinical trials, with the expectation that such combination might result in a better therapeutic index producing more effective inhibition of clonal cells and also, due to the modest toxicity of mTOR inhibitors against normal cells, by reducing unwanted side effects of JAK2 inhibitors administered at lower, yet effective, dose.

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