



From Palliation to Epigenetic Therapy in Myelofibrosis

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Myelofibrosis shows a progressive clinical course and usually a poor, lethal prognosis. The molecular pathogenesis of this disease largely remains to be fully understood but the identification of the *JAK2V617F* mutation in more than half of patients was a major improvement in our understanding of the disease biology and may represent the first biologic marker useful for risk stratification, independently from conventional clinical predictors. After many elusive efforts, new effective treatment strategies are becoming available for this disease. Allogeneic transplanta-

tion following reduced-intensity conditioning programs, at least in some patients, may induce not only a hematologic response but also a molecular remission, thus supporting the hope of a possible, definitive eradication of the disease. Moreover, new innovative drugs, targeting either the *JAK2V617F* mutation or more general oncogenic mechanisms, may provide widely applicable, effective treatments to many patients for whom allogeneic transplantation is not feasible.

Introduction

Myelofibrosis is a clonal proliferative disease of hematopoietic stem cells, leading to an inappropriate cytokines release, fibrosis of the bone marrow, constitutive mobilization of committed progenitor cells into the peripheral blood and extramedullary hematopoiesis.^{1,2} This disease may present either as idiopathic (primary myelofibrosis, PMF) or as transformation of an antecedent polycythemia vera (PV) or essential thrombocythemia (ET). Myelofibrosis is characterized by a progressive clinical course and a shortened life expectancy, with median survival after diagnosis of less than 5 years. Established prognostic factors including age, hemoglobin level, and white blood cell count have been used for risk assessment, but these characteristics do not fully explain the risk of death or major clinical events. Morbidity and mortality are usually the result of leukemic transformation, spleno-portal hypertension, and infections, as well as thrombosis and hemorrhage.³

A major improvement in our understanding of the disease biology and future therapeutic perspectives has recently come from the identification of the somatically acquired Janus kinase 2 (*JAK2*) mutation (V617F) which is detectable in more than half of the patients with primary myelofibrosis and post-ET myelofibrosis and in nearly all of those with a secondary form following a previous PV.⁴ Despite its crucial pathogenetic role, the clinical relevance of *JAK2V617F* in myelofibrosis is not completely understood. It has been suggested that patients with *JAK2V617F* mutants usually have higher white blood cell counts and a history of thrombosis or pruritus and are less likely to require transfusion during follow-up. Divergent results were

obtained as far as the effect of *JAK2V617F* mutation on survival and evolution to acute leukemia.⁵⁻⁸ In a recent large retrospective survey we reported that the *JAK2V617F* mutation plays a significant and independent influence on the disease phenotype and showed that many clinical manifestations are correlated with the expansion of clonal hematopoietic cells harboring the *JAK2V617F* mutant allele. Any level of mutant alleles was found to favor higher concentration of hemoglobin and aquagenic pruritus. Low levels of mutant alleles, as in the heterozygous state, favors higher platelet count, whereas high levels, as in the homozygous state, are positively associated with a hyperproliferative profile with higher white blood cell count, larger splenomegaly, and greater need of cytoreductive therapy. Most importantly, the strongest influence of the mutated genotype is on the risk of leukemic transformation that was 5.2 times as great as in non-mutated patients. Thus, *JAK2V617F* mutation should be considered a prognostic risk factor independent from the conventional predictors and may represent a target of novel drugs directed against the constitutively active *JAK2* kinase.⁹

The definition of important aspects of the molecular pathogenesis of this disease and the availability of innovative and potentially curative treatment modalities has also promoted international scientific initiatives for the definition of new, rigorous and accurate criteria for the response assessment of patients with myelofibrosis.¹⁰ The International Working Group (IWG) has recently defined not only 3 main clinical response categories (CR, PR, and clinical improvement) but also recommended baseline and follow-up evaluation of cytogenetic and molecular remission. These response

criteria are now of paramount importance particularly when considering drugs which may target key molecular lesions involved in the pathogenesis of the disease.¹¹

Palliative Treatments for Splenomegaly and Anemia

Until recently, the main treatment goal of most patients with myelofibrosis has been the palliation of symptoms related to massive splenomegaly and anemia. Splenic enlargement must be treated when progressive in order to prevent the development of massive, unmanageable splenomegaly. Cytotoxic chemotherapeutic drugs such as busulfan, chlorambucil, 6-thioguanine, melphalan and hydroxyurea can be useful. However, concern has been raised as to their role in promoting blast transformation. Interferon- α has also been used, but despite its similar cytoreductive effects and less transforming activity, it may not be as well tolerated as hydroxyurea, which remains the most commonly used and best tolerated cytoreductive treatment. Although no formal evaluation of efficacy has been done by using internationally agreed response criteria, a 40% response on splenomegaly is expected with the use of hydroxyurea. However, the clinical response achieved with chemotherapy is usually short, lasting a median of only 4.5 months, and only 16% of patients on long-term maintenance therapy enjoy sustained relief of symptoms.

Splenic irradiation has been used for treatment of the big spleen syndrome. Irradiation in fractions of 0.15 to 1 Gy administered daily or by an intermittent fractionation schedule (i.e., 2 or 3 times per week) to a total dose per treatment course of 2.5 to 6.5 Gy may be effective. Responses are transient, lasting an average of 3.5 months, and hematopoietic toxicity is frequently significant. Radiation therapy is considered as a temporary measure to be employed in patients who are too ill to tolerate splenectomy or chemotherapy.

Splenectomy may also be indicated for patients with painful splenomegaly, recurrent splenic infarctions, documented hemolysis, transfusion-dependent anemia or refractory thrombocytopenia. Improvements in constitutional symptoms, transfusion-dependent anemia, and portal hypertension may be achieved, but splenectomy is associated with a postoperative morbidity rate of 15% to 30% and a mortality rate of almost 10%, both largely due to episodes of bleeding, infection, and thrombosis. Rapidly progressive hepatomegaly and thrombocytosis may also occur in about 20% of patients after splenectomy.

Anabolic steroids such as danazol remain a well-tolerated and effective treatment of anemia, with a favorable response achievable in about 40% of patients and only moderate and transient adverse events.^{12,13} A pooled analysis of the results obtained with recombinant human erythropoietin showed response rates ranging from 16% to 60%.¹⁴ Serum erythropoietin levels <125 U/L, favorable cyto-

netic abnormality (13q- or 20q-), absence of homozygous *JAK2V617F* mutation, low β 2-microglobulin serum levels and slight to moderate splenomegaly were associated with a favorable response.¹⁵ However, a recent analysis raised concern as to the possible role of danazol and erythropoietin in promoting leukemic transformation of myelofibrosis.¹⁶

Hematopoietic Stem Cell Transplantation

Allogeneic stem cell transplantation is the only curative approach in patients with myelofibrosis, but the most appropriate conditioning regimen for the transplant procedure has not been established yet. After standard, myeloablative conditioning, 5-year survival rates of about 50% has been reported but at the cost of a treatment-related mortality exceeding 30%.¹⁷ In a recent updated Seattle study, it has been reported that patients conditioned with a myeloablative targeted dose of busulfan plus cyclophosphamide (tBUCY) had a 68% probability of survival and a non-relapse mortality of 34% after 5 years.¹⁸ To reduce the high treatment-related mortality and to increase the eligibility to the transplant procedure non-myeloablative preparative regimens have been tested. Using a conditioning regimen based on reduced busulfan (10 mg/kg), fludarabine (180 mg/m²) and antithymocyte globulin (ATG), Kröger and co-workers reported 3-year overall and disease-free survival of 84% and treatment-related mortality of 16% at 1 year.¹⁹

In another retrospective analysis Rondelli reported 21 patients whose median age at transplantation was 54 years and who received different conditioning regimens. Interestingly, post-transplant evaluation showed 100% stable chimerism in 86% of patients, whereas 10% achieved complete chimerism only after donor lymphocyte infusion (DLI). Relapse or progression of the disease was observed in 14% while 85% were alive a median 31 months after transplantation.²⁰ After the busulfan/fludarabine/ATG-based conditioning regimen, a complete or nearly complete regression of bone marrow fibrosis was seen in 59% at day +100, in 90% at day +180, and in 100% at day +360 with no correlation between occurrence of acute graft-versus-host disease and fibrosis regression.²¹ With other conditioning regimens, however, a progressive reduction of marrow fibrosis and spleen size can still be ongoing after 36 months following allogeneic transplantation.²⁰

For patients who are candidates for transplant the role of splenectomy is still matter of debate. In patients with a spleen greater than 30 cm in length prior to transplant, a delayed neutrophil and platelet engraftment has been observed. A pretransplant splenectomy might therefore facilitate the time to engraftment but can also be associated with serious postoperative complications.²² For patients bearing the *JAK2V617F* mutation, a highly sensitivity (at least 10⁻⁴), real-time PCR based monitoring of minimal residual disease has been developed. By this approach it is

possible to quantitatively evaluate the presence of the JAK2V617F-mutated allele after allogeneic stem cell transplantation and to demonstrate the achievement of a molecular remission in more than 70% of the patients receiving a reduced conditioning. The molecular follow-up data correlated very well with the clinical course and proved very effective to determine the quality of remission as well as to guide the optimal timing for immunologic treatment strategies such as DLI.²³ However, identifying patients who should be offered allogeneic transplantations remains difficult. Most likely, allogeneic transplantation should be considered for all patients under the age of 50 years and a myeloablative conditioning should be considered only for those younger than 40 to 45 years, while a reduced-intensity approach is for older or unfit patients.²⁴ However, age is not the only crucial prognostic factor and comorbidities, or cytogenetic abnormalities should also be included in the decision-making process. Younger patients must be monitored very carefully and allogeneic transplant offered even to low-risk patients if clinical evidence of disease progression is documented. On the other hand, if some of the new promising new molecule prove to be active in inducing good quality remission, allogeneic transplantation will probably remain a second-line therapy limited only to those patients with an incomplete or absent response to the medical treatment.²⁵

To develop an active treatment strategy to control massive hepato-splenomegaly without causing undue morbidity and mortality and for patients lacking a suitable donor, the feasibility of autologous transplantation has also been investigated. In a pilot study, 27 patients with a median age of 59 (range 45-75) were mobilized at steady state (n = 2), after granulocyte colony-stimulating factor (G-CSF) alone (n = 17), or after anthracycline-cytarabine induction plus G-CSF (n = 8). A median of 11.6×10^6 CD34⁺ cells per kilogram were collected so that most patients could undergo myeloablation with oral busulfan alone (16 mg/kg) followed by autologous peripheral blood stem cell infusion. However, the hematologic engraftment was slow and a back-up PBSC infusion was necessary in 40% because of delayed neutrophil or platelet recovery. Although symptomatic splenomegaly improved in 70% of patients and hemoglobin response occurred in 59% of anemic patients, the early mortality rate due to non-relapse causes (14%) and rapid disease progression (14%) were not negligible.²⁶

Targeted Therapy

Thalidomide, lenalidomide and other drugs targeting angiogenesis

Thalidomide is an anti-angiogenic and immunomodulatory drug that inhibits several pro-inflammatory cytokines. Thalidomide may induce improvement of anemia and thrombocytopenia particularly if combined with prednisone. With

this regimen, 62% of patients had an objective and sustained response in the anemia and 40% became transfusion independent.²⁷ Other studies did not obtain similarly good results, possibly due to the use of different doses of thalidomide or patient selection.²⁸ Notably, the use of high-dose thalidomide (100-600 mg/day) may be remarkably poorly tolerated, particularly due to sedation, neuropathy and constipation, and the dropout rate may exceed 60% of patients.

Compared to thalidomide, lenalidomide is a second-generation derivative with 2000-fold higher activity in terms of TNF- α inhibition and most importantly with a superior toxicity profile. When given at 10 mg/day for 3 to 4 months, the overall response rate was 22% for anemia, 33% for splenomegaly and 50% for thrombocytopenia. Additional treatment effects observed in some patients included resolution of leukoerythroblastosis, reduction in medullary fibrosis and angiogenesis, and occasionally, cytogenetic remission and reduction in JAK2V617F mutation burden.²⁹ Another thalidomide analog, pomalidomide (CC-4047), is up to 10,000 times more potent at inhibiting TNF- α than thalidomide and is currently under testing both in United States and Europe.

PTK787/ZK 222584 (PTK/ZK) is a tyrosine kinase inhibitor of the vascular endothelial growth factor receptor (VEGFR), the platelet-derived growth factor receptor (PDGFR), c-Kit and c-FMS. In a recent Phase II study, 29 patients received a continuous dosing schedule of PTK/ZK (500-750 mg twice daily) and 1 patient achieved complete remission. However, the overall clinical activity of this molecule was considered modest.³⁰

Sunitinib is an anti-angiogenic drug that inhibits PDGF receptors, VEGF receptors and stem cell factor receptor (c-Kit), thus making it an attractive molecule for myelofibrosis. It also blocks the proliferation and induces apoptosis of acute myeloid leukemic cells by inhibiting phosphorylation of FLT3-ITD, FLT3-Asp835, and FLT3-WT.³¹

Bevacizumab, commercially marketed as Avastin, is a monoclonal antibody currently used for patients with metastatic colon cancer. For its activity against VEGF it may be also an effective agent for the treatment of PMF. To evaluate the safety and the efficacy of bevacizumab treatment in patients with PMF a Phase II study has been recently launched within the Myeloproliferative Disorders-Research Consortium (MPD-RC). Administration will be as a continuous IV infusion given at the dose of 15 mg/kg every 3 weeks for at least 4 cycles before assessment of clinical response.

Signal transduction inhibitors

Based on its ability to inhibit PDGFR signaling, imatinib was tested at doses ranging between 200 and 800 mg/day. However, the use of this drug not only failed to translate into clinical benefit but was also poorly tolerated since treatment was halted in 70% due to side effects such as

neutropenia, musculoskeletal pain, edema and, surprisingly, thrombocytosis. This latter effect is probably related to a direct stimulation of bone marrow progenitors and megakaryopoiesis.³²

Tipifarnib (R115777; Zarnestra™; Ortho Biotech Products, L.P., Bridgewater, NJ) is a farnesyl transferase inhibitor with significant in vitro antiproliferative activity on myeloid and megakaryocytic hematopoietic progenitor cells from patients with myelofibrosis.³³ In a recent Phase II clinical trial, tipifarnib was given to 34 symptomatic patients with either primary or post-PV/ET myelofibrosis, but early termination of the trial was necessary in 56% of patients due to disease progression (21%) and adverse drug effects (18%), mainly myelosuppression and neuropathy. The response rate was 33% for hepato-splenomegaly and 38% for transfusion-requiring anemia. Clinical response did not correlate with either degree of colony growth or measurable decrease in quantitative JAK2V617F, and no favorable changes occurred in bone marrow fibrosis, angiogenesis or cytogenetic status.³⁴

MK-0457 is a potent and selective inhibitor of the Aurora family of serine/threonine kinases, which are essential to control the cell cycle progression, and is able to inhibit JAK2 at nanomolar concentrations.³⁵ Clinical trials have been launched in patients with chronic myeloproliferative disorders,³⁶ but safety issues are now under consideration.

GX15-070MS is a small-molecule antagonist of the BH3-binding groove of the Bcl-2 family of proteins that can inhibit the interactions of Bcl-2 with proapoptotic molecules. GX results in early and vigorous induction in vitro of apoptosis of CLL cells. For its ability to inhibit Bcl-2 the drug has also been proposed for a Phase II single-agent study for patients with myelofibrosis.

The proteasome inhibitor bortezomib

The extensive fibrosis and osteosclerosis observed in myelofibrosis have been hypothesized to be secondary to a reactive process mediated by inflammatory mediators, including transforming growth factor β (TGF- β) and osteoprotegerin (OPG). Hematopoietic cells from patients with myelofibrosis have recently been identified as having activation of the NF- κ B pathway, which in turn activates the production of TGF- β , and interleukin (IL)-1, a stimulator of OPG. Based on these observations and results obtained in a murine model³⁷ clinical trials have been launched to test the efficacy and safety of bortezomib in patients with myelofibrosis. In a recent Phase II clinical study, however, therapy with bortezomib was found substantially ineffective and, according to the International Working Group for Myelofibrosis Research and Treatment response (IWG-MRT) criteria, no patient achieved a clinical improvement.³⁸ In another Phase I-II study performed by the MPD Research Consortium, 12 patients with myelofibrosis, refractory or not suitable to first-line chemo-

therapy, were treated with bortezomib 0.8-1.3 mg/m² given at days 1, 4, 8, and 11 every 21 days \times 6 cycles. Dose-limiting toxicity (DLT) occurred in 1 patient treated with bortezomib in the 1.3 mg/m² cohort, consisting of respiratory distress syndrome. The maximum tolerated dose was 1.3 mg/m² for 4 days every 3 weeks. No complete, major or moderate responses according to the EUMNET response criteria were documented. Therefore, these results do not seem to support the initial enthusiasm about this drug given the preclinical demonstration of myelofibrosis inhibition in the murine model.

JAK2 Inhibitors

The development of JAK2 inhibitors is an obvious and rational approach for a therapeutic intervention to inhibit JAK/STAT-signaling in MF patients who have a JAK2 activation not only as a consequence of the JAK2V617F mutation but also mutations in MPL (MPLW515L/K)³⁹ or other alternative mechanisms. Several small molecule inhibitors of JAK2 have been synthesized and are actively tested as potential new drugs for the treatment of ET, PV and myelofibrosis (**Table 1**).

TG101209 and TG101348

TG101209 is an orally bioavailable small molecule that potently inhibits JAK2, FLT3 and RET kinases, with significantly less activity against other tyrosine kinases, including JAK3. TG101209 may induce cell-cycle arrest, apoptosis, and inhibition of JAK2V617F, STAT5 and STAT3 phosphorylation in a human JAK2V617F-expressing acute myeloid leukemia cell line. Furthermore, TG101209 suppressed growth of hematopoietic colonies from primary progenitor cells harboring not only JAK2V617F but also MPL515 mutations. Interestingly, TG101209 induced a significant animal survival when tested in an in vivo mouse model of JAK2V617F disease, and the clinical benefit correlated with inhibition of JAK2V617F activity in vivo.⁴⁰ TG101348 is a related, ATP-competitive inhibitor designed and synthesized using structure-based drug design methods to inhibit JAK2, but not other closely related kinases.⁴¹ TG101348 inhibits colony growth in patients with chronic myeloproliferative disorders (CMPD) more potently than in healthy controls, reflecting its potential therapeutic window. Endogenous colony growth is inhibited at nanomolar concentrations in cells obtained from CMPD patients harboring not only the JAK2V617F but also MPLW515L/K or JAK2 exon 12 mutations. In addition, TG101348 inhibits proliferation and induces apoptosis in a human erythroblast leukemia (HEL) cell line that harbors the JAK2V617F mutation, as well as in the murine Ba/F3, pro-B cell line expressing human JAK2V617F.^{42,43}

TG101348 was able to selectively inhibit the formation of erythroid colonies from lentiviral transduction of cord blood progenitors with JAK2V617F.⁴⁴ Moreover, in a

Table 1. New drugs under clinical development in myelofibrosis.

Drug	Company	Phase	Indication	Route	Primary target
JAK2 inhibitors					
INCB01842447	Incyte	Phase I/II	Myelofibrosis	Oral	JAK2
XLO1945	Elelixis	Phase I	Myelofibrosis	Oral	JAK2
TG10134841	TargeGen	Phase I/II	Myelofibrosis	PMF	JAK2
HDAC inhibitor					
ITF235767	Italfarmaco	Phase II	Essential thrombocythemia, Polycythemia vera, Myelofibrosis	Oral	HDAC
Methyltransferase inhibitor					
5-azacitidine ⁵⁸	Pharmion	Phase II	Myelofibrosis	Subcute	DNA methyltransferase
Drugs targeting angiogenesis					
CC-4047 (Actimid [®])	Celgene Corp.	Phase I/II	Myelofibrosis	Oral	angiogenesis
Bevacizumab (Avastin [®])	Genentech	Phase II	Myelofibrosis	I.V.	VEGF
Sunitinib	Pfizer	Phase II	Myelofibrosis	Oral	PDGFR VEGFr, FLT-3, c-KIT
Others					
MK-0457 (VX680)	Merck	Phase I	Relapsed/refractory hematological malignancies, including JAK2617V>F-positive MPD	I.V.	Aurora kinases (A,B,C)
CEP-70149	Cephalon	Phase II	Myelofibrosis	Oral	FTL3
AT9283	Astex Therapeutics	Phase I/II	Myelofibrosis	I.V.	Aurora kinases
GX15-070MS	Gemin X	Phase II	Myelofibrosis	I.V.	Bcl-2

murine bone marrow transplant assay of established PV, animals treated with TG101348 showed a dose-dependent reduction in the degree of splenomegaly, extramedullary hematopoiesis and reduction of bone marrow reticulin. A dramatic survival advantage was documented for animals treated with TG101348 compared with animals treated with placebo. These findings indicate that efficacious treatment of the PV like syndrome may result in reversion of myelofibrosis.⁴¹

XL019

XL019 has a potent selective and reversible inhibitory activity on JAK2. XL019 downregulates STAT signaling in cell lines expressing both wild-type and activated forms of JAK2. IC₅₀ for inhibition of STAT5 phosphorylation by XL019 ranged from 623 nM (on the HEL cell line) to 3398 nM (on KG-1 cell line). Based on these in vitro as well as in vivo data using a murine xenograft model with the human HEL cell line (maximum tumor growth inhibition of 60%), XL019 is being evaluated in subjects with primary or post-PV/ET myelofibrosis in a Phase I dose escalation study. Preliminary evidence of clinical activity was observed in most subjects, including reduction in spleen size, reduction in erythropoietin-independent colony formation and relief of constitutional symptoms, including pruritus, fatigue, back pain and abdominal fullness. Adverse events

were mild and included grade 1 nausea, headaches, equilibrium imbalance, dizziness, chest discomfort, visual disturbances, fatigue and hypertension.⁴⁵

INCB018424

INCB018424 is a potent, selective, and orally bioavailable inhibitor of JAK2. INCB018424 inhibits the proliferation of BaF/3 cells expressing JAK2V617F with an IC₅₀ of 100–130 nM and this correlates with the inhibition of the JAK/STAT pathway. INCB018424 inhibited the cytokine-independent formation of erythroid progenitor colonies from patients with PV with an IC₅₀ of 67 nM while normal colony formation from healthy donors was inhibited 50% at > 400 nM. In a murine model where implantation of BaF/3 cells expressing JAK2V617F results in rapid organomegaly and reduced survival, oral administration of INCB018424 was well tolerated, eliminated neoplastic cells from the spleen, liver, and bone marrow, and prolonged survival.⁴⁶ In a recent Phase I/II study conducted in patients with primary or post-PV/ET myelofibrosis at the dose of 25 to 50 mg PO BID, INCB018424 resulted in a rapid and marked reduction in splenomegaly. No dose-limiting toxicities or other significant adverse events occurred in any patients to date and the pharmacokinetic analysis demonstrated that administration of INCB018424 resulted in plasma drug concentrations suf-

ficient to markedly inhibit JAK2. In some patients the percentage of JAK2V617F-positive cells detectable in the peripheral blood decreased but did not disappear.⁴⁷

CEP-701

CEP-701 (also called lestaurtinib, Cephalon, West Chester, PA) belongs to a class of compounds identified as indolocarbazole alkaloids. This orally available compound is a potent FLT3 inhibitor and is cytotoxic to several FLT3-expressing human leukemia-derived cell lines and to primary bone marrow-derived AML blasts harboring FLT3 mutations.⁴⁸ Moreover, CEP701 is also a potent inhibitor of wild-type and mutated JAK2 tyrosine kinase and induces a significant growth inhibition of the JAK2V617F mutated HEL cell line. For this reason, a phase II study of CEP701, at the dose of 80 mg PO BID, was designed for patients with primary and post-PV/ET myelofibrosis who harbor JAK2V617F mutation. These patients had a median aged 56 years and had been previously treated with a median 3 therapies. Except for grade 2 nausea in 1 patient, no significant toxicities were registered.⁴⁹

Epigenetic Therapy

Although genetic lesions have been the focus of cancer research for many years, it is now evident that aberrant epigenetic modifications also play a major role in cancer pathogenesis.⁵⁰

Hypomethylating agents

The best-known epigenetic marker is DNA methylation and in humans, DNA methylation occurs in cytosines that precede guanines, called dinucleotide CpGs, which are rather frequent within the promoter region of many genes. Methylation of such sequences occurring in the 5' end of tumor suppressor genes may lead to their inactivation.⁵¹ Very little is known about the role of aberrant DNA methylation in the pathobiology of myelofibrosis, but in some patients hypermethylation of calcitonin, RAR α 2, p15INK4b, p16INK4a, histone 2A, TNF, TNF Receptor1 and FGF14 genes has been reported.^{52,53} Moreover, not only protein-coding genes undergo these modifications since CpG island promoters of noncoding microRNAs were shown to be hypermethylated in tumors.⁵⁰ Notably, a defined microRNA profile that distinguishes primary myelofibrosis (PMF) granulocytes from those of normal subjects and, partially, also from patients with PV or ET has been recently reported.⁵⁴ Interestingly, unlike genetic lesions such as translocations, duplications or mutations, DNA methylation is reversible and it is possible to re-express DNA-methylated genes in cancer cells by using demethylating agents. In a recent *in vitro* study, Shi and co-workers analyzed the effect of a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5azaD), in combination with the histone deacetylase inhibitor trichostatin A (TSA) on the behavior

of CD34⁺ cells isolated from normal donors and patients with myelofibrosis. Under 5azaD/TSA treatment normal CD34⁺ were led to expand whereas the number of CD34⁺ cells from patients was decreased. Exposure of myelofibrosis CD34⁺ cells to 5azaD/TSA resulted in a significant reduction of both JAK2V617F-positive hematopoietic colonies and the number of colonies that contained chromosomal abnormalities in 2 JAK2V617F-negative IM patients. Finally, exposure of myelofibrosis CD34⁺ cells to 5azaD/TSA was shown to restore CXCR4 expression on the cell surface of progenitor cells and their migratory response to SDF-1.⁵⁵ This seems particularly important when considering that the constitutive mobilization of CD34⁺ cells in the peripheral blood, a peculiar finding observed in patients with myelofibrosis, has been attributed to a proteolytic degradation of SDF-1 and reduced CXCR4 expression on hematopoietic progenitor cells.⁵⁶ Moreover, it has been recently shown that CD34⁺ cells from patients with PMF, unlike those from normal subjects, present hypermethylation of CXCR4 promoter CpG island 1, which can be almost completely reverted *in vitro* by treatment with 5-AzaD.⁵⁷ All in all, these data provide a rationale for sequential therapy with chromatin-modifying agents for patients with myelofibrosis. In a recent Phase II trial specifically designed to test the efficacy of 5-azacitidine, patients with either relapsed/refractory or newly diagnosed myelofibrosis with poor prognosis were treated with 5-azacitidine at the dose of 75 mg/m² subcutaneously daily for 7 days, every 4 weeks. The median duration of 5-azacitidine therapy was 5.5 months and a clinical response has been observed in 8 (24%). Only 1 patient met all the criteria for complete remission except for the achievement of bone marrow histological remission. Responses were observed both in patients with and without JAK2V617F mutation. Therapy with 5-azacitidine resulted in gradual and significant decline of the methylation from the baseline, but the degree of global DNA hypomethylation achieved during treatment was not different between responders and non-responders. Myelosuppression was the major adverse effect, with grade 3-4 neutropenia in 29% of patients. In summary, although 5-azacitidine was well tolerated, its clinical use was followed by modest clinical activity.⁵⁸

Histone deacetylase inhibitors

Interestingly, DNA methylation occurs in the context of histone proteins, and epigenetic silencing is also strictly associated with histone deacetylation. Remodeling of chromatin between open and closed forms has a key role in epigenetic regulation of gene expression. The opposing activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) tightly regulate gene expression through chromatin modification. HATs transfer acetyl groups to amino-terminal lysine residues in histones, which results in local expansion of chromatin and increased ac-

cessibility of regulatory proteins to DNA, whereas HDACs catalyze the removal of acetyl groups, leading to chromatin condensation and transcriptional repression.⁵⁹ Consequently, there has been considerable effort to develop HDAC inhibitors (HDACi) and several such molecules have been synthesized and shown to affect the growth, differentiation and survival of tumor cells of different origin including multiple myeloma, acute myelogenous leukemia and other hematological malignancies.⁵⁹ One class of HDACi is the hydroxamate family of compounds whose prototype is suberoyl anilide hydroxamic acid (SAHA, Vorinostat)⁶⁰ which inhibits class I and class II HDACs and is being tested in Phase I and Phase II trials for solid and hematological cancers.^{61,62} Vorinostat has recently been approved by the U.S. Food and Drug Administration for the treatment of cutaneous T-cell lymphomas.⁶³ We recently described the potent antiproliferative and pro-apoptotic activity against cells of acute myelogenous leukemia and multiple myeloma of a new synthetic HDAC class I inhibitor (ITF2357, Italfarmaco, Cinisello Balsamo, Italy).⁶⁴ Despite its *in vitro* and *in vivo* antitumor activity, ITF2357 shows little toxicity against normal cells such as mesenchymal stem cells, hepatocytes and peripheral blood mononuclear cells (MNCs), and thrombocytopenia and gastrointestinal toxicity represent the most common side effects after its use in normal volunteers and patients.⁶⁵ Of note, ITF2357 similarly to other HDAC inhibitors induces a significant upregulation of the cell cycle inhibitor p21. Interestingly, recent data also suggest that HDAC activities are elevated in patients with myelofibrosis so that the clinical use of HDAC inhibitors in clinical trials for treating these patients has been hypothesized.⁶⁶ For all these reasons, we investigated the activity of ITF2357 on cells carrying the JAK2V617F mutation obtained from patients with PV or ET. In these experiments we were able to show that cells obtained from patients with PV or ET carrying the JAK2V617F mutation are sensitive in colony assays to a 100- to 500-fold lower dose of ITF2357 as compared to cells bearing wild-type JAK2. We also provide evidence that ITF2357 promotes the outgrowth of normal colonies over that of JAK2V617F mutated cells *in vitro*. Noteworthy, this new HDACi induces downmodulation of the JAK2V617F in HEL cells but not JAK2 wild-type protein in three different cell lines, and finally, we show that JAK2V617F inhibition takes place at the post-transcriptional level and is followed by downmodulation of the phosphorylated STAT5 and STAT3 proteins and of PRV-1 gene expression (**Figure 1**; see Color Figures, page 491).⁶⁷ Moreover, an intriguing property of HDAC inhibitors relies on their ability to downmodulate several soluble cytokines secreted by blood cells or from accessory cells of the bone marrow microenvironment.^{68,69} The autocrine and paracrine secretion of different cytokines may play an important role

for the neoplastic proliferation of myeloid precursor cells, thus representing a possible relevant target of new antineoplastic drugs. Most strikingly, ITF2357 inhibits production of IL-6, VEGF and interferon (IFN)- γ , with an IC₅₀ similar to that required for apoptosis induction of leukemic cells (0.25-0.5 μ M). These data are in agreement with the ability of the drug to specifically inhibit lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF)- α , IL-1 α and IL-6 but not IL-8 production by peripheral blood mononuclear cells.⁶⁹ Finally, spontaneous secretion of IL-6 and VEGF is also inhibited by ITF2357.⁶⁴ All in all, these data prompted us to launch a prospective Phase II clinical study with ITF2357 for PV, ET and myelofibrosis patients carrying the JAK2V617F mutation.

Concluding Remarks

After many elusive efforts, effective new treatment strategies are becoming available for patients with either primary or post-PV/ET myelofibrosis. This has been primarily due to the development of effective and reasonably well tolerated, reduced-intensity allogeneic transplantation programs by which some patients may achieve a durable molecular remission and possibly a definitive eradication of the disease. Moreover, new innovative drugs, targeting either specific lesions such as the JAK2V617F mutation or more general oncogenic mechanisms, are now in the early phase of clinical development and promising results have been reported. These new molecules may provide widely applicable and effective pharmacologic treatments to many patients for whom the allogeneic stem cell transplantations are not feasible due to advanced age, the presence of multiple and severe comorbidities or the lack of suitable donors.

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Off-label drug use: The off-label use of thalidomide, lenalidomide, tipifarnib, 2-chlorodeoxyadenosine, imatinib, etanercept, bortezomib, INCB018424, XL019 and ITF2357 are discussed.

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