Chronic Myeloproliferative Disorders: From Molecular Pathogenesis to Targeted Therapy

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Introduction

The elucidation of the molecular pathogenesis of a variety of serious or life-threatening conditions has led to the expectation that the development of safe and effective therapies will soon follow. This principle has previously been demonstrated in malignant hematology by the effectiveness of *ABL* kinase inhibitors in chronic myelogenous leukemia (CML). A new opportunity to deliver on this premise is underway in the closely related non-CML myeloproliferative neoplasms (MPNs).

The MPNs are a group of hematologic malignancies characterized by the clonal or oligoclonal proliferation of one or more myeloid lineages that arise from a polyclonal stem cell pool. The WHO classification (Swerdlow et al. 2008) of MPNs includes chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic neutrophilic leukemia, chronic eosinophilic leukemia not otherwise specified, mastocytosis, and MPN unclassifiable. This chapter will focus on the insights, research developments, and recent clinical trial results related to PV, ET, and PMF.

The modern classification of PV, ET, and PMF can be traced back to the description in 1892 by Louis Henri Vasquez of a patient with marked erythrocytosis and hepatosplenomegaly (Vasquez 1892). Vasquez postulated that this polycythemia vera resulted from a hematopoietic cell proliferation. Gustav Hueck added to the field the first description of the presence of bone marrow fibrosis and extramedullary hematopoiesis (Hueck 1879). In 1934, Emil Epstein and Alfred Goedel

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J. Tao and E. Sotomayor (eds.), *Hematologic Cancers: From Molecular Pathobiology to Targeted Therapeutics*, Cancer Growth and Progression 14,
DOI 10.1007/978-94-007-5028-9_12,

(Epstein and Goedel 1934) described a distinct clinical syndrome characterized by thrombocytosis in the absence of marked erythrocytosis. The association of these three diseases as a set of phenotypically related "myeloproliferative disorders" was contributed by William Dameshek (1951), who also noted that PV patients can later develop bone marrow fibrosis, leuko-erythroblastic changes, and increasing splenomegaly. Dameshek drew the conclusion that these changes were consistent with progression to "terminal myelofibrosis" and that it may be useful to consider the myeloproliferative disorders as "closely interrelated" disorders of bone marrow proliferation related to an "undiscovered stimulus."

The discovery of the Philadelphia chromosome and its later refinement as a balanced 9;22 translocation and the BCR/ABL transcript provided direction for understanding the molecular pathogenesis of CML (Nowell and Hungerford 1960; Rowley 1973). Subsequently, the stem cell origin of the malignant clone in CML could be clearly demonstrated by the detection of either the characteristic t(9;22) cytogenetic abnormality and its BCR/ABL transcript in all hematopoietic lineages by fluorescent in situ hybridization (FISH) or reverse transcription-polymerase chain reaction (RT-PCR) techniques. Targeting of the BCR/ABL transcript served as the rationale for the development of imatinib. Imatinib therapy has subsequently been shown to result in a reversal of the clonal dominance supported by the BCR/ABL fusion protein and results in normalization of cell morphology, peripheral blood counts, and molecular cytogenetics (including RT-PCR). A dramatic improvement in survival has been demonstrated with most therapy-compliant patients having sustained clinical remission, many with chromosomal and molecular remission. The increased understanding of the etiology of CML and the eventual therapeutic developments for CML drew a clear diagnostic and management separation between CML and the other classically described MPDs.

The magnitude of success that stemmed from targeting the BCR/ABL fusion protein offers promise that that this approach can be successful if applied to other clonal diseases associated with a unique somatic genetic lesion. To be true, three key requirements should be met. The first is the identification of a drugable target that is responsible for initiating and maintaining the disease phenotype. To achieve maximal benefit, interference with the function of that target must result in both cessation and reversal of the disease process (i.e. tumor cells can no longer survive or replicate and relevant disease associated consequences need to be reversible). Second, the intended target must be a common event present in most of the individuals affected by that disease. The frequency of the disease needs to be sufficient enough to support the clinical, scientific, and financial challenges that are inherent in drug development and clinical practice. A third key feature is the ability to selectively interfere with the unique processes and metabolism of the tumor and thus avoid unintended consequences (Walgren et al. 2005). The ease of achieving this requirement is target dependent and tissue dependent. Some targets may play significant roles in both normal homeostasis and in the neoplastic process. Other targets may have little that differentiates them from non-mutated or closely homologous forms. An inability to selectively target the form involved in the disease process may lead to dose limiting toxicities and thereby reduce the ability to maximize clinical

effectiveness. This is a potential challenge that may have two different effects when one considers genetic lesions. On one hand, the ability to selectively target only the aberrant cellular protein or process limits toxicity, improves efficacy rates, and potentially enhances the ability to modulate (for example silence) the target for improved efficacy in a selected population. On the other hand, this high degree of selectivity potentially restricts therapeutic benefit to only those patients with that particular target. The sections that follow will first briefly review characteristics of PV, ET, and PMF and then discuss the ongoing research efforts while attempting to illustrate how well these therapeutic challenges are being addressed for the non-CML MPN patients.

Non-CML MPN Disease Characteristics

Polycythemia Vera

Polycythemia vera is a chronic neoplasm of the blood that is characterized by increased red blood cell production that occurs independently of normal regulatory mechanisms. PV has been described to have three phases:

1. a prodromal phase during which a mild erythrocytosis may be detected (often retrospectively). However, some patients with early PV have normal blood counts yet may present with typical PV complications such as Budd-Chiari syndrome and the full PV phenotype eventually develops.
2. an overt polycythemic phase with a noticeably increased red blood cell mass and common symptoms and signs such as rubor and pruritus.
3. a post-PV myelofibrotic phase (post-PV MF, a form of secondary MF) in which patients exhibit disease transformation characterized by progressive cytopenias, bone marrow fibrosis, and extramedullary hematopoiesis.

Prominent clinical features of PV include hypertension, vascular abnormalities such as rubor due to high RBC mass, venous or arterial thromboses such as MI, stroke, DVT, PE, portal or splenic vein thrombosis, headache, visual disturbances, protracted dizziness, pruritus, gout, and erythromelalgia. The clinical phenotype of PV is dominated by the vascular complications. A minority of PV patients may also experience an evolution of their disease into post-PV MF (the third phase of the disease) or acute leukemia (Passamonti et al. 2004).

Original reports of disease survival suggested a median survival of less than 2 years in non-phlebotomized patients (Tefferi 2003). Changes in medical practice now facilitate much earlier diagnosis and with modern therapies and improved supportive care the median expected survival is reported in the range of 10 years or more. However, even with these improvements in care, patients with PV have been reported to have a 1.6-fold higher risk of death than the general population and a 3.3-fold higher risk of death has been reported for patients who are younger than

50 years of age at the time of disease diagnosis (Passamonti et al. 2004; Gruppo Italiano Studio 1995; Anía et al. 1994). Moreover, all currently available therapies have significant side effects. For example, alkylating myelosuppressive agents are associated with a >3-fold increase in cancer-related mortality (see Current Therapy Section for further discussion) (Gruppo Italiano Studio 1995). While transformation to acute leukemia is a rare event in PV patients, occurring with an estimated incidence of about 5 per 1,000 person-years, the outcome is disappointingly poor when leukemic transformation does occur, with a median survival of 2.9 months, independent of treatment strategy chosen (best supportive care or intensive chemotherapy) (Passamonti et al. 2005). The short median survival of these patients impedes their ability to successfully identify a suitable donor and receive hematopoietic stem cell transplant.

Essential Thrombocythemia

Similar to PV, essential thrombocythemia is a chronic neoplasm of the blood. However, ET is associated with marked elevation of peripheral platelet counts in the absence of an increased red blood cell mass. Beginning with the onset of the disease, there is an increased frequency of both major hemorrhagic events and major thrombotic events. In the absence of opportunities for routine complete blood cell count screening, many ET patients will first present with a potentially life threatening vascular complication. Major thrombotic events have been found to be prevalent in 7.6–29.4% of ET patients upon diagnosis and in 5.3–30.7% of patients during follow-up, with the occurrence of arterial thrombosis accounting for more than two-thirds of thrombotic events. Ischemic stroke still remains the most common thrombotic event among patients with ET (Papadakis et al. 2010). A retrospective study by GIMEMA (Gruppo Italiano Malattie Ematologiche dell'Adulto) found the recurrence of thrombosis in PV or ET patients with a history of major thrombosis (n = 494) to be high, with a probability of 17.7% at 2 years after the first thrombotic event. The risk of recurrence increased further to 30.8% at 5 years, and 49.9% at 10 years. Despite medical diagnosis and management of the ET patients, the nature of recurrent thrombosis continues to be life threatening. In their study, De Stefano et al. report that the most frequent first recurrent thrombosis was cerebrovascular disease (i.e. ischemic stroke or transient ischemic attacks) in 191 cases, followed by venous thromboembolism (160/494), acute coronary syndrome (106/494), and peripheral arterial thrombosis (44/494) (De Stefano et al. 2008).

The survival of patients with ET is primarily defined by disease-related complications, the primary ones being vascular disease (thrombosis and hemorrhage) and transformation to either myelofibrosis or leukemia (Cervantes et al. 2008). In a study conducted in Olmsted County, Minnesota, 5 and 10-year survival were a respectable 74.4 and 61.3%, correspondingly, but both were significantly lower than expected for age-matched controls. The cause of death was related to complications of ET, such as leukemia transformation, thrombotic complications, and gastrointestinal

hemorrhage (Mesa et al. 1999). Similar to the disease history of PV, as the time from diagnosis extends, the cumulative risk of transformation to leukemia and myelofibrosis is increased for patients with ET. In one study (Tefferi 2003), the cumulative probability for AML transformation was reported to be 1.4% at 10 years and 8.1% at 20 years, with a median time to AML transformation of 13.8 years. A higher risk was seen for transformation to myelofibrosis (i.e. post-ET MF) of 3.8% at 10 years and 19.9% at 20 years with a median time to transformation of 12.4 years.

Primary Myelofibrosis

Primary myelofibrosis (PMF) is a clonal neoplasm of the blood which is characterized by a progressive evolution or worsening of bone marrow fibrosis. The bone marrow of PMF patients may also have proliferation of megakaryocytes and granulocytes. In the fibrotic stage of the disease, the bone marrow has marked reticulin or collagen fibrosis often with osteosclerosis. Concurrently, the fibrotic stage may be characterized by leukoerythroblastosis and extramedullary hematopoiesis associated with splenomegaly caused by clonal circulating progenitor cells as demonstrated by the concordance between bone marrow and splenic cytogenetic clones (Mesa et al. 2001; Wolf and Neiman 1987). Some clinical investigators recognize an early stage of the disease, termed the "prefibrotic phase", that may present with a hypercellular bone marrow, thrombocytosis, and atypical megakaryocytes with minimal or no appreciable reticulin fibrosis.

A diagnosis of PMF is associated with a poorer prognosis than that for PV or ET. PMF is associated with a substantial reduction in life expectancy, on the order of 31% compared to gender- and age-adjusted controls (Cervantes et al. 2008; Rozman et al. 1991), with reports of median overall survival ranging from 3.5 to 7 years (Cervantes et al. 1997, 2001, 2008, 2009; Cervantes and Barosi 2005; Tefferi and Elliott 2007). The largest prognostic study reported for PMF included over 1,000 patients from 7 institutions with a reported median overall survival of 5.7 years. At the time the study was reported, 517 deaths had occurred. Among the 276 cases where the cause of death was reported, the most frequent cause of death was transformation to acute leukemia (86 cases, 31%), followed by progression of myelofibrosis without leukemic transformation (50 cases, 18%), thrombosis and cardiovascular complications (37 cases, 13%), infection (n = 29, 11%), bleeding outside of the setting of acute transformation (n = 14, 5%), portal hypertension (n = 12, 4%), and other causes (n = 48, including 12 cases of second neoplasias) (Cervantes et al. 2009).

A study of 337 patients with primary or secondary myelofibrosis in chronic phase found the risk of progressing into blast phase (persistent elevation of blasts ≥20% in blood or bone marrow) to be 4, 11, and 22% at 1, 3, and 5 years, respectively (Tam et al. 2009). The results are more disappointing when one considers that progression into blast phase is uniformly fatal (Vaidya et al. 2009). Analysis of the PMF population

from the Olmsted County, MN study reveals a median time to progression (defined as a decrease in hemoglobin of 2 g/dL, progressive splenomegaly, or onset of hypercatabolic symptoms) of 7 months, and 3-year survival of 52.4%, which was significantly lower than expected for age-matched controls (Mesa et al. 1999). Also concerning is the observation that the risk of developing a second disease of the marrow is high in PMF patients. In a Swedish study of 1,368 patients with PMF spanning the interval from 1958 to 2004, the standardized incidence ratio (SIR; calculated as the ratio of observed to expected number of cases) for any second leukemia was 26.6 with a median time of 2 years. Of the reported secondary leukemias, acute myeloid leukemia was the most frequent and was diagnosed a median of 3 years after PMF, with a SIR of 73.0 (Hemminki et al. 2009).

Allogeneic or syngeneic hematopoietic stem cell transplantation (HSCT) is the only identified curative treatment for patients with PMF (Hoffman et al. 2007). There are a number of associated challenges that limit the widespread use of HSCT including availability of a suitable matched donor, selection of appropriate conditioning or graft versus host disease (GVHD) prophylaxis regimens, and selection of recipients. In addition, the average age at diagnosis for PMF is 67, and even nonmyeloablative allogeneic HSCT is rarely done after age 65. Unfortunately, no randomized prospective clinical trials are available to facilitate these decisions and the optimal use of HSCT is not clear. Retrospective analyses of published data indicate that HSCT risks are significant (reviewed in Mesa 2010) and depend on patient age, donor compatibility, conditioning regimen, and other factors. The frequency of treatment related mortality ranges between 10 and 30%. Rates of acute GVHD and chronic GVHD are higher at 10–60% and up to 85% respectively, and overall survival ranges from 30 to 60%. Interestingly, the use of HSCT has been associated with resolution of two key chronic features of MF – splenic enlargement and bone marrow fibrosis (Ciurea et al. 2008). In a small case series, there was a progressive resolution of bone marrow fibrosis noted at 3 and 12 months post-HSCT (reducing from a median pre-treatment of grade 2–3 to a 12 month median < grade 1). Similarly, spleen sizes were noted to have "progressively decreased in every patient evaluable at each time point." While HSCT has not yet become an optimized or even a broadly applicable treatment modality, these observations suggest that with effective elimination of the PMF clone these two key chronic and debilitating features of PMF may be reversible.

MPN Research

In 1974, the observation was made that bone marrow cells from PV patients, but not normal volunteers, were able to give rise to erythroid colonies in the absence of exogenous cytokines. This capacity is defined as endogenous erythroid colony (EEC) formation (Prchal and Axelrad 1974). EEC formation was also observed in subsets of ET and PMF patients. These results provided new insights into the pathophysiology of MPNs and served to confirm the clinical conjectures from the

proceeding century. The wider availability of sequencing and array techniques in the past decade provided new opportunities to gain insight into the pathophysiology of MPN. Studies examining gene expression profiles led to the identification of several genes that were dysregulated in MPN such as *MPL* (Moliterno et al. 1998), *BCL-XL* (Silva et al. 1998), and *PRV-1* (Temerinac et al. 2000). In 2005, results emerged from several labs that pointed toward one frequent genetic lesion as a common etiology across PV, ET, and PMF.

Using a liquid culture system to study CD34+ PV cells, Ugo et al (2004) observed that whereas erythroid differentiation was an erythropoietin (EPO)-independent phenomenon, it was still mediated by signaling pathways identical to those in EPO-induced differentiation. This signaling pathway was sensitive to inhibitors of EPO-induced differentiation such as the PI3K inhibitor LY294002, the Src kinase family inhibitor PP2, and the JAK2[1] inhibitor AG490. As the highest upstream kinase identified, the role of JAK2 gained further attention. Use of short interfering RNA (siRNA) decreased JAK2 protein levels, and in cells from PV patients, JAK2 siRNA impaired spontaneous erythroid differentiation while markedly inhibiting EEC formation thus confirming the results seen with the small molecule inhibitors (James et al. 2005). These results prompted the same group, led by William Vainchenker, to look for activating mutations in the *JAK2* gene and directly lead to the identification of a G-to-T mutation at nucleotide 1849. This coding mutation leads to a substitution of phenylalanine for valine at position 617 (V617F). Confirmation of this result demonstrated that the same mutation was present in samples from 40 to 45 PV patients. However, the mutation was not observed in 15 controls, nor was it found in samples from 35 patients with secondary erythrocytosis. Further examination showed that this mutation was an acquired phenomenon.

Independently, Anthony Green and his colleagues (James et al. 2005) sequenced the coding exons of *JAK2* in 73 PV patients, 51 ET patients, 16 PMF patients, and 90 controls. The V617F mutation was not observed in any controls, but it was identified in 97, 57, and 50% of the PV, ET, and PMF patients, respectively. Their results also agreed that the gain of this mutation is an acquired event that arose in a multipotent progenitor that is capable of giving rise to erythroid and myeloid cells. They were able to demonstrate that the V617F mutation was present in all EPO-independent erythroid colonies.

Based on the speculation that loss of heterozygosity (LOH) could be a molecular basis of PV, Kralovics et al. (2002) found that LOH on the short arm of chromosome 9 (9pLOH) was a recurrent event in MPN suggesting that 9p could harbor a mutation responsible for the clonal expansion of hematopoietic cells in MPN. To test their hypothesis, Kralovics et al. (2005) performed microsatellite mapping of the 9pLOH region and DNA sequencing in 244 patients with myeloproliferative disorders

[1] Using HUGO Gene Nomenclature, human gene symbols are italicized, with all letters in uppercase (*JAK2*). Protein designations are the same as the gene symbol but are not italicized (JAK2). Mouse gene symbols are italicized, with only the first letter in uppercase (*Jak2*). Murine proteins are designation in the same fashion but are not italicized (Jak2).

Table 1 Incidence rate and frequency of mutation of MPD

Disease	US age-adjusted incidence rate/100,000[a]	V617F mutation frequency
CML	1.5	
Non-CML MPN (PV + ET + PMF)	1.57	
PV	0.79	~95%[b]
ET	0.53	60%[b]
PMF	0.25	63.5%[c]

Data source: [a]SEER and NAACCR (Rollison et al. 2008; Ries et al. 2008)
[b]GIMEMA-MPD WP (Vannucchi et al. 2007)
[c]GIMEMA-Italian Registry of MF (Barosi et al. 2007)

(128 PV, 93 ET, and 23 PMF). Their mapping identified a 9pLOH region that included *JAK2*. When the 9pLOH was present, *JAK2* had a homozygous G to T transversion, resulting in the V617F substitution. All 51 patients with 9pLOH had the V617F mutation. Their functional studies indicated that the V617F mutation provided hematopoietic precursors with proliferative and survival advantages. Furthermore, the patients they studied with the V617F mutation had a significantly longer duration of disease, a higher rate of complications (fibrosis, hemorrhage, and thrombosis), and more frequently required treatment with cytoreductive therapy than patients with wild-type *JAK2*.

Using a high-throughput DNA resequencing technique to evaluate the kinome of MPN patients, a group lead by Gilliland also reported this recurrent non-synonymous variant of the *JAK2* gene (Levine et al. 2005). Their results demonstrated that the *JAK2* V617F mutation was present in 74% of their granulocyte DNA samples from 164 PV patients and approximately one-third of these individuals were homozygous for the mutation. In their sample set, the mutation was also present in granulocyte DNA samples from 37 of 115 ET and 16 of 46 PMF patients, but was not observed in 269 normal individuals.

Regardless of the approach used, each group arrived at the conclusion that an acquired point mutation in *JAK2* resulting in a valine to phenylalanine substitution was a common event in PV, ET, and PMF. The exact frequency of this mutation in each disease is debatable as evidenced by the reported frequencies in these initial reports. In part, this may be due to factors such as sample sizes, referral biases, and methods of detection. Subsequent studies suggest that frequencies may be higher (Table 1). In terms of feasibility for new therapy development, it was suggestive that the frequency of this mutation was sufficient to warrant additional research investment from academic and industry groups. However, did this observation reduce the attractiveness of this indication as a potential target or did this potentially drugable target have a large enough population to support trial enrollments and recovery of development costs? This translates to the basic question of how many patients have this disease? Data on the incidence and prevalence of MPNs are limited. Availability of this information in the European Union is variable across member nations. US population-based registries (SEER and the NAACR) began monitoring MPDs in 2001 when these disorders were officially reclassified

as neoplasms. As a result, these sources are able to provide incidence rates but are not yet able to provide prevalence rates. The age-adjusted incidence of PV, ET, and PMF are similar and the combine incidence approximates that of CML (Table 1) (Rollison et al. 2008). In contrast to the prognosis of CML before imatinib therapy, the median survival of MPNs is longer, especially for PV and ET, and as a result the prevalence of MPNs is likely larger based on the multiple of incidence and median survival time. Hence, the results reported in 2005 demonstrated the existence of a new potential therapeutic target for a majority of MPN patients and new questions began to feed research interests.

Wild-Type and Mutant JAK2 Function

The JAK protein family is a group of cytosolic non-receptor tyrosine kinases that facilitate signal transduction from activated cytokine receptors. Upon cytokine stimulation, JAK protein interaction with the ligand bound receptor results in autophosphorylation of the JAK protein at its activation loop, in the kinase domain, resulting in an activated JAK. Activated JAK proteins subsequently phosphorylate the cytoplasmic domains of cognate receptors and additional signalling proteins such as signal transducer and activator of transcription family (STAT), the MAPK pathway, and PI3K-Akt (Yamaoka et al. 2004; Vainchenker et al. 2008). The mammalian JAK proteins, comprising JAK1, JAK2, JAK3, and TYK2, are structurally unique among protein tyrosine kinases (PTK) and are characterized by sharing a complex multidomain structure which consists of seven distinct domains termed the JAK homology (JH1–JH7) domains. Two of these domains at the C-terminal end (JH1 and JH2) are similar but not identical domains. The JH1 domain is a highly conserved PTK domain that is responsible for ATP binding and is critically important for the protein's physiological function. The cis JH2 domain, also called the pseudokinase domain or kinase-like domain, lacks catalytic activity but plays a crucial role in the regulation of the JH1 PTK domain (Boudeau et al. 2006; Saharinen and Silvennoinen 2002; Saharinen et al. 2000, 2003). When expressed *in vitro*, the JAK2 V167F protein has constitutive kinase activity in the absence of cytokines and is constitutively phosphorylated unlike wild-type JAK2 protein (Levine et al. 2005). The wild-type JAK2's phosphotransferase activity of the kinase domain is inhibited by the JH2 domain and deletion of this region in JAK2 or JAK3 significantly increases kinase activity. This JH2-mediated inhibition appears to be an inherent structurally dependent feature of the JAK protein family that does not require other regulatory proteins. The aminoterminal FERM domain plays an additional role in regulating JAK2 function both through interactions with the EPO-receptor (Funakoshi-Tago et al. 2008). The JH2 domain is capable of inhibiting the kinase activity noncompetitively, decreasing the enzyme's maximum velocity (V_{max}) without changing the substrate affinity (K_m) (Saharinen et al. 2000). The non-synonymous V617F mutation in this autoinhibitory JH2 pseudokinase domain abrogates this cis negative regulation by lowering its K_m value for substrates (Zhao et al. 2010) (Fig. 1).

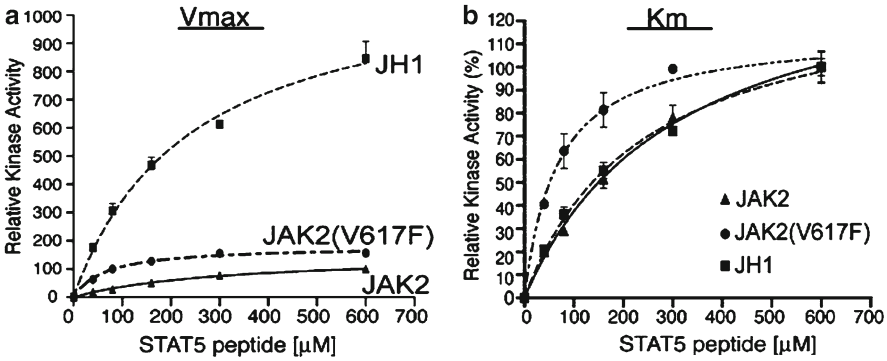

Fig. 1 JAK2(V617F) has a lower K_m towards a STAT5-derived peptide than JAK2 or JAK2 kinase domain (From Zhao et al. (2010)) (**a**) Comparison of V_{max} values for HA–JAK2, HA–JAK2(V617F) and HA–JH1. Data were normalized to maximal wild-type JAK2 activity. (**b**) Comparison of K_m values for HA–JAK2, HA–JAK2(V617F) and HA–JH1. The activity of the kinases was normalized to the maximal activity of each construct

Using biochemical methods to characterize enzymatic activity of JAK2 wild type and V617F mutant enzymes, Zhao et al (2010) demonstrated that the K_m of the V617F mutant was much lower than that of either the intact wild-type protein or the JH1 fragment across a number of substrates. In the case of STAT5 the K_m was 67 μM for JAK2 V617F, 306 μM for JAK2, and 239 μM for the JH1 domain. A similar trend was seen for a JAK2-derived peptide (a surrogate for measuring autophosphorylation). Interestingly, while the wild-type construct lacking the FERM domain was more active than the intact wild-type JAK2 (suggesting the FERM domain has an autoinhibitory role in JAK2), the activity of the JAK2 V617F mutant construct lacking the FERM domain was dramatically lower than the intact JAK2 V617F. Collectively, these results help to explain the constitutive activity seen in the V617F mutant through the demonstration that under normal intracellular conditions where concentrations of substrates are typically below saturation, the JAK2 V167F enzyme exhibits hyperactivity compared to the wild-type JAK2 enzyme. Moreover, the demonstration of selectivity in affinity between the wild-type and mutant proteins raises the possibility for selective inhibition of the mutant at the catalytic site.

How the JH1 and JH2 domains interact is not known precisely. Only a portion of the Jak2 (murine) kinase domain has been crystalized (Lucet et al. 2006). Computational models have been generated to aid in understanding this knowledge gap, and they suggest a possible JH1-JH2 interface between residues D994-E1024 in the JH1 domain and V617-E621 in the JH2 autoinhibitory domain (Lindauer et al. 2001; Giordanetto and Kroemer 2002).

An additional reported change in the regulation of JAK2 V617F is the ability to escape normal negative feedback regulation. For example, the suppressor of cytokine signalling proteins, SOCS1 and SOCS3, are normally capable of binding to wild-type JAK2 and inhibiting its kinase activity (Nicholson et al. 1999; Sasaki et al. 2000). In stark contrast, expression of SOCS3 appears to paradoxically increase

Table 2 Cytokine receptors and Janus kinase binding preferences

Receptor sub-type	Cytokine receptor	Janus kinase
Type I		
Homodimeric cytokine receptors	EPOR, TPOR, GHR, PRLR, G-CSFR	JAK2
Cytokine receptors sharing βc subunit	IL-3R, IL-5R, GM-CSFR	JAK2
Cytokine receptors sharing gp130 subunit	IL-6R, IL-11R, OSMR, LIFR	JAK1, JAK2, TYK2
Cytokine receptors sharing γc subunit	IL-2R, IL-4R, IL-7R, IL-9R, IL-15R, IL-21R	JAK1, JAK3
Type II		
Interferon & others	IFNα, IFNβ, IFNγ, IL-10R, IL-19R, IL-20R, IL-22R, IL-24R, IL-28R, IL-29R	JAK1, JAK2, TYK2

JAK2 V617F protein stability resulting in increased SOCS3 phosphorylation and increased JAK2 V617F phosphorylation (Hookham et al. 2007).

Three Diseases from One Genetic Lesion?

Based on the observation that the *JAK2* V617F mutation is not present in all of the PV, ET, and PMF patients one could question if this is a causal lesion. However, in aggregate the data suggest that there are multiple pathways for activation of JAK2 that appear to give rise to a functionally similar etiology that is defined by dysregulation of JAK2. The source for three different disease manifestations of this acquired genetic lesion appears to rest on two contributors – genetic background and gene dosage.

One consideration that has implications on this question is the diversity of roles that JAK2 plays in normal hematopoiesis. JAK2 is a binding partner for numerous Type I and Type II cytokine receptors (Table 2). Given the range of cytokine receptors and associated ligands that rely on JAK2, either alone or in combination with other Janus kinases, it is easy to see the crucial role that JAK2 can play in hematopoiesis starting from the level of long-term and short-term hematopoietic stem cell differentiation to the more lineage committed differentiation (Fig. 2). It is interesting to note that the role JAK2 plays is more limited along the avenues of differentiation that give rise to lymphocytes, both T-cells and B-cells.

A number of studies have demonstrated that retroviral expression of Jak2 V617F in murine bone marrow transplant assays results in significant PV-like phenotype in the transplant recipient mice (James et al. 2005; Lacout et al. 2006; Wernig et al. 2006; Zaleskas et al. 2006; Bumm et al. 2006). Recipient animals demonstrate a peripheral erythrocytosis which can progress to myelofibrosis, but thrombocytosis was not demonstrated in these studies. Leukocytosis was observed in the Balb/C

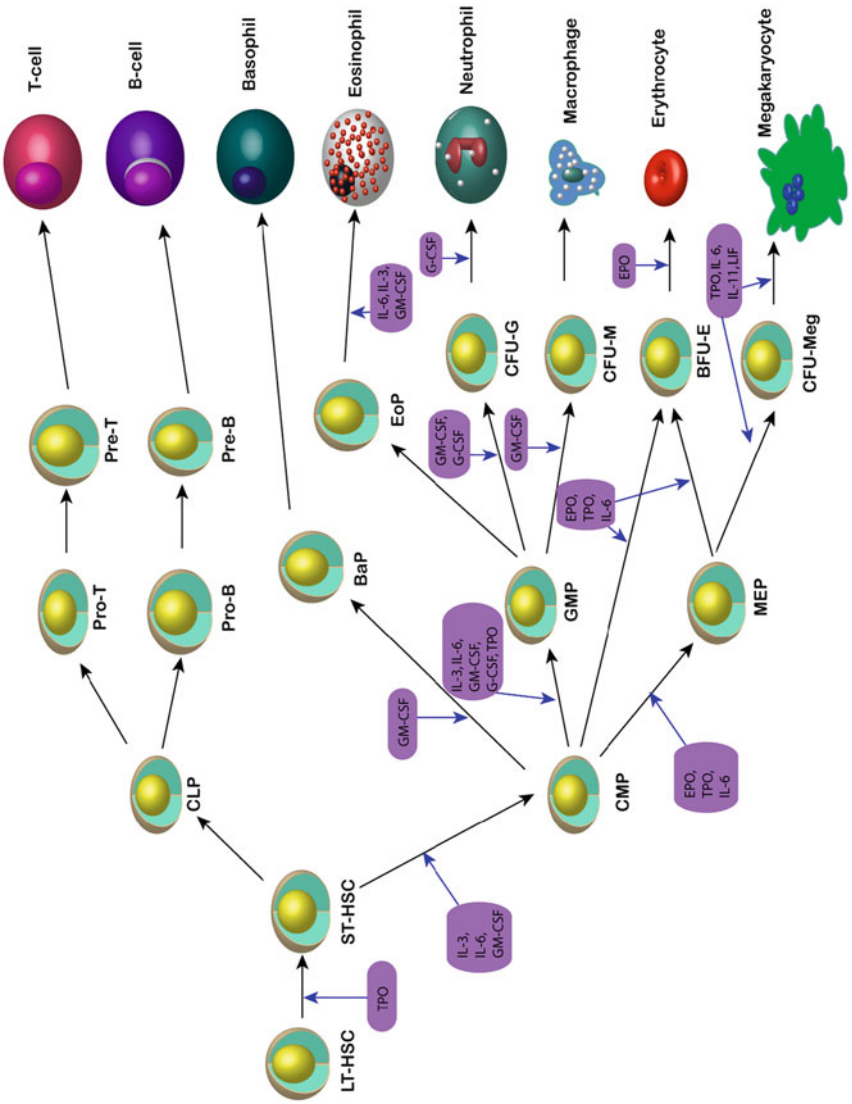

Fig. 2 Role of JAK2 dependent cytokine signaling in hematopoiesis

strain but was not present in studies with the C57Bl/6 strain. It has been suggested that the failure to observe an ET-like phenotype in these studies together with the observed difference in susceptibility to leukocytosis between genetic strains could stem from the need for additional genetic events to support the development of the different MPN phenotypes. Potential criticisms of these studies include their use of a murine *Jak2* cDNA in which the G to T mutation at codon 617 was introduced and the failure to evaluate the role of the natural promotor for *JAK2*. To address these issues, Tiedt et al. (2008) employed an inducible transgenic technique to demonstrate that human *JAK2* V617F is also capable of inducing an MPN phenotype in mice. Through the use of a combination of inducible promotor systems, they were able to develop transgenic strains with varying human JAK2 V617F expression levels. In mice with expression levels of JAK2 V617F that were lower than the endogenous wild-type Jak2, the resulting phenotype bore resemblance to ET with elevated platelet counts and moderate neutrophilia. In a transgenic strain that expressed JAK2 V617F at a level approximating that of the wild-type Jak2, a PV-like phenotype was observed with increased hemoglobin, thrombocytosis, and neutrophilia.

Employing a transgenic approach combined with the use of a tissue specific promoter, Xing et al. (2008) generated murine lines that model ET, PV, and PMF. *JAK2* V617F copy number varied in these three derived transgenic lines and was determined to be 13 ± 1.4, 1.9 ± 0.3, and 24 ± 2.7, respectively, for their A, B, and C mice. Transgenic line A mice, which had a high level of JAK2 V617F expression, demonstrated a marked increase in platelet number as well as increases in white blood cell counts, hemoglobin, and hematocrit, thus having a phenotype resembling that of ET or PV. The transgenic line B mice exhibited more moderate elevations in cell counts that did not reach the levels more characteristic of ET or PV. Spleen weights in the line A mice were 2 to 8-times larger than control animals and 10 of 26 line A mice developed fibrosis in the bone marrow and spleen after 30 weeks of age. These findings were not noted in examination of animals less than 30 weeks of age. Development of fibrosis was reported to be a much less frequent event in the lower copy number line B mice. The line C founder mouse, which had the highest gene copy number, demonstrated a more aggressive phenotype, and this animal died at 4 weeks. At that time the line C mouse was noted to have a spleen 10 times the size of comparably aged control mice.

Examination of heterozygous *Jak2* V617F Gene knock-in mice demonstrated constitutive Jak2 activation and autonomous erythroid progenitor cell growth. These mice manifested a severe PV-like disorder that progressed to PMF with decreased bone marrow cellularity and splenomegaly associated with marked increases in erythroid (88-fold) and myeloid (82-fold) precursors (Marty et al. 2010). Interestingly, the embryonic development of this disease was not fatal; however, the authors noted that the severity of the disease could contribute to embryo-lethality of a similar inherited disorder in humans. Akada et al. (2010) independently employed a Gene knock-in mouse model yielding heterozygous and homozygous Jak2 V167F expression patterns. Heterozygous expression resulted in a phenotype characterized by polycythemia due to excessive production of erythrocytes despite low serum EPO levels, increased hematocrit and

hemoglobin, leukocytosis with neutrophilia, thrombocytosis, and extramedullary hematopoiesis with splenomegaly. These results demonstrate that heterozygous Jak2 V617F expression is sufficient for induction of PV. Homozygous expression was associated with greater increases in reticulocytes and peripheral cell counts as well as a more pronounced fibrosis of the bone marrow and splenomegaly. Basal activation of the Stat5, Akt, and Erk1/2 pathways was greater in the homozygous state suggesting that wild-type Jak2 may be able to compete with the mutant Jak2 V617F when coexpressed.

Greater understanding of the influence of genetic complexity of MPN is also emerging. Standard karyotyping techniques have shown abnormal cytogenetics are present in approximately 30% of MPN patients, occurring more frequently in PMF but less frequently in PV and ET (Gangat et al. 2008, 2009; Hussein et al. 2009; Panani 2007; Reilly 2008). As previously described in this chapter, microsatellite studies identified uniparental disomy of the short arms of both chromosomes 9 and 1 (9pUPD and 1pUPD), contributing to the identification of *JAK2* V617F and mutations in *MPL* respectively (Kralovics et al. 2002). The sensitivity of cytogenetic evaluation that is now possible has increased with the availability of high-resolution DNA microarrays. This has facilitated identification of deletions and UPDs of 4q associated with *TET2* (Delhommeau et al. 2009; Tefferi et al. 2009), as well as abnormalities in 11q associated with *CBL* (Dunbar et al. 2008; Sanada et al. 2009). In contrast with the 9p and 1p UPDs, which are known to be associated with enhance JAK2 signaling, the influence of mutations in *TET2* and *CBL* is presently not clear. Additional mutations in *ASXL1* (Carbuccia et al. 2009) and *IDH1/2* (Green and Beer 2010) and deletions in *IKZF1* (Jager et al. 2010) have been identified in association with leukemic transformation (post-MPN AML).

When higher resolution techniques have been employed the frequency of genomic aberrations appears to be more common. In a sample of 408 MPN patients (162 PV, 80 ET, 79 PMF, 29 post-MPN AML, and 58 patients with secondary MF or accelerated phase) analyzed by high-resolution SNP microarrays only 37.5% of patients had a wild-type karyotype and the remainder harbored at least one chromosomal aberration (Klampfl et al. 2011). Of these patients, 297 (72.8%) exhibited a *JAK2* mutation and all patients with 9pUPD (n = 169) were positive for the *JAK2* V167F mutation. Six of the seven patients with 1pUPD were positive for *MPL* W515L. An additional 25 aberrations were recurrent in 3 or more patients.

When Klampfl et al. (2011) further examined the frequency of chromosomal aberration and other disease factors, they noted no association with disease duration. However, patient age at the time of sample collection was positively associated with the number of defects. Interestingly, patients with *JAK2* mutations were not found to carry more chromosomal aberrations then their *JAK2 V617F* negative counterparts. No aberration was specifically associated with *JAK2* V617F negative MPN. *JAK2* V617F homozygosity (9pUPD) was associated with secondary myelofibrosis or accelerated phase (sMF/AP) suggesting that gene dosage may predispose for a higher risk of secondary myelofibrosis in both PV and ET (post-PV MF or post-ET MF). Chromosome 1q amplifications, all of which amplified *MDM4*, were also associated with sMF/AP as well as post-MPN AML. The *MDM4* gene

product is an inhibitor of p53 and its amplification may set the stage for MPN disease progression as loss of p53 function is associated with a number of malignancies and has been shown to participate in leukemic transformation in MPN (Beer et al. 2010; Laurie et al. 2006; Riemenschneider et al. 2003).

Current Therapy

Polycythemia Vera (PV)

Current treatment practices do not cure PV but, rather, attempt to control the disease related symptoms and decrease the likelihood of complications. Historically, agents such as radioactive phosphorus and alkylating agents (melphalan and busulfan) were used to reduce red blood cell mass. Use of these agents is associated with an increased risk of leukemic transformation (Gruppo Italiano Studio 1995), and as a result, these agents are typically reserved for use in patients >70 yo. Currently, phlebotomy is the frontline modality employed by many practitioners with the goal of reducing the hematocrit to ≤45%. However, a prospective study of 1,638 PV patients found no difference in risk of death, thrombotic events, or hematologic progression when hematocrits were in a range between 35 and 55. Thus, the role of hematocrit in PV thrombosis and benefits of phlebotomies are unclear (Di Nisio et al. 2007). Findings from this same population did suggest that high platelet counts might be associated with a decreased risk of hematologic transformation and myelofibrosis. When compared to patients with a platelet count of $\leq 300 \times 10^9$/L, patients whose platelet counts were in the range of either $301–500 \times 10^9$/L or $>500 \times 10^9$/L, the risk of myelofibrosis was 54% lower (HR 0.46; 95% CI: 0.21–1.02, $P = 0.550$) and 66% lower (HR 0.34; 95% CI: 0.12–0.97, $P = 0.431$), respectively. This later finding is of particular interest in light of the previously discussed murine MPN model studies that demonstrated loss of thrombocytosis and more rapid progression of myelofibrotic features in models with higher gene dosage. However, when platelet count exceeds 100×10^{10}/L, there is an increased risk of bleeding attributable, at least in part, to an acquired von Willebrand disease (Michiels 1999).

After the initial reduction in red blood cells is achieved, patients are typically treated with maintenance phlebotomy with the goal to maintain the same target hematocrit. During maintenance therapy, the frequency of phlebotomy requirements will typically decline as patients develop an intentional iatrogenic iron deficiency state. The development of iron deficiency may assist in subsequent control of red blood cell counts. Phlebotomy may increase the number of platelets, and while it may attenuate some disease related symptoms, it typically does not reduce the size of an enlarged liver or spleen. Thus, even patients who respond to phlebotomy may also require pharmacologic intervention.

Non-specific cytoreductive therapy can be achieved with hydroxyurea (hydroxycarbamide). Myelosuppression with hydroxyurea offers a greater reduction in symptoms than phlebotomy. Although hydroxyurea can help to decrease splenomegaly,

it rarely results in complete resolution. Treatment with hydroxyurea usually is well-tolerated but does require routine monitoring of cell counts and often requires dose modification or holidays to avoid significant myelosuppression. In some studies hydroxyurea therapy has been associated with poor tolerance due to the development of leg ulcers, buccal aphthous ulcers, gastric pain, or diarrhea (Najean and Rain 1997a, b). In addition, when used for many years there is concern that hydroxyurea therapy may increase the risk of transformation to leukemia. Despite numerous attempts to define this risk it remains the subject of debate (Finazzi et al. 2005). It is unclear whether this leukemogenesis is secondary to hydroxyurea or results from progression of the underlying MPN disease.

Alternative drugs for lowering the number of platelets, such as interferon-alpha and anagrelide, are sometimes used in younger people who may need treatment for long periods. The use of interferon and its pegylated forms for the management of MPNs is an area of active research. In exploratory studies, pegylated interferon-alpha has been reported to result in hematologic improvements in up to 80% of PV and ET patients. Pegylated interferon-alpha may reduce the allele burden of *JAK2* V617F and may restore polyclonal hematopoiesis with extended therapy (Liu et al. 2003; Kiladjian et al. 2006, 2008; Quintas-Cardama et al. 2009).

Low-dose aspirin is frequently used in PV as an antiplatelet therapy intended to reduce the risk of thrombotic events. However, use of aspirin has been controversial. At one time aspirin therapy was avoided due to the observation by the Polycythemia Vera Study Group that a high incidence of gastrointestinal bleeding occurred in patients who received 900 mg of aspirin daily (Tartaglia et al. 1986). A pilot study (Gruppo Italiano Studio Policitemia (GISP) 1997) suggested that the use of low-dose aspirin was not associated with bleeding complications in PV patients, and these results enabled further study of low-dose aspirin by the European Collaboration on Low-Dose Aspirin in Polycythemia Vera (ECLAP) (Finazzi 2004; Landolfi et al. 2004). The ECLAP projected included 1,638 patients, enrolling 1,120 into a prospective, observational cohort study. The remaining 518 patients were enrolled in a parallel randomized, double-blinded, placebo-controlled trial to assess the efficacy and safety of enteric coated low-dose aspirin (100 mg per day). Patients were excluded from the randomized trial and directed to the observational study if they had a recognized need for anti-thrombotic therapy, a contraindication to aspirin, or were unwilling to participate in the randomized study. In the observational study (Finazzi 2004), 40% of deaths were due to a cardiovascular event, and the incidence of fatal, major and minor thrombosis was 5.5 events per 100 patients per year. Factors associated with an increased risk of cardiovascular events included age greater than 65 years, history of thrombosis, smoking, hypertension, and congestive heart failure. Antiplatelet therapy was the only variable associated with a lower risk of thrombosis. In ECLAP's randomized study (Landolfi et al. 2004), treatment with aspirin was associated with a reduced, but not statistically significant, risk of nonfatal myocardial infarction, nonfatal stroke, or death from cardiovascular causes (RR 0.41, 95% CI 0.15–1.15; $P = 0.09$). However, when secondary analysis also included pulmonary embolism and major venous thrombosis in the combined endpoint, the

relative risk was significantly decreased (RR 0.40, 95% CI 0.18–0.91; $P=0.03$). This study did confirm the pilot study's finding that low-dose aspirin was not associated with a significant risk of major bleeding episodes (RR 1.62, 95% CI 0.27–9.71). In a systematic review of the literature that include the ECLAP study, Squizzato et al. (2008), concluded that the use of aspirin in PV was associated with a lower, but not statistically significant, risk of fatal thrombotic events in PV patients (OR 0.20, 95% CI 0.03–1.14).

Stemming from increased turn-over of nucleic acids and an associated increased production of uric acid, PV and other MPN patients may develop hyperuricemia, gout, or renal injury that can be managed with allopurinol therapy. Other drugs may be considered to assist in management of symptom burden such as treatment of itching with antihistamines, and use of NSAIDs for headaches, bone pain, and burning sensations in the hands and feet.

Essential Thrombocythemia (ET)

Therapy for ET is primarily focused on management of peripheral cell counts with cytotoxic agents and reduction of arterial and venous thrombotic events. Drugs frequently used include hydroxyurea, anagrelide, and interferon-alpha. Treatment with one of these drugs and aspirin is typically started when clotting complications develop. Therapy may be initiated prophylactically when indicated by a risk assessment. Independent predictive risk factors include: age, platelet count $>1{,}000\times10^9$/L, leukocyte count $>12\times10^9$/L, prior history of thrombosis, smoking, and diabetes (De Stefano et al. 2008; Tam et al. 2009; Landolfi et al. 2006; Elliott and Tefferi 2005). While not yet independently validated, De Stefano et al (2008) have also reported the presence of the *JAK2* V617F mutation to be associated with a 3.8-fold enhanced risk for thrombosis in patients less than 60 years of age. If drug treatment does not sufficiently slow platelet production in emergent situations drug therapy may be replaced by platelet pheresis.

The use of either hydroxyurea and aspirin or anagrelide and aspirin has been compared in a large randomized study. In high-risk patients with ET, hydroxyurea with aspirin was demonstrated to have an overall superiority at the composite endpoint of risk of arterial thrombosis, venous thrombosis, serious hemorrhage, or death from thrombotic or hemorrhagic causes (Harrison et al. 2005). While both treatment assignments were equivalent in long-term control of platelet counts, the anagrelide plus aspirin treatment was associated with significantly higher rates of arterial thrombosis (OR 2.16, 95% CI 1.27–3.69, $P=0.004$), serious hemorrhage (OR 2.61, 95% CI 1.27–5.33, $P=0.008$), and transformation to myelofibrosis (OR 2.92, 85% CI 1.24–6.86, $P=0.01$). Hydroxyurea plus aspirin was noted to be better tolerated with a lower treatment discontinuation rate. In contrast, anagrelide was noted to have better activity preventing venous thrombosis (OR 0.27, 95% CI 0.11–0.71, $P=0.006$). Looking at the risk of hematologic transformation, 16 of 405 ET patients receiving anagrelide plus aspirin transformed to post-ET MF compared to

5 of 404 on the hydroxyurea plus aspirin arm. The estimated actuarial risk of secondary myelofibrosis 5 years after trial entry was 2% for the hydroxyurea group (95% CI 0–5) and 7% for the anagrelide group (95% CI 3–10).

Myelofibrosis (MF)

Although classified differently by the WHO, primary myelofibrosis (PMF) and secondary myelofibrosis are clinically managed similarly. Generally speaking, outcomes in MF are determined by the degree of retained bone marrow function, the risk of arterial and venous cardiovascular events, and by the risk of leukemic transformation. MF may progress slowly as some individuals may live for 10 years or longer. In others with higher risk or lower levels of bone marrow function, the disorder can worsen rapidly. A number of risk stratifications techniques are available to help individualize a patient's risk assessment and treatment strategy. The Lille scoring system has been a widely used method of risk stratification based on two adverse prognostic factors (Dupriez et al. 1996). Scoring based on the presence of a hemoglobin <10 g/dL and a WBC of either <4 or $>30\times10^9$/L, allows separation into one of three groups with *low* (0 factors), *intermediate* (1 factor), and *high risk* (2 factors), associated with respective median survivals of 93, 26, and 13 months. Other more recent risk stratification tools for MF include the International Prognostic Scoring System (IPSS) (Cervantes et al. 2009), the Dynamic International Prognostic Scoring System (DIPSS) (Passamonti et al. 2010) and the Dynamic International Prognostic Scoring System Plus (DIPSS Plus) (Gangat et al. 2011). The IPSS uses five adverse risk factors determined at the time of diagnosis. Age >65 years, the presence of constitutional symptoms, hemoglobin <10 g/dL, leukocyte count $>25\times10^9$/L, and circulating blasts ≥1%. Each risk factor is assign 1 adverse risk point. Detection of 0, 1, 2, and ≥3 risk factors defines *low*, *intermediate-1*, *intermediate-2*, or *high-risk* and the corresponding association with median survivals of 11.3, 7.9, 4, and 2.3 years. The limitation of this tool is its restriction for use only at the time of initial diagnosis.

The introduction of the DIPSS, which was also developed by the International Working Group for Myeloproliferative Neoplasms Research and Treatment, allows application of the tool throughout the course of disease (Passamonti et al. 2010). DIPSS scoring assesses the same risks used in the IPSS, but DIPSS assigns a risk score of 2 points for hemoglobin <10 g/dL and adjusts the assignment of risk category to the following score totals: *low* (0 adverse points), *intermediate-1* (1 or 2 points), *intermediate-2* (3 or 4 points), and *high risk* (5 or 6 points). With the DIPSS, the corresponding median survivals were not reached for the low risk group, and were 14.2, 4, and 1.5 years respectively for intermediate-1, intermediate-2, and high risk scores. Whereas the DIPSS was validated for use during clinical course to assist in treatment decisions, the DIPSS does not allow modification of risk scoring for cytogenetic abnormalities, platelet counts, or transfusion status. These deficiencies have been addressed in the DIPSS Plus with the assignment of 1 point each for unfavorable karyotype, platelets lower than 100×10^9/L, and need for red cell

transfusion (Gangat et al. 2011). Unfavorable karyotypes were defined as complex karyotype or single or two abnormalities including +8, −7/7q−, i(17q), −5/5q−, 12p−, inv(3), or 11q23 rearrangement. In this tool, categories were assigned as *low risk* with 0 points, *intermediate-1* with 1 point, *intermediate-2* with 2–3 points and *high risk* with 4 or more points. The median survival times associated with each DIPSS Plus risk group are 180, 80, 35, and 16 months respectively. Each of these models has merits for application with current treatment options, and yet each has omitted potentially predictive factors in order to reduce complexity and maintain usability. The future will undoubtably present new refinements to these models and new models are likely, especially as new treatment options and refinements in disease understanding become available.

Once the risk is assessed, treatment options for MF are limited and not satisfactory. Currently, only stem cell transplantation can cure the disorder. However, HSCT is not a treatment option for most MF patients and is often performed under research protocols. Alternate treatments used in MF are intended to relieve symptoms or prevent complications and little is available to delay the progression of the disorder.

A prominent disease feature requiring treatment is anemia. Anemia in MF is often a challenge to treat due to its complex origins. In a disease characterized by excessive JAK2-STAT signalling it is not surprising that EPO stimulating agents are often ineffective (Huang and Tefferi 2009). Use of single agent or combination use of an androgen and/or prednisone temporarily lessens the severity of the anemia in about one third of people with myelofibrosis (Cervantes et al. 2007). The immunomodulatory IMiDs (i.e. thalidomide, lenalidomide, and pomalidomide) which inhibit TNF-α and have been tested for their ability to improve anemia in myelodysplastic syndrome are being explored for use in MF (Begna et al. 2011; Lacy and Tefferi 2011; Mesa et al. 2010). Use of the DNA hypomethylating agent 5-azacitidine has also been examined in phase II studies in MF patients. Despite demonstrating the induction of global hypomethylation, 5-azacitidine therapy was associated with limited clinical activity in MF patients (Mesa et al. 2008; Quintas-Cardama et al. 2008). Although potential therapies for anemia are available, the efficacy of these therapies in MF is limited, and many patients will require red blood cell transfusions. Platelet transfusions may also be required to treat thrombocytopenia with bleeding. The degree of transfusion requirements may also be a confounding issue for individuals who are transplant candidates due to the development of transfusion-associated allosensitization. As with other neoplasms, MF related myelosuppression can predispose patients to bacterial infections and the resultant need for antibiotic therapy.

Symptomatic splenomegaly is another characteristic problem in MF that is thought to develop from a combination of factors including sequestration of immature circulating myeloid progenitors and proliferation of extramedullary hematopoiesis (Mesa et al. 2001; Zhang and Lewis 1989). Available treatment options include hydroxyurea, surgery, and splenic irradiation. Use of hydroxyurea has been discussed in the sections on PV and ET above. Many of the same tolerability and safety issues apply in the management of MF and may even be exacerbated by the need to use higher doses of hydroxyurea (i.e. 2–3 g/day) (Mesa 2009). The resulting cytopenias

may in fact be problematic limiting the ability to continue high doses. Even with the use of higher doses, it is not common to achieve a sustained 50% reduction in spleen size which is a bench mark criteria for clinical improvement as defined by the International Working Group for Myelofibrosis Research and Treatment (Tefferi et al. 2006). Therapy with alkylating agents can be considered for control of splenic enlargement in some MF patients. This approach should be undertaken with caution due to the potential risk of therapy associated leukemia. With oral melphalan therapy, two-thirds of patients may have reductions in spleen size, but in one study, 26% of treated patients developed acute leukemia (Petti et al. 2002).

Therapeutic splenectomy is another potential option. Removal of the spleen may increase the number of red blood cells and reduce the need for transfusions in some patients. Splenectomy has no clear positive impact on survival or disease course and may, in fact, have a net negative effect due to the associated rates of complication (27.7%) and fatality (6.7%) (Mesa et al. 2006).

A third option for management of splenomegaly is radiotherapy. Extramedullary hematopoiesis is as sensitive to radiation as is medullary hematopoiesis. Thus, involved sites such as the spleen, lungs, or paraspinal masses are sites suitable for external beam radiotherapy. Unfortunately, the associated myelosuppression from this procedure can be severe, the long term benefit is often limited, and adjacent tissue injury can create additional problems (e.g. abdominal visceral adhesions) (Mesa 2009).

Evolution in Therapeutic Understanding

Whether JAK2 dysregulation is the initial disease defining event or a consequence remains unanswered. However, the evolving *in vivo* pre-clinical data does clearly illustrate that constitutive activity of JAK2 V617F is sufficient to recapitulate the MPN phenotypes. As such, there is interest in determining whether inhibition of JAK2 or selective inhibition of JAK2 V167F could result in clinical benefits. In 2007, the first JAK inhibitor was tested in MF patients. This is a relatively quick response time compared to that for CML where decades passed between the identification of the genetic lesion and the introduction of a targeted inhibitor. The ability to rapidly move an experimental therapeutic into an MPN focused clinical trial was facilitated by the availability of multitargeted kinase inhibitors that had activity against the Janus kinase family. At the time that JAK inhibitors started clinical testing it was unclear whether responses in MPN patients would parallel that observed in CML with imatinib. To date this has not been the case. However, lack of selectivity may limit the ability to adequately inhibit the mutant clones due to toxicities stemming from non-selective inhibition. Recognizing this possibility, other drug development groups have taken an approach of identifying new molecules that possess higher degrees of selectivity for JAK2 (TG101348) or for the JAK2 V617F mutant (LY2784544) (Hood et al. 2007; Ma et al. 2010).

Non-clinical testing of the JAK2 inhibitors has been based on a combination of *in vitro* enzymatic biochemical assays, *in vitro* cell based assays, *in vitro* model testing,

and *ex vivo* testing of patient samples such as the endogenous erythroid progenitor (EEC) assay. Testing of potential candidate molecules typically begins with biochemical enzyme screening. Molecules of interest are then selected for further testing. Most cell based proliferation studies have verified *in vitro* enzyme assay results, demonstrating effective inhibition of JAK2 V617F expressing cells. The observed potency in enzymatic assays is often higher (i.e. lower IC_{50}) than what is observed in cell based assays (Table 3). Interrogations of signalling pathways, also, typically confirm reductions in JAK2 dependent signalling pathways such as STAT3 or STAT5 phosphorylation. Several agents (including TG101348 (Lasho et al. 2008), CEP-701 (Hexner et al. 2008), SGI-1252 (Ahmed et al. 2011), LY274544 (Florensa et al. 2010), CYT387 (Pardanani et al. 2009)) have demonstrated the ability to inhibit growth-factor independent progenitor cell growth in patient derived samples.

Much of the available non-clinical study results for the JAK2 targeting molecules currently in clinical development come from *in vitro* work and less information is available regarding testing in the *in vivo* murine models. In part, this is a reflection of the fact that development of many of these molecules occurred before the development of the murine models. As a result, few of these agents have been tested in the context of different gene dose levels or across multiple genetic backgrounds.

Using a transgenic mouse model, ruxolitinib (INCB018424), an inhibitor of JAK1 and JAK2 (Table 3), was shown to decrease splenomegaly and eliminate neoplastic cells from the bone marrow, spleen and liver (Fridman et al. 2007). XL019 and SGI-1252 have been studied in a tumor xenograft model with human erythroid leukemia cells (HEL), a *JAK2* V617F homozygous cell line, injected subcutaneously in athymic nu/nu mice. In this model, a reduction in STAT5 phosphorylation but not total STAT5 levels was observed when SGI-1252 was dosed at 400 mg/kg orally 3 times a week (Ahmed et al. 2011). Dosing with XL019 twice daily led to an increase in apoptosis and decreased tumor vasculature (Verstovsek et al. 2007). TG101348 was studied in mice who had received bone marrow cells from donor mice after the cells had been retrovirally transfected with either *Jak2* wild-type or *Jak2* V617F. Transplanted mice who received TG101348 at 120 mg/kg oral gavage twice a day demonstrated a decrease in hematocrit, splenomegaly, and reticulin fibrosis in the bone marrow and had a prolonged survival (Wernig et al. 2008).

Another MPN model employs the use of SCID mice injected with GFP-positive BaF/3 cells expressing JAK2 V617F. With this model, normal murine peripheral blood cell counts (i.e. endogenous wild-type bone marrow derived cells) are suppressed. In addition, BaF/3 *JAK2* V617F cells preferentially localize to the spleen, resulting in splenomegaly. Oral treatment with TG101209 at 100 mg/kg twice daily for 10 days prolonged survival and reduced STAT5 phosphorylation (Lasho et al. 2008). Oral administration of LY2784544 for 7 or 14 days in the BaF/3 *JAK2* V617F xenograft model demonstrated dose-dependent reductions in STAT5 phosphorylation, reductions in splenic enlargement, and a decreased BaF/3 *JAK2* V617F-GFP tumor burden with a TED50 of 13.7 mg/kg (Ma et al. 2010). LY2784544, demonstrated an apparent dose dependent selectivity for the mutant JAK2 V617F kinase (Table 3) and in the BaF/3 *JAK2* V617F xenograft model, treatment with LY2784544 showed no effect on normal murine erythroid progenitors (CD71/Ter119 positive

Table 3 Kinase selectivity of JAK2 inhibitors in clinical development

Inhibitor	Assay type	Literature reported IC_{50} (μM)					Other reported targets	References
		JAK1	JAK2	JAK2 V617F	JAK3	TYK2		
XL019	Enzyme	0.130	0.002		0.250	0.340		Paquette et al. (2008) and Verstovsek et al. (2007)
ruxolitinib (INCB018424)	Enzyme	0.0033	0.0028		0.428	0.019		Quintas-Cardama et al. (2010)
	Cell prolif.			0.127				
lestaurtinib (CEP701)	Enzyme		0.001				FLT3, PDGFR, Trk-A, RET	Dobrzanski et al. (2006) and Verstovsek (2010)
TG101348	Enzyme	0.105	0.003		0.996	0.405		Hood et al. (2007) and Wernig et al. (2008)
	Cell prolif.			0.270				
SB1518	Enzyme		0.023	0.019			FLT3	Verstovsek et al. (2010a)
LY2784544	Cell prolif.		1.36	0.003				Ma et al. (2010)
CYT387	Enzyme	0.011	0.018		0.155	0.017		Pardanani et al. (2009) and Tyner et al. (2010)
	Cell prolif.		1.424	1.5				

cells) or neutrophils/monocytes (CD11b/Gr-1 positive cells) after treatment for 7 days. Treatment with LY2784544 at 80 mg/kg restored the murine platelet counts to the levels seen in untreated non-tumor bearing animals.

In a murine model of MPN in which lethally irradiated Balb/c mice were transplanted with bone marrow donor cells that had been retrovirally transduced with *JAK2* V617F, twice daily oral gavage treatment with CYT387 was associated with a dose-dependent reduction or normalization of white cell counts, hematocrit, and spleen size (Tyner et al. 2010). Treatment at the highest dose (50 mg/kg twice daily) was associated with a statistically significant reduction in mutant positive cells in the spleen but failed to eliminate the *JAK2* V617F/GFP-positive cell population.

Emerging Clinical Trial Results

Clinical trial results for a number of the JAK2 targeting agents are emerging in abstract form, and peer-reviewed results have been published describing the exploratory experience in primary and secondary MF with three of these agents (lestaurtinib, ruxolitinib, and TG101348) (Verstovsek et al. 2010b; Pardanani et al. 2007; Santos et al. 2009).

Clinical development of lestaurtinib (CEP-701) in MF began in phase II prior to development as a FLT-3 inhibitor for use in AML. Studies in AML had previously defined a recommended oral dose regimen of 80 mg daily with the most frequently observed adverse events being nausea, emesis, anorexia, and diarrhea (Smith et al. 2004). In the first MPN phase II study of lestaurtinib (NCT00494585) 22 *JAK2* V617F positive primary or secondary (post-PV or post-ET) MF patients were studied (Santos et al. 2009). All but two had received prior therapy. Six previously treated subjects had a clinical improvement response by the International Working Group Myelofibrosis Research and Treatment criteria (IWG-MRT). The IWG-MRT is a composite set of MF disease response criteria that define complete remission (CR), partial remission (PR), clinical improvement (CI), progressive disease (PD), stable disease (SD), and relapse based on physical examination, laboratory, and histologic evaluation elements (Tefferi et al. 2006). Of the patients who received CEP-701 and achieved a response, three had a decrease in spleen size greeter than 50%, two became transfusion independent, and another had a greater than 50% decrease in spleen size with an improvement in platelet and neutrophil counts. No patients had improvements in bone marrow fibrosis. Three of the patients who achieved a response had an abnormal karyotype, but none demonstrated a cytogenetic response to therapy. The lestaurtinib therapy study also failed to demonstrate an effect on *JAK2* V617F allele burden. GI toxicities were the most commonly reported study related adverse event with 72% having diarrhea of any grade, 50% nausea (grade 1–2), 27% vomiting (grade 1–2) and 23% with flatulence (grade 1–2). Of those treated, eight experienced grade 3 or 4 toxicities of thrombocytopenia

(23%), anemia (14%), and diarrhea (9%) with both thrombocytopenia and diarrhea resulting in dose reductions in some patients.

Ruxolitinib (INCB018424) was examined in a phase I-II trial (NCT00509899) in primary or secondary MF patients regardless of *JAK2* V617F status (Verstovsek et al. 2010b). Study participation required being refractory to or relapsing after prior therapy, or having intermediate or high risk according to the Lille risk score (Dupriez et al. 1996), or having splenomegaly or hepatomegaly if post-splenectomy. Dose exploration began with a standard 3 + 3 dose escalation schema but transitioned to a dose titration paradigm after establishing maximum tolerated doses of 25 mg twice daily or 100 mg once daily. Thrombocytopenia was identified as the dose limiting toxicity and at the 100 mg daily dose level, 2 of 6 patients developed clinically significant thrombocytopenia during the second month of therapy. Further exploration established that a 15 mg twice daily oral starting dose followed by individualized dose titration was the most tolerated regimen. Of the studies patients treated with this regimen, 52% achieved a ≥50% reduction in splenomegaly (occurring during the first 3 months of therapy) with <10% experiencing grade 3 or 4 toxicities. Splenic responses were durable beyond 1 year in the subset of patients with data for 1.5–2 years of follow-up. Although the best splenic response was seen in the group who received 25 mg twice daily, 60% of these patients required a dose reduction due to thrombocytopenia. Subgroup analysis revealed no differences in response between patients with or without the *JAK2* V617F mutation. No difference was seen among patients with primary or secondary MF. In addition to the objective improvements observed in spleen size, ruxolitinib therapy was associated with significant improvements in total and individual symptom scores as collected by the Myelofibrosis Symptom Assessment Form (MFSAF). These symptom improvements were durable through 6 months of therapy.

Within the group of patients receiving the highest ruxolitinib dose intensity regimens (either 15 mg twice daily or 25 mg twice daily), 28 patients were transfusion dependent at baseline. Four of these patients achieved transfusion independence after a median duration of 12 weeks. Study eligibility criteria excluded MF patients with a platelet count of $\leq 100 \times 10^9$/L and those with an absolute neutrophil count of $\leq 1.5 \times 10^9$/L. As a result the study could not evaluate the ability of ruxolitinib therapy to improve these two disease associate features. Across all assigned doses, 23% of the patients who were transfusion-independent at baseline developed anemia. A dose dependent association was suggested by the observation that the frequency of study related anemia ranged from 8% among the patients treated at 15 mg twice daily to 27% among the patients who received 25 mg twice daily.

Results of the TG101348 phase I dose escalation study have been reported (NCT00724334) (Pardanani et al. 2007). This trial also employed a 3 + 3 cohort design followed by a cohort expansion for dose-confirmation to study once daily oral administration of TG101348 in high- or intermediate-risk primary or secondary MF patients across the range of doses from 30 to 800 mg. Intrapatient dose escalation was permitted after completion of three treatment cycles at the assigned starting dose. Risk assessment was determined by the Mayo PSS (Elliott et al. 2007) which is similar to the previously described risk assessment tools. The Mayo PSS defines high-risk as having two criteria and intermediate-risk as having one criteria where

criteria include: hemoglobin <10 g/dL, WBC count <4 or >30 × 10^9/L, platelet count <100 × 10^9/L, absolute monocyte count ≥1 × 10^9/L. The study enrolled 59 patients (75% PMF, 20% post-PV MF, and 5% post-ET MF), and the majority (86%) were *JAK2* V617F positive.

The study's declared maximum tolerated dose was 680 mg daily based on the development of reversible grade 3 or 4 hyperamylasemia at 800 mg. Across all patients, the most common adverse events were related to the gastrointestinal system. In the MTD cohort (680 mg/d), frequent GI events included nausea (77.5% grade 1–2, 5% grade 3–4), diarrhea (62.5% grade 1–2, 12.5% grade 3–4), and vomiting (67.5% grade 1–2, 2.5% grade 3–4). Hyperlipasemia was also frequent at this dose level (37.5% all grades, 15% grade 3–4). Treatment related grade 3 and 4 hematologic adverse events were observed. Of the patients treated with 680 mg per day who were not transfusion dependent at baseline, 54.2% developed grade 3 or 4 anemia and 27.5% developed grade 3 or 4 thrombocytopenia.

Sixty-one percent of the cohort who received 680 mg per day experienced a minimum 25% decrease in palpable splenomegaly within the first two cycles and 45% of patients receiving this dose had spleen size reduction qualifying as a criteria for clinical improvement by IWG-MRT (i.e. at least a 50% reduction lasting at least 8 weeks). This response was durable at cycle 12 with 50% having a clinical improvement (CI) defined by the spleen response. In addition, 72% of the patients with a baseline leukocytosis who received 680 mg/d for 6 cycles (13/18) achieved a normal WBC.

The TG101348 phase I trial is the first to report a statistically significant reduction in *JAK2* V617F allele burden. In patients harboring the *JAK2* V617F mutation, the allele burden at base line ranged from 3 to 100% with a median of 20%. Across all baseline values, without respect to study drug dose, there was a statistically significant reduction of allele burden to 17% ($P = 0.04$) and 19% ($P = 0.01$) after 6 and 12 cycles respectively. Looking only at those individuals with a baseline allele burden above 20%, the median was 60% prior to therapy and decreased to 31% after 6 cycles ($P = 0.002$) and 32% at 12 cycles ($P = 0.002$). In this group 45% (9 patients) had a ≥50% reduction in *JAK2* V617F allele burden, but 20% exhibited an increase.

Conclusions and Path Forward

Each of the three early clinical development studies for patients with MF discussed illustrate that the first group of experimental JAK2 inhibitors may offer clinical benefits through reductions in disease related symptoms and control of splenomegaly. To date, the studies have failed to demonstrate evidence of disease modification. These three studies have not yet reported a clinically significant ability to improve histiologic changes in bone marrow fibrosis, nor have they demonstrated a significant positive impact on cytopenias. Apparent compound-related adverse events are being observed during use of these agents.

The MPNs remain diseases where the approved therapy is primarily palliative, minimally effective, and complicated by safety concerns. In this setting the advances seen with the first wave of JAK2 inhibitors in development are suggestive of significant therapeutic progress and may offer new hope to patients. The results have led to interests in the study of combination therapies as a next step in the advancement of MPN therapy. The iterative effort of combining non-selective JAK2 inhibitors with other agents currently being studied in MPN such as the IMiDs or hypomethylation agents is a promising approach. However, our growing awareness of the molecular pathophysiology of this disease suggests that these efforts may not cure the MPNs, and these combinations are likely to be accompanied by at least additive side effect profiles.

The results of our current clinical data set may be looked at in a different light. HSCT has shown that effective eradication of the malignant clone can improve disease related symptoms, splenomegaly, and reverse the accumulated bone marrow fibrotic injuries. JAK2-directed therapy has demonstrated that disease symptoms are improved with inhibition of the JAK2/STAT pathway and the resultant modulation of cytokine signaling. In addition, JAK2 therapies have demonstrated that there is a relationship between dose, target inhibition, improvements in splenomegaly, and modulation of peripheral cell counts. Thus, it appears that maximally effective disease modification will require two things: (1) selective inhibition or eradication of the tumor cells and (2) retention or restoration of normal marrow reconstitution. Achieving both may lead to reversal of the disease manifestations similar to what has been demonstrated in HSCT. In the published clinical trials reviewed here, the ability to test this hypothesis was limited by target dependent inhibition of normal marrow. The observed dose limiting toxicities suggest that effective disease targeting will require exploration of the role of selective *JAK2* V617F inhibitors.

A potential additional confounder for defining effective therapy is the underlying heterogeneity of the MPN phenotypes. A single therapy used only at a single dose or regimen may not achieve the same degree of clinical effect in all subjects. This observation is supported by the observed genetic variability of the disease as well by the variability of responses seen with the available JAK2 therapies. This heterogeneity suggests that tailoring of therapy will be required for optimal care. Different patients may require potentially different therapies for first- or second-line therapy. With introduction of effective therapy it is predictable that some patterns of resistance will be identified requiring combination therapy or changes in drug selection. Genetic heterogeneity has implications for trial designs, regulatory approval, and companion diagnostic needs. There is debate about the appropriateness of current diagnostic classifications systems and whether they should be reconsidered in light of the new understandings of molecular pathogenesis (Harrison 2010).

There are additional challenges related to patient selection and evaluation of clonal burden reductions. Newer risk models such as the DIPSS and DIPSS Plus offer promise for risk assessments and benchmarks for evaluating the effect of therapy on overall survival. This is an important consideration given the duration of time and number of patients needed to demonstrate an improvement in overall survival or progression-free survival in MPN. An unavoidable challenge in the

evaluation of any potentially disease modifying therapy is the need to evaluate the long term effects of therapy. This is especially true for the MPNs where there is diversity in overall survival times and potential heterogeneity for disease responsiveness. It is clear that the most advanced MF patients have a significant reduction in overall survival. It is not clear, if the disease process is equally amenable to therapy for both a newly diagnosed MPN patient or for an advanced MPN patient. In the case of CML, patients in chronic phase are more responsive to imatinib therapy then those in blast phase. Tools analogous to the DIPSS are needed to appropriately define and identify those ET and PV patients who are at greatest risk from their disease. Hopefully, the availability of patient selection discoveries will help to mitigate some of these issues, but this is not likely to resolve the conflict between ongoing and immediate need for therapy vs need for long-term outcome evaluations.

Non-clinical studies have suggested that gene dosage plays a role in defining disease phenotype and clinical complication risks. This will almost certainly be of interest in evaluating and selecting potential therapies. However, the ability to effectively validate whether gene dosage plays a role in the human disease process is hampered by the lack of a standardized reference test to measure *JAK2* V617F allelic burden in patient samples. This problem is more confounding to drug efficacy evaluations. The published JAK2 clinical trials have used different tests and each has its own unique characteristics such as differences in cell population analyzed (granulocytes vs mononuclear cells), source of control (cell lines vs plasmids), range of control alleles used in standardization, primers, etc. As a result, each *JAK2* assay has a different performance profile including variations in limits of detection, sensitivity, and specificity. While different clinical laboratories often pragmatically use such diverse tests, regulatory approvals that depend on laboratory test results for efficacy evaluation or patient stratification may require more rigorous validation.

MPN patients have a clear need for better therapy today. The basic and clinical research progress in the MPN field has truly accelerated in the past decade. More importantly, this progress looks poised to deliver new treatment options for MPN patients. It appears that soon, the first wave of JAK2 inhibitors will be reviewed for regulatory approval. If approved, patients and physicians could have access to a new tool for disease and symptom burden reduction. Based on the available data, this should not be a stopping point but should rather be viewed as the front runner of even more promising future therapies which are capable of meeting the needs of MPN patients and their families.

Author's Note

Following completion of this chapter, the US FDA granted approval in November 2011 for ruxolitinib phosphate for the treatment of patients with intermediate or high-risk myelofibrosis, including primary myelofibrosis, post-polycythemia vera myelofibrosis and post-essential thrombocythemia myelofibrosis.

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