SPOTLIGHT EDITORIAL

***BCR-ABL1*-positive CML and *BCR-ABL1*-negative chronic myeloproliferative disorders: some common and contrasting features**

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Introduction

‘.....to put together such apparently dissimilar diseases as chronic granulocytic leukaemia, polycythemia, myeloid metaplasia and di Guglielmo's syndrome may conceivably be without foundation, but for the moment at least, this may prove useful and even productive. What more can one ask of a theory?’

So ended the editorial entitled ‘Some Speculations on the Myeloproliferative Syndromes,’ which William Dameshek published in one of the early issues of *Blood* in 1951.¹ He speculated that these various conditions, which he had termed ‘myeloproliferative’, were all somewhat variable manifestations of excessive proliferative activity of marrow cells, perhaps because of a hitherto undiscovered stimulus. More than half a century later, Dameshek, were he still alive, would probably have been pleased to learn that much has been learned about the cellular defects that cause these various disorders and the term he introduced, myeloproliferative, is still in daily use. It is true that we do now talk of the chronic myeloproliferative disorders (CMD), a category into which Di Guglielmo's syndromes do not really fit, but the other haematological disorders that Dameshek grouped together do now seem to share more cellular and molecular characteristics than might have been expected—and many of the unanswered questions in the different disorders are also very similar. It is worth noting that the 2008 World Health Organization classification scheme for myeloid neoplasms groups the chronic myeloproliferative

disorders under a single subheading, ‘myeloproliferative neoplasms’ (MPNs), and this term may well displace CMD in the near future.²

Of the chronic myeloproliferative disorders, or myeloproliferative neoplasms, perhaps the major distinguishing factor until recently was whether they did or did not have a Ph chromosome. The issue of whether chronic myeloid leukaemia (CML) is truly a CMD or should be regarded as a leukaemia *sui generis* is obviously largely semantic, but for the moment, one possible solution is simply to refer to Ph-positive and Ph-negative CMD, which makes categorization of the disorders under consideration somewhat clearer. But many of the issues in both categories of CMD are very similar. In both categories, one may ask whether the major molecular lesions, *BCR-ABL1* and *JAK2-V617F*, respectively, are primary or secondary. In both categories, genomic instability (GI) seems to increase the risk of disease progression. In both categories, deregulated protein tyrosine kinases seem to be seminal, but the mechanisms of activation are quite different and the signal transduction pathways involved probably differ, though they are still incompletely defined. *BCR-ABL1*-positive CML responds remarkably well, in most but not all cases, to tyrosine kinase inhibitors. Early results suggest that comparable results may be achievable in some cases by inhibiting *JAK2*. These are just some of the issues addressed at a recent meeting of investigators interested in the biology and treatment of some of the disorders that Dameshek grouped together. This paper summarizes some of the recent data discussed at the meeting and includes reference to some data presented or published after the meeting.

The initiating molecular events in the CMDs

Does BCR-ABL1 come first?

For some years after the discovery of what came to be known as the Philadelphia (originally Ph¹, but now Ph) chromosome in 1960, it was possible to argue that it was merely a marker of the leukaemia cell with no pathogenetic significance. This view was weakened, but by no means disproved, by the demonstration by Fialkow *et al.*³ in 1967 using an ingenious new technique that CML appeared to be clonal. They studied three female patients

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who were heterozygous for the two isoenzymes, A and B, of glucose-6-phosphate dehydrogenase (G6PD), and showed that granulocytes appeared uniformly to be of one or other isoenzyme type, but never both. Subsequently, they studied lymphocytes from female G6PD heterozygotes, and showed those of the same isoenzyme type as the leukaemia lacked a Ph chromosome. In 1991, they described results of studying EBV-induced cell lines from one CML patient heterozygous for G6PD.⁴ Of the Ph-negative B-cell lines, those with the same G6PD isoenzyme as the patient's own leukaemia showed multiple non-Ph cytogenetic abnormalities, whereas those with the non-leukaemia G6PD isoenzyme were all cytogenetically normal. This work was extended in 1993 by Raskind *et al.*⁵ who showed that the EBV-induced cell lines were Ph-negative, but of the same G6PD isoenzyme type in 5 of 14 G6PD heterozygous patients with CML. Fialkow's series of studies supported the notion that clonal haematopoiesis preceded the acquisition of the Ph chromosome—at least in some patients – and implied that molecular abnormalities might precede the acquisition of *BCR-ABL1*.^{3,4}

Fialkow's studies, however, are not definitive. The extent to which lymphocytes from patients with CML are Ph-positive has been a matter of debate for many years. The weight of the evidence suggests that some B-lymphocytes are part of the leukaemia clone in most patients, and Ph-positive T-lymphocytes can also be identified.⁶ However, the frequency of Ph-positive cells decreases, when one compares both T- and B-cell progenitors with their more mature progeny, suggesting that Ph-positive cells are eliminated during differentiation.⁶ Moreover, Ph-positive EBV-transformed B-cell lines are difficult to establish and usually short-lived.⁷ These data are consistent with the notion that the Ph chromosome involves a pluripotential stem cell. The loss of the Ph chromosome in mature lymphocytes (by an unclear mechanism), and the difficulty in establishing stable Ph-positive lymphoblast lines using EBV certainly complicates the interpretation of Fialkow's data. However, it seems plausible that at least in some patients, the acquisition of a Ph translocation is superimposed on a preexisting clonal abnormality, and the underlying question is whether some molecular lesion *always* preexists.

The question of whether *BCR-ABL1* can act as an oncogene to induce CML has been addressed in murine models. Retroviral transduction of the *BCR-ABL1* cDNA into murine bone marrow is sufficient to induce a CML-like myeloproliferative disease with a short latency.^{8–10} With enhanced infection efficiency, the disease that results from retroviral infection is polyclonal, suggesting that the expression of *BCR-ABL1* alone is sufficient to induce the disorder in mice.¹¹ Furthermore, using transgenic mice in which the *BCR-ABL1* oncogene is conditionally expressed in response to doxycycline, disease induction occurs rapidly and can be extinguished rapidly in response to the antibiotic, again supporting a solo function for *BCR-ABL1* in mediating disease.^{12,13}

Although data in mouse models suggest that *BCR-ABL1* can represent an initiating lesion that is sufficient to induce disease, only data from patients can fully address the issue of what molecular lesions come first, and whether cooperating genetic lesions are essential for disease pathogenesis. Further evidence for an event preceding the acquisition of a Ph chromosome has been reported recently by Zaccaria *et al.*¹⁴ They collected cytogenetic data from five CML patients who had presented in Italy with Ph-positive CML, but with additional cytogenetic abnormalities in their Ph-positive cells. After treatment with imatinib, these five patients achieved haematologic remissions with Ph-negative marrow metaphases, but some of the original non-Ph abnormalities persisted in each case. The authors

speculated that their observations were best explained by postulating that, at least in these patients, the Ph translocation must have been acquired in a clone that already bore a cytogenetic marker. In most patients with CML, however, treatment with imatinib induces cytogenetic remissions with polyclonal haematopoiesis.^{15,16} This alone is the strongest evidence that *BCR-ABL1* is the central oncogenic lesion, whether acting alone or in concert with cooperating genetic or epigenetic events.

Thus, although there is evidence that *BCR-ABL1* can initiate a myeloproliferative disease in experimental animals, there is circumstantial evidence to suggest that at least in some human patients, the acquisition of a Ph chromosome associated with a *BCR-ABL1* fusion gene may not be the initial molecular event.

Does JAK2-V617F come first?

In many ways, the function of the *JAK2-V617F* in Ph-negative CMD resembles that of *BCR-ABL1* in CML, but with some interesting differences. The *JAK2-V617F* mutation is present in the majority of patients with polycythemia vera (PV), and about half of the patients with essential thrombocythemia (ET) and primary myelofibrosis.^{17–20} Similar to the conclusion reached for *BCR-ABL1*, expression of *JAK2-V617F* alone is sufficient to cause CMD phenotypes in mouse models. Retroviral *JAK2-V617F* expression in bone marrow cells resulted in PV with a very short latency and a tendency to later develop myelofibrosis within 3–6 months.^{17,21–24} More recently, transgenic mouse models confirmed that the *JAK2-V617F* is sufficient to cause MPD and showed that the expression of *JAK2-V617F* can also generate an ET phenotype with massive thrombocytosis.^{25–27} The phenotype appears to be dependent on the ratio of mutant *JAK2-V617F* expression versus wild-type *JAK2* (*JAK2wt*).²⁵ Thus, in mouse models, there is no need to postulate an event that precedes the acquisition of *JAK2-V617F*, similar to the data in CML. However, this single step model is difficult to reconcile with all the data available on human CMD. Several lines of evidence suggest that in a subset of CMD patients, there are molecular events that occurred before *JAK2-V617F*. However, at present, there are no data to make one believe that these events are mandatory for the phenotypic manifestation of MPD.

Familial cases of MPD have been described that manifest PV and/or ET phenotypes with an acquired *JAK2-V617F* mutation present in haematopoietic cells only.^{28,29} In most cases, the inheritance is compatible with an autosomal dominant trait with low penetrance, in which only the predisposition to acquiring additional somatic mutations is inherited in these families. Affected family members have clonal haematopoiesis and display growth of endogenous erythroid colonies in methylcellulose.³⁰ Typically, most, but not all, affected family members carry the *JAK2-V617F* mutation in exon 14, but in some families, a *JAK2* exon 12 mutation or a *BCR-ABL1*-positive CML was found.^{29,31} Given the low incidence of MPD and *JAK2-V617F* in the general population (approximately 1/100 000 per year), the germ-line mutation appears to increase several hundred-fold the likelihood of acquiring *JAK2-V617F*, and can also lead to the acquisition of a *JAK2* exon 12 mutation or *BCR-ABL1* fusion gene, although with a much lower probability. The prevalence of a hereditary component in MPD is likely being underestimated because of the low penetrance of the phenotypic manifestation. Moreover, there appears, at least in Sweden, to be an increased risk of PV, ET and myelofibrosis in first degree relatives of patients with known myeloproliferative neoplasms regardless of *JAK2* mutation status.³²

A second unexpected finding in patients with CMD is that many patients with ET, but also some with PV and idiopathic

myelofibrosis (IMF), display low allelic ratios of *JAK2*-V617F. Analysis of granulocytes from female ET and PV patients with an allelic ratio of the mutant allele below 25% revealed that the granulocytes negative for the mutation were in most cases clonal by X-chromosome inactivation pattern.³³ Furthermore, in two del(20q)-positive patients, the *JAK2*-V617F clone was considerably smaller than the del(20q)-positive clone, suggesting that in these patients del(20q) preceded the acquisition of *JAK2*-V617F.³³ Additional indirect evidence for the presence of clonal events that precede the acquisition of the *JAK2*-V617F mutation comes from the studies of erythroid progenitors in methylcellulose. Analysis of single colonies revealed that some endogenous erythroid colonies in PV patients can grow in the absence of *JAK2*-V617F.³⁴ In some patients, two different mutations can be detected simultaneously, for example, *JAK2*-V617F and *MPL*-W515L, *JAK2*-V617F and *BCR-ABL1* or *JAK2*-V617F and *JAK2* exon 12.^{35–37} In one case, the analysis of single erythroid colonies established that the presence of *JAK2*-V617F and *JAK2* exon 12 mutation was mutually exclusive. Thus, the *JAK2*-V617F and *JAK2* exon 12 mutations occurred independently and not sequentially.

Finally, secondary leukemic transformation in patients with *JAK2*-V617F at the diagnosis of MPD frequently results in an absence of *JAK2*-V617F in leukemic blasts.^{38,39} Although it is possible that in these cases, the acute leukaemia arose *de novo*,⁴⁰ the fact that leukaemic transformation is more frequent in CMD patients than in an age-matched control population would be difficult to explain by chance alone. Thus, the alternative explanation that the MPD and AML populations share a common clonal origin remains a valid hypothesis.

The currently available data suggest that mutations predisposing to *JAK2*-V617F and other somatic mutations occur in a subset of patients with CMD. These mutations could be inherited through the germline or acquired in haematopoietic cells only. The proportion of patients with and without such predisposing mutation and the function of these mutations in leukaemic transformation remain to be determined.

How does *JAK2*-V617F cause three different clinical phenotypes?

Early insight into this conundrum came from the Medical Research Council's PT-1 trial.⁴¹ Analysis of prospective data from 776 ET patients from this study demonstrated that *JAK2* mutation status defines two biologically distinct subtypes of ET with differences in presentation, outcome and response to therapy.⁴² *JAK2*-negative patients do not become *JAK2*-positive suggesting that these two groups represent distinct diseases rather than separate stages of the same disorder. Mutation-positive patients displayed multiple features resembling a *forme fruste* of PV, and analogous results have been reported in idiopathic myelofibrosis.^{38,43,44} Mutation-positive ET and PV, therefore, appear to form a phenotypic continuum, with the degree of erythrocytosis and thrombocytosis determined by physiological or genetic modifiers, and similar arguments may explain interindividual differences in reticulin fibrosis.

Approximately 5% of patients with PV lack a V617F mutation in exon 14. Recently several different somatic gain-of-function mutations affecting *JAK2* exon 12 have been identified.⁴⁵ These mutations are present in the majority of patients with V617F-negative PV and have revealed a novel myeloproliferative syndrome, with features distinct from V617F-positive PV. Retroviral transduction studies demonstrated that BaF3/EpoR cells carrying exon 12 mutations were able to proliferate

without added interleukin-3, and exhibited increased *JAK2* and *ERK1/2* phosphorylation relative to *JAK2*wt and *JAK2*-V617F. Three of the exon 12 mutations generated a K539L substitution, which produced a myeloproliferative phenotype, including erythrocytosis, in a murine retroviral bone marrow transplantation model. *JAK2* exon 12 mutations define a distinctive myeloproliferative syndrome, which includes patients currently diagnosed as PV or idiopathic erythrocytosis.^{31,45,46} The different clinical phenotypes associated with the *JAK2* exon 12 and V617F mutations may reflect, at least in part, stronger ligand-independent signalling by the former.⁴⁵

What determines whether the acquisition of a *JAK2*-V617 mutation will result in PV or ET? One major factor appears to be mitotic recombination giving rise to homozygosity for the mutation. Analysis of individual erythroid colonies demonstrated that the vast majority of patients with PV harbour subclones homozygous for the mutation, whereas this is rare in ET.⁴⁷ These results suggest that an increased level of mutant *JAK2* signalling results in more erythrocytosis and less thrombocytosis. This concept is consistent with results from murine retroviral transplant studies,²¹ and also with the observation that compared to V617F mutations exon 12 mutations are associated with higher levels of ligand-independent signalling and are accompanied by a more extreme erythrocytosis.⁴⁵ CMD patients who lack a *JAK2* mutation are likely to represent a heterogeneous group, a concept supported by the identification of *MPL* mutations in approximately 3–9% of V617F-negative IMF patients^{48–50} and in 1% of ET patients.⁵⁰

Taken together, these data suggest that the conventional division of the *BCR-ABL1*-negative CMDs into PV, ET and IMF is no longer appropriate. Instead, current evidence is more consistent with a new molecular classification,⁵¹ which recognizes the similarities between the *JAK2*-positive CMDs together with the significance of the level of *JAK2* signalling, and suggests that patients labelled as having IMF may in fact be presenting in accelerated phase of a preexisting occult CMD.

Genomic instability

What do we know about genomic instability in CML?

The reciprocal chromosomal translocation [t(9;22)] generating the Ph chromosome encoding *BCR-ABL1* kinase arises as a result of an unfaithful repair of spontaneous DNA double-strand breaks (DSBs), which is most probably induced by oxidative stress, radiation, genotoxic chemicals and/or replication stress.^{52,53} Reports from numerous laboratories documented that CML cells display GI resulting in accumulation of point mutations and chromosomal aberrations, which contribute to the development of resistance to *BCR-ABL1* kinase inhibitors (small molecular inhibitors, e.g., imatinib) and, perhaps, to blastic transformation.^{52,54,55} Indeed, CD34⁺/CD38[−] stem cells isolated from untreated chronic phase (CP) CML patients present mutation(s) in the *BCR-ABL1* kinase domain (KD),⁵⁶ suggesting that the increased GI is an intrinsic characteristic of quiescent CML stem cell pool. Work from the Skorski and Rassool groups indicates that the increased GI in Ph-positive progenitor cells strongly depends on *BCR-ABL1* kinase activity, but the contribution of preexisting conditions responsible for the generation of t(9;22) is not excluded.^{52–55, 57} Consistent with the pivotal role of *BCR-ABL1* in inducing GI is the observation that additional chromosomal and molecular abnormalities occur much frequently during blastic transformation than in CP CML^{53,55} and this strongly and directly correlates with the levels of *BCR-ABL1* kinase activity that increases when the

disease progresses. Thus, the burning question is: to what extent is *BCR-ABL1* kinase activity the cause of the GI?

BCR-ABL1 kinase increases the levels of reactive oxygen species leading to the accumulation of oxidized bases and DSBs.⁵⁸ In addition, *BCR-ABL1*-positive leukaemia cells acquire more DNA lesions after genotoxic treatment.⁵⁹ Recent findings from Skorski's lab suggest that the elevated levels of reactive oxygen species-induced oxidative DNA lesions are present in CD34⁺ CML stem/progenitor cells.⁶⁰ Therefore, *BCR-ABL1* kinase predisposes CML stem/progenitor cells to GI by increasing the amount of DNA damage.

DNA lesions caused by oxidized bases can be repaired by base excision repair mismatch repair and nucleotide excision repair pathways.⁶¹ A report by Skorski and Penserga⁵² and unpublished results from his group presented at the Meeting suggest that *BCR-ABL1* kinase may exert a negative effect on base excision repair and mismatch repair, while stimulating nucleotide excision repair; the latter effect generated point mutations. *BCR-ABL1*-mediated mechanisms responsible for these effects are not known. Altogether, *BCR-ABL1* kinase-dependent inhibition of base excision repair and mismatch repair, and stimulation of mutagenic nucleotide excision repair may contribute to point mutations in *BCR-ABL1* and p53 genes, which are responsible for the resistance to small molecular inhibitors and may contribute to malignant progression of CML.⁶²

DSBs are repaired by homologous recombination (HR), non-homologous end-joining (NHEJ) and single-strand annealing.⁶³ *BCR-ABL1* kinase stimulates the efficiency, but compromises the fidelity of HR and NHEJ.^{64–67} Induction of HR in leukaemia cells is driven by *BCR-ABL1*-mediated stimulation of RAD51 protein, a key element in HR.⁶⁵ Point mutations and large deletions are introduced into the products of HR and NHEJ in leukaemia cells, but not normal counterparts, but the precise mechanisms responsible for the infidelity are not known. Brady and colleagues⁶⁴ suggested that the aberrant expression of DNA repair proteins involved in NHEJ may generate extensive deletions in *BCR-ABL1*-positive leukaemias. In addition, work from Skorski's group indicates that *BCR-ABL1* kinase may also stimulate a very rare, but extremely unfaithful, single-strand annealing. Thus, *BCR-ABL1* kinase appears to aberrantly enhance all three DSB repair mechanisms (HR, NHEJ and single-strand annealing); however, it also compromises the fidelity of repair.

In this scenario, *BCR-ABL1*-induced unfaithful regulation of DSBs repair can contribute to point mutations in HR products^{58,66} and chromosomal aberrations such as translocations and partial deletions detected by spectral karyotyping.⁶⁸ In addition, *BCR-ABL1*-mediated modification of centrosomal integrity may lead to aneuploidy.⁶⁹

On the basis of these considerations, elevated levels of DNA lesions accompanied by inefficient or enhanced, but unfaithful, DNA repair mechanisms seem to depend primarily on *BCR-ABL1* kinase activity. Furthermore, it also appears that a direct correlation exists between the number and type of DNA lesions and the levels of *BCR-ABL1*. Thus, a simplistic conclusion would be that the suppression of *BCR-ABL1* kinase activity by tyrosine kinase inhibitor (TKI) would prevent GI in CP and blast crisis (BC) CML. This, although plausible, does not seem to be the ideal strategy for preventing the acquisition of additional molecular and chromosomal abnormalities in all types of CML patients as pharmacologic inhibition of the normal Abl1 function compromises DNA-damage response and allows an increase in the mutation frequency in non-leukemic cells.⁷⁰ In conclusion, it is clear that a preexisting background of GI might facilitate developing CML; however, as increased GI correlates with CML disease progression, the remaining critical question is:

'Does TKI treatment in CP CML suppress or just significantly delay its transformation into a classical Ph-positive or worse, a Ph-negative BC?'

What do we know about genomic instability in JAK2-V617F disorders?

Genetic instability is associated with all fusion tyrosine kinases that result in a constitutive kinase activation.⁷¹ This is the case for *TEL-JAK2* in which the *TEL* domain induces an oligomerization of the KD and an activation of *JAK2*, though *TEL-ABL1* also deregulates normal ABL1 function in rare cases of leukaemia.⁷² The link between genetic instability and constitutive activation of a kinase is related by three complementary mechanisms:

- First, an increase in radical oxygen species as a consequence of proliferation.
- Second, stimulation of the RAS/MAP kinase, PI3K and Stat pathways that regulate DNA repair by mechanisms remaining greatly unknown.
- Third, inhibition of apoptosis permitting the accumulation of genetic abnormalities.

The *JAK2-V617F* is a gain-of-function mutation that leads to the constitutive activation of *JAK2-STAT5*, PI3K and MAPK/ERK downstream pathways.^{17–20} However, in contrast to *TEL-JAK2*, oligomerization of *JAK2-V617F* may require a cytokine receptor to constitutively activate the kinase, and it has been suggested that only homodimeric cytokine receptors have this property,⁷³ although this remains extremely controversial. Thus, in contrast to fusion tyrosine kinases, *JAK2-V617F* is a subtle mutation that can be still exquisitely and finely regulated explaining why the diseases induced by *JAK2-V617F* have a milder evolution than CML. One of the main hypotheses explaining how a single mutation may give rise to three different diseases is based on the level of the constitutive signalling mediated by the mutated *JAK2* kinase. There is strong evidence that one of the major mechanisms is a *JAK2-V617F* gene dosage occurring by duplication or trisomy 9.⁷⁴ In addition, it has been reported that the number of chromosomal abnormalities increases during ET and PV progression to myelofibrosis with the presence of trisomy and deletion, but the precise role of these genetic abnormalities in disease progression remains to be determined.^{75,76} Nevertheless, the occurrence of several genetic abnormalities strongly suggests that *JAK2-V617F* is capable of inducing a genetic instability that may lead to changes in the CMD phenotype rather than in true leukaemia and, therefore, *JAK2-V617F* contrasts with other constitutively activated kinases. Among the mechanisms leading to genetic instability, it has been already demonstrated that *JAK2-V617F* induces reactive oxygen species production and inhibits apoptosis through induction of Bcl-xL.^{77,78} Recently Plo et al.⁷⁹ have shown that *JAK2-V617F* increases HR at a level close to *TEL-JAK2*. By introducing a specific substrate of HR in the genome of a Ba/F3/EpoR cell line overexpressing *JAK2-V617F*, it could be shown that HR was increased 20-fold in the presence of erythropoietin (Epo) in comparison to the parental cell line. When *JAK2wt* was overexpressed, an increase in HR was also seen in the presence of Epo, but at a much lower level (fivefold). This means that the effect of *JAK2* on HR may depend on the level of signalling or, alternatively, that *JAK2-V617F* may have some that the *JAK2wt* lacks. In agreement with these results and by a similar approach, HR activity was found increased in CD34⁺ cells derived from PV or primary myelofibrosis (PMF) after cytokine stimulation in comparison to control CD34⁺ cells. In addition, increased spontaneous RAD51 foci formation

was observed in cultured cells. The precise mechanism leading to the increased HR remains unknown. Furthermore, in the Ba/F3 cell line, *JAK2-V617F* induces centrosomal abnormalities associated with aneuploidy. Finally, 100- and 10-fold increases in mutagenesis at HPRT and Na/K ATPase loci, respectively, were found suggesting that *JAK2-V617F* induces events that are more often deletions than point mutations.

The increase in HR may explain the numerous chromosomal abnormalities and the high frequency of *JAK2-V617F* homozygosity observed in MPD. It has been shown that, in 30% of PV with very high *JAK2-V617F* burden, 9pLOH (loss of heterozygosity) and *JAK2-V617F* homozygosity are related to mitotic recombination, an event usually processed by HR.²⁰ Moreover, in about 50% of PV, the *JAK2-V617F* burden is much lower because of the presence of *JAK2-V617F* homozygous and heterozygous clones as well as wt clones.⁸⁰ In these cases, it is possible that mechanisms other than mitotic recombination, such as gene conversion, may be responsible for the 9pLOH. It is noteworthy from the work of Plo *et al.*⁷⁹ that an over signalling by *JAK2wt* may also induce genetic instability, although at a lower level than *JAK2-V617F*. Furthermore, it is expected that the *MPL-W515* mutations (*W515K/L/A*) should also be able to induce increased HR through constitutive activation of *JAK2*. This may explain the presence of several cases of homozygous mutations, although the precise mechanisms have not been described yet.

It remains an intriguing question why *JAK2-V617F* generates an increase in HR leading to genetic instability in a manner similar to CML. About 10% of PV or ET cases progress to leukaemia, whereas it is an invariable event in CML.^{53,81} Moreover, more than 50% of post-PV and post-ET leukaemias do not bear *JAK2-V617F*, whereas all CML-derived leukaemias are *BCR-ABL1* positive.^{39,53} There are three main hypotheses. Firstly, it is unknown whether *JAK2-V617F* is able to modify other DNA repair mechanisms such as NHEJ, BER and nucleotide excision repair as described for p210-*BCR-ABL1*.⁵² Secondly, *JAK2-V617F* is a weak gain-of-function protein, especially in its heterozygous state. Thus, in contrast to *BCR-ABL1*, it is possible that this mutation has a proliferative activity that is insufficient to be synergistic with a genetic event that would alter differentiation. In contrast, genetic instability may permit the selection of genetic events that will finally increase the haematopoietic proliferation. Thirdly, it has been shown in PV that the amplification of the *JAK2-V617F* clone occurs mainly at the postprogenitor stage, whereas it takes place at the progenitor stage in CML. In consequence, the *JAK2-V617F* burden is low in haematopoietic stem cells and progenitors in many PV and ET. This may explain that the probability to develop leukaemia remains low, despite a genetic instability. However, during the disease progression, and especially in myelofibrosis, the *JAK2-V617F* burden becomes high and the probability to develop leukaemia markedly increases. For example, this probability is estimated to be about 20% in PMF with *JAK2-V617F*.⁴⁴

Interestingly, at least seven cases have been reported to be positive for both *JAK2-V617F* and *BCR-ABL1* (reviewed in Krämer⁸²). Some evolved from a *V617F*-positive CMD to CML (with acquisition of *BCR-ABL1* at the CML stage), whereas in others, *V617F*-positive IMF or PV arose in CML patients after remission induction with imatinib. Colony analysis of one case clearly demonstrated that *BCR-ABL1* arose as a secondary event on a preexisting *V617F*-positive clone,⁸³ but whether the same sequence of events occurred in the other cases is unclear. The probability of both abnormalities arising completely independently in a single individual is minute, and thus, it seems likely *BCR-ABL1* arose on a background of *V617F*-induced GI.

Finally, we need to understand further mechanisms that lead to the formation of *JAK2-V617F*. The replacement of a valine by a phenylalanine resulting from G:C to T:A transversion is highly suggestive of a mutation induced by an oxidized guanine (8-oxoG), which has not been adequately repaired by base excision repair.⁸⁴ It may thus be hypothesized that *JAK2-V617F* MPDs may be favoured by any chronic proliferative or inflammatory stress involving the haematopoietic stem cells. Familial forms of MPDs do not correspond to germline mutation in *JAK2* or in other genes directly involved in MPDs, but they do constitute predisposition states.²⁹ Future studies aiming at the identification of MPD susceptibility genes that may correspond to genes involved in reactive oxygen species metabolism, DNA repair or the control of HSC cell cycle will be very important to understand the mechanisms predisposing to the sporadic MPDs.

'New' mutations in *BCR-ABL1*

Why do they occur?

The most common new genetic alterations found in CML patients at the onset of resistance to imatinib are point mutations in the *BCR-ABL1* kinase domain (KD).^{85–92} These mutations impair imatinib binding either by disrupting critical contact sites between Bcr-Abl and the inhibitor—for example, T315I, F317L and F359V—or by favouring a conformation of the kinase to which the inhibitor binds less effectively or not at all—for example, mutations G250E, Y253H or E255K/V in the P-loop or H396P in the activation loop.^{86,87}

It is likely that mutations arise in the KD quite independently of imatinib therapy in rare Ph⁺ cells.^{93,94} These cells would subsequently undergo Darwinian selection in the presence of the inhibitor, if the mutation they harbour is able to confer a survival advantage. But at what hierarchical level do the mutations originate? Several studies have indicated that primitive/quiescent CML stem cells are inherently resistant to imatinib. Exposure to imatinib *ex vivo* has been shown to block proliferation, but it does not induce apoptosis in this cell population.^{95–97} Clinical experience now confirms that CML stem cells do indeed persist after imatinib treatment *in vivo*. This is supported by several lines of evidence: few patients achieve a complete molecular remission on imatinib; discontinuation of treatment even in the rare cases of complete molecular responders often causes disease recurrence;⁹⁸ patients in complete cytogenetic response have easily detectable Ph-positive CD34⁺ cells, colony-forming cells and long-term culture-initiating cells.⁹⁹ This has led to the hypothesis that *ABL1* KD mutations may arise at the stem cell level, and may be one of the mechanisms underlying the incomplete eradication of malignant progenitors. Results of the recent study by Jiang *et al.*⁵⁶ (mentioned above) suggest that CP CML stem cells are already highly genetically unstable and begin accumulating mutations spontaneously and at a high frequency. Using highly sensitive approaches like allele-specific oligonucleotide PCR and sequencing of cloned *BCR-ABL1* transcripts, freshly isolated CD34⁺CD38[–] cells from imatinib-naïve CP CML patients were found to already harbour KD mutations. Further accumulation of mutations was observed when these cells were cultured *in vitro* for 3–5 weeks both in the presence or absence of imatinib. Besides single and multiple point mutations inside and outside the KD, small insertions, small deletions and premature stop codons were also observed. Some of these alterations have never been reported in patients and are likely to be clinically irrelevant, as they may be neutral or even reduce the growth advantage of the clone, but mutations known to be associated

with imatinib resistance were also detected. These mutations are likely to predispose patients to subsequent TKI therapy failure. But if the mutation rate in CP CML stem cells is so impressively high, why are relapse rates in CP patients so low?¹⁰⁰ And why are relapses in this clinical setting explained by *ABL1* KD mutations in no more than one-third of cases?⁹¹ The frequency of *ABL1* KD mutation detection both before starting imatinib and at the time of imatinib resistance has been reported to correlate with the 'age' of the leukemic population.^{91,95} Might Jiang's results have been biased by the fact that the patients from whom stem cells were isolated had been diagnosed with CML up to 10 years earlier?

In addition, two previous studies searching for *ABL1* KD mutations at the stem cell level had yielded contrasting results.^{97,101} In one study,¹⁰¹ double-gradient denaturing-gradient gel electrophoresis failed to detect *ABL1* KD mutations in CD34+CD38- or CD34+CD38+ cells isolated from a newly diagnosed CP CML patient who later developed a Y253H and imatinib-resistance, nor in their long-term culture-initiating cell-derived progeny. In contrast, the same mutation observed at the mononuclear cell level at the time of imatinib resistance in a late CP, and an accelerated phase patient was also found in the CD34+CD38+ cell compartment and in the long-term culture-initiating cell-derived progenies from CD34+CD38- and CD34+CD38+ cells. In the other study,⁹⁷ direct sequencing of CD34+CD38- cells isolated from newly diagnosed CP patients did not show evidence of mutations. Can these discrepancies in results be attributable only to differences in sensitivity of the mutation detection methods employed in the three studies? Could it be that the CML stem cell, unlike its progeny, is not 'oncogenically addicted' to *BCR-ABL1*? The issue remains controversial, highlighting the need for further investigations. It is also unclear whether analogous *JAK2* mutations will be revealed by the use of *JAK2* inhibitors. Whether detection of clinically relevant mutations (i.e., T315I, E255K and so on) in the CD34+ subpopulation do always predict for subsequent development of resistance needs to be confirmed. If so, the recent advent of next generation sequencing technologies allowing sensitive, high-throughput and cost-effective mutation analysis of cancer specimens^{102,103} might prove a valuable tool to detect KD mutations in newly diagnosed patients before imatinib start.

What is their clinical significance?

Over 90 mutations in the KD of *BCR-ABL1* have been reported in patients taking imatinib.^{104–106} Most of these mutations are uncommon and the degree to which they alter imatinib sensitivity is in many cases unknown. However, nine common KD mutations accounting for over 60% of cases where mutants have been detected. These nine mutations have been well characterized.

Several years ago, the prognosis for patients with KD mutations within the P-loop was reported as being worse than the prognosis for patients with mutations in other regions.^{107–109} A possible explanation for this inferior prognosis has now been provided, at least for the E255K and Y253F mutants. Recent studies have suggested that these mutants may confer increased transformation activity to the *BCR-ABL1* protein.^{110–112} Griswold *et al.*¹¹¹ looked at the transformation potency of five common mutants and found variable transformation potency, which in some cases was actually less than the transformation potency of the native form of *BCR-ABL1*. Shah *et al.*¹¹⁰ demonstrated that in the case of compound mutants where two mutations are present within the same *BCR-ABL1* gene,

greater potency was observed even though both of the mutants, when present on their own, were predicted to confer weaker potency.

The clinical relevance of newly detected mutations in the absence of a significant increase in the level of *BCR-ABL1* as measured by real-time quantitative PCR has not been established. However, in patients where newly detected mutations are seen in the setting of rising *BCR-ABL1* levels, they frequently herald clinically significant disease progression.^{108,113,114} Some patients will respond, at least in the short term, to a dose increase in imatinib but others are known to be resistant to high doses of imatinib and a switch to a second generation kinase inhibitor is more likely to be effective.

Response to the second generation kinase inhibitors, dasatinib and nilotinib, may be influenced by the type of mutation present at the time of switching. Mutations that are less sensitive to dasatinib include F317L, Q252H and E255V/K.^{115,116} For nilotinib, *in vitro* studies predict that E255V/K, Y253H and F359V/C may be the least responsive to therapy.^{117–118}

The spectrum of mutations that will emerge on either dasatinib or nilotinib therapy appears to be much more restricted than with imatinib. *In vitro* mutagenesis screens have identified T315 and F317 as the common sites for dasatinib-resistant mutations.^{119–122} Additionally, some mutations are emerging on dasatinib that are imatinib and nilotinib sensitive, raising the possibility that the combination therapy might be more effective. Similar *in vitro* screens for nilotinib-resistant mutations identified a limited number of resistant mutations.

As response dynamics associated with specific mutations become better documented in clinical studies, it should become possible to develop recommendations about which mutations are more likely to respond to either nilotinib or dasatinib and which mutations may predict poor response to both drugs. Where allografting is a possible second- or third-line therapy, this knowledge may help when deciding whether to embark on an allograft regardless of early response to second-line therapy or only if early response is suboptimal.

Newer investigational approaches

Gaps in our knowledge of disease pathogenesis

All CML cases are positive for *BCR-ABL1* by definition, and recently it has become apparent that all or virtually all patients with PV harbour activating mutations in *JAK2*. However, the molecular pathogenesis of half of ET and PMF, plus the majority of atypical MPDs remains obscure. For ET and PMF, roughly 50% of cases are positive for *JAK2*-V617F and 1–5% have *MPL* S115 mutations. For atypical CMDs, a small proportion of cases are positive for one of >40 known tyrosine kinase fusions that structurally and functionally resemble *BCR-ABL1*.¹²³ Other cases are positive for *JAK2*-V617 or *NRAS* mutations, but collectively these abnormalities account for only a minority of affected individuals. Finally, the pathogenesis of disease progression for both *BCR-ABL1*-positive and *BCR-ABL1*-negative diseases is poorly understood. Several strategies are being pursued to try and identify new abnormalities in these areas.

Gene expression arrays

The advent of tyrosine kinase inhibitors has radically changed the treatment of CML. Nevertheless, resistance and relapse are not rare in patients with CP disease treated with imatinib, and are the rule for patients with advanced phase disease (accelerated and blast phase). We do not fully understand the biology that

drives progression.^{53,55} We know that progression is associated with functional changes such as changes in cell proliferation, blocking of differentiation and decreased apoptosis, whereas a few gene mutations have been identified in BC (e.g., p53), few occur commonly.¹²⁴ By simultaneously scanning the changes in gene expression across the entire transcriptome, gene array methods offer a promising approach to look the deregulation of entire sets of genes and pathways. Thus, one can study changes in the transcriptome that occur in CML progression (comparing CP to blast phase) or response (comparing responders to non-responders) to discover (1) genes associated with progression and response, and (2) specific pathways involved in progression and response. Potentially, these results could yield biomarkers for progression and response, and pinpoint new drug targets to prevent or treat advanced phase disease.

Gene and pathway dysregulation

Thus far, gene expression studies have provided some insight into the biology of progression. Several interesting genes associated with progression have a biological 'track record' that makes them of particular interest to study. For example, *SOCS2*, thought to normally play a part in negative regulation of proliferation, has been found to be upregulated in advanced phases of CML.^{125,126} Elastase (*ELA2*) and *BMI2* have been found in microarray studies to be implicated in progression, and these may be associated with disease response in CP patients.^{125,127} Data from several sources seem to be converging on the Wnt/ β -catenin pathway as critical for the evolution of CML. Wnt/ β -catenin signalling is thought to be important in cell self-renewal, and mutations in β -catenin have been found in various epithelial solid tumours. Aberrant Wnt/ β -catenin signalling has thus been demonstrated in CML and T-ALL; activation of the WNT/ β -catenin pathway was observed in primary cell samples from patients with CML, with levels increasing with progression.^{125,128,129} The aberrant regulation of transcription factors can affect scores of downstream genes and hence are an efficient method to cause functional changes in cells. In addition, several transcription factors appear to be involved in CML progression. Jun-B knockout mice develop a myeloproliferative disorder similar to CML.¹³⁰ Jun-B has been shown to be downregulated in CML progression.¹²⁵ Genes controlled by the transcription factors *MZF1* and δ -*EF1* appear to be deregulated in CML progression.^{125,131} *MZF1* is a member of the Kruppel family of zinc-finger proteins originally cloned from a cDNA library from a BC CML patient,¹³¹ and have a critical function in haematopoietic stem cell differentiation, including the modulation of CD34 and *c-MYB* expressions,¹³² and *MZF1*^{-/-} knockout mice display an increase in haematopoietic progenitor proliferation, which continues in long-term culture conditions.¹³³ Moreover, *MDFI*, an inhibitor of myogenic basic helix-loop-helix transcription factors found overexpressed in CML progression, both interacts with axin, and influences Jun signalling, thus perhaps linking these two pathways implicated in CML progression.^{125,134,135}

Although gene expression provides a very sensitive measure of a cell state, it nevertheless is a limited look at the complexities of progression. However, with new techniques of mapping DNA structure (e.g., comparative genomic hybridization) and protein identity and levels (mass spectroscopy), a more complete look at the biology of BC is possible. In addition, the discovery of microRNAs (miRNAs or miRs, see below), which appear to regulate both RNA and protein expression, is an attractive link from DNA structural changes to protein expression. One can imagine studies where CML cells have DNA, miRNA, mRNA

and protein studies performed. The mapping of all of these systems will more clearly define the link between Bcr-Abl1 expression, GI,¹³⁵ DNA damage and altered gene/protein regulation. Such studies should clarify the underpinnings of response and progression.

microRNAs in CML

Altered miR expression contributes to aberrant post-transcriptional regulation of gene expression in different type of cancers,¹³⁶ but their role in the pathogenesis and progression of CML from CP to BC is still largely unknown. Perrotti and colleagues^{137,138} reported that microarray and Northern analysis show that expression of a discrete number of miRs is altered in an imatinib-sensitive manner in CML-BC^{CD34+} compared to CML-CP^{CD34+} progenitors and in *BCR-ABL1*-expressing haematopoietic cell lines. Interestingly, the same group also showed that the downregulation of some of these miRs is likely important for blastic transformation, as their expression directly interferes with the activity of *BCR-ABL1*-regulated RNA-binding proteins, which translationally inhibit myeloid differentiation in BC CML by suppressing expression of the C/EBP α transcription factor.^{129,137} These data not only reinforce the importance of *BCR-ABL1* in the post-transcriptional control of gene expression,¹³⁹ but also suggest a new function for miRNAs as functional regulators of RNA-binding proteins involved in the control of malignant cell growth, survival and differentiation. A group from Madrid has reported very recently that a 7-Mb fragile site on chromosome 14q32, which includes miR-203, is deleted in some haematologic malignancies and inactivated by hypermethylation in others.¹⁴⁰ miR-203 controls the expression levels of *ABL1* and also of *BCR-ABL1*, so its functional absence is associated with increased levels of *BCR-ABL1* expression in CML. Re-expression of miR-203 reduces proliferation of BCR-ABL1-dependent tumour cells. They conclude that miR-203 functions as a tumour suppressor gene; when it is deleted or silenced, induced re-expression might be of therapeutic benefit in some cases.

Genomic arrays

Array comparative genomic hybridization (CGH) can detect amplifications and deletions, some of which may be associated with cryptic fusion genes. However, using custom-designed tiling arrays that target the tyrosine kinome, no novel recurrent tyrosine kinase fusion genes have been identified thus far in *BCR-ABL1*-negative MPDs. In CML, genome-wide array CGH has identified several copy-number changes that appear to be associated with disease progression.¹⁴¹

Single nucleotide polymorphism arrays are an alternative technique for detecting copy-number changes but can also identify acquired uniparental disomy, thought to mark the presence of an activated oncogene or inactivated tumour suppressor gene. It has emerged that large tracts of acquired uniparental disomy are common in atypical CMDs with several different chromosomes being affected, indicating substantial genetic heterogeneity in these diseases. Recurrent abnormalities of chromosomes 7 and 11 are particularly prominent and characterization of the 11q locus has identified a number of different transforming mutations that impact on intracellular signalling pathways (NCP Cross, unpublished data). In contrast to atypical CMDs, acquired uniparental disomy is uncommon in V617F-negative classical CMDs and also CML in transformation. High-density single nucleotide polymorphism arrays have recently pinpointed deletions of the *Ikaros* gene as a critical

event in lymphoid transformation of CML as well a Ph chromosome-positive ALL.¹⁴²

Other approaches

High-throughput sequencing or mutation scanning is becoming more widely applied as the cost of sequencing falls and was one of the routes by which *JAK2*-V617F was identified, as well as the finding of *MPL*515 mutations.¹⁴³ However, it has become apparent that the majority of sequence changes identified by sequencing are in fact unimportant 'passenger' mutations that happened to be present in a cell that acquired other oncogenic changes. Identification of the relatively small number of pathogenetic 'driver' mutations requires functional proof of their activity. Other possible techniques include proteomic analysis to detect aberrantly tyrosine phosphorylated proteins,¹⁴⁴ and systematic short hairpin RNA screens that may be used to individually knock down the expression of target genes, for example, the tyrosine kinome.¹⁴⁵ These combined approaches are likely to make significant inroads into our understanding of the basic pathogenesis of CMDs in the next 1–2 years.

New approaches to therapy

BCR-ABL1 and T315I mutant clones (Table 1)

Imatinib (Novartis Pharma, Basel, Switzerland), nilotinib (Novartis) and dasatinib (Bristol-Myers Squibb, Wallingford, CT, USA), as well as the investigational agents bosutinib (Wyeth, Cambridge, MA, USA) and INNO-406 (Innovive Pharmaceuticals, NY, USA), all target *BCR-ABL1* by binding within the ATP-binding site of the *ABL1* KD.¹⁴⁶ All five of these inhibitors make hydrogen-bond interactions with the side-chain hydroxyl group of the Thr315 gate-keeper residue, which make important contributions to their activity. As discussed above, the most frequently encountered mechanism of secondary drug resistance in both CML and Ph+ ALL involves the emergence of leukaemic clones expressing mutant forms of *BCR-ABL1*, in which amino-acid residues within the *ABL1* KD are exchanged with alternative residues that maintain enzymatic activity, but result in a much reduced binding affinity to the inhibitor. One such mutant, encountered in $\approx 10\%$ of cases, involves the exchange of the Thr315 residue with Ile (T315I), which lacks the side-chain hydroxyl group necessary to bind the currently available inhibitors, is resistant to these drugs and represents a particular unmet medical need.

Two potent Aurora kinase inhibitors, MK-0457 (Merck & Co, Whitehouse Station, NJ, USA) and PHA-739358 (Nerviano Medical Sciences, Nerviano, Milan, Italy), which possess some

degree of activity against T315I *BCR-ABL1*, are under investigation in phase II clinical trials in resistant CML patients.^{147,148}

Both of these agents have shown haematologic responses following intravenous infusion at doses, which transiently inhibit CRKL phosphorylation. There are also two multikinase inhibitors, which inhibit T315I *BCR-ABL1* in early clinical development. The Exelixis (San Francisco, CA, USA) company is evaluating XL-228, which inhibits wt (Ki 5 nM) and T315I (Ki 1.4 nM) *ABL1*, as well as Aurora-A, IGF-1R and the SRC-family kinases. In a phase I trial, 63 patients with CML or Ph+ ALL received XL-228 administered weekly for 1 h through i.v. infusion.¹⁴⁹ At 0.45 and 0.9 mg/kg, mean C_{max} values were 166 and 461 ng/ml, respectively, with no drug-related serious adverse events being reported. Kyowa Pharmaceuticals (Tokyo, Japan) is developing KW-2449, an orally bioavailable, multi-kinase inhibitor against wt (IC₅₀ 14 nM) and T315I (IC₅₀ 4 nM) *ABL1*, Aurora-A (IC₅₀ 46 nM), *FLT3* (IC₅₀ 6.6 nM), *FGFR1* (IC₅₀ 36 nM) and *SRC* (IC₅₀ 400 nM).¹⁵⁰ In a phase I trial, patients ($n=29$) with CML, Ph+ ALL, AML or MDS have received KW-2449 at 200 mg b.i.d., without an MTD being established. Ariad Pharmaceuticals (Cambridge, MA, USA) have also been researching dual *BCR-ABL1*/*SRC*-family kinase inhibitors, which maintain activity against mutant forms of *BCR-ABL1*, including the T315I; AP24534 potently inhibits wt (IC₅₀ 12 nM) and T315I (IC₅₀ 58 nM) *ABL1*, *VEGFR-1*, -2 and -3 (IC₅₀ 3–58 nM), *FGFR-1*, -2, -3, -4 (IC₅₀ 0.4–58 nM), *LYN* (IC₅₀ 3 nM), *SRC* (IC₅₀ 2 nM), Tie-2 (IC₅₀ 3 nM) and *FLT3* (IC₅₀ 26 nM), but not (IC₅₀ > 1000 nM) Aurora-A, IGF-1R, InsR or CDK2. This compound maintains potency in cells and shows oral activity in K562 cells and T315I-Ba/F3 xenograft models in mice.¹⁵¹ AP24534 is expected to enter phase I clinical trials in haematologic malignancies (including refractory CML) and in solid tumours during 2008.

Although multitargeted kinase inhibitors might possess advantages in the treatment of patients with genetically unstable, advanced CML, such agents could also be liable to a higher incidence of side effects resulting from the inhibition of kinases other than *BCR-ABL1*.

Utilizing fragment and structure-based drug design, SGX Pharmaceuticals (San Diego, CA, USA) have recently reported success in identifying agents, which can selectively inhibit *BCR-ABL1* and maintain activity against the T315I mutant. Thus, compounds from a 7-azaindole series bind to the *ABL1* KD in the DFG-inactive conformation, but in a different manner to dasatinib and bosutinib, which provides for potent inhibition of both wt and T315I *BCR-ABL1* and a high degree of selectivity over other tyrosine kinases.¹⁵² SGX393 (also called SGX70393) is one such compound, which following oral administration of 50 mg/kg q24 h inhibited T315I-driven tumour growth and reduced levels of pCrKL in tumour tissue in a murine xenograft model.¹⁵³

Other approaches towards selective inhibition of wt and mutant *BCR-ABL1* involve targeting allosteric-binding sites. Structural studies suggest that autoregulation of *ABL1* might involve the stabilization of an assembled inactive conformation of the kinase through an intramolecular interaction between an *N*-terminal myristoyl group (not present in the *BCR-ABL1* fusion protein) with a binding site near the C-terminus.¹⁵⁴ Gray and colleagues¹⁵⁵ have described small molecule inhibitors of *BCR-ABL1*, which are believed to work by interacting with this myristoyl-binding site, and these studies therefore show promise for the identification of selective allosteric inhibitors of both wt and mutant forms of *BCR-ABL1*, including T315I.¹⁵⁵ Deciphera Pharmaceuticals (Lawrence, KS, USA) are developing a different class of allosteric inhibitors of *BCR-ABL1*, which are believed to interact with a pocket, which can regulate switching between the catalytically active and inactive conformations of the KD

Table 1 Agents that selectively target *BCR-ABL1*, *BCR-ABL1*-T315I or *JAK2*

BCR-ABL1	BCR-ABL1-T315I	JAK2
Imatinib	MK-0457	TG101348
Nilotinib	PHA-739358	INCB018424
Dasatinib	XL-228	XL019
Bosutinib	KW-2449	Lestaurtinib (CEP701)
	AP24534	AZD1480
	SGX393	Erlotinib
	DCC-2036	SB1518

Agents in Roman type have entered clinical trials; agents in italics have not been reported yet as studied in any patient with a myeloproliferative disease. This table is not intended to be comprehensive, because there are various other new agents now under study.

and show selectivity towards *ABL1*, *BCR-ABL1*, *FLT3* and *SRC* family kinases. The lead compound, DCC-2036, inhibits the proliferation of Ba/F3 cells transformed with either wt *BCR-ABL1* or a number of *BCR-ABL1* mutants including T315I, G250E, M351T and Q252H at GI_{50} values below 100 nM, although a number of other mutants, such as Y253H, E255K/V and F359V, are less sensitive. In a CML model induced in mice by injection of T315I-Ba/F3 cells, oral administration of 100 mg/kg/day of DCC-2036-reduced peripheral blood leukocyte counts and significantly prolonged animal survival in comparison to vehicle-treated control mice.¹⁵⁶

Although several non-selective kinase inhibitors with T315I *BCR-ABL1* activity are under clinical investigation, it will be difficult to establish what degree of activity results from target inhibition and which off-target (non-*BCR-ABL1*) activities are beneficial. On the basis of their mechanisms of action and preclinical activities, selective inhibitors of T315I are expected to show clinical efficacy at well-tolerated doses and might have potential for use in combination with other *BCR-ABL1* inhibitors to reduce the emergence of drug-resistant forms of the enzyme. However, in patients whose disease has progressed and GI is 'well established' in their CML cells, responses to selective T315I inhibitors are unlikely to be durable and clones expressing other drug-resistant mutants may well emerge. Therefore, in some cases at least, less selective multitargeted kinase inhibitors could prove advantageous.

JAK2-V617F

The identification of the *JAK2* pathway as the main molecular lesion involved in the pathology of classical MPD, through the characterization of mutations affecting *JAK2* or *MPL*, has promoted the development of therapeutic *JAK2* inhibitors. Preclinical or clinical studies using orally available small molecule inhibitors developed by several companies have now been reported. These *JAK2* inhibitors can be divided in two groups: those designed to specifically inhibit *JAK2* (XL019 from Exelixis, SB1518 from S*Bio Pte Ltd (Singapore) TG101348 from TargeGen Inc (San Diego, CA, USA), AZD1480 from AstraZeneca (Macclesfield, UK), and those inhibiting other kinases, including other *JAK* family members (INCB018424 from Incyte Corporation (Wilmington, DE, USA), Lestaurtinib (CEP701) from Cephalon Inc (Frazer, PA, USA),¹⁵⁷ erlotinib from Genentech Inc (South San Francisco, CA, USA)¹⁵⁸ and MK-0457 from Merck.¹⁵⁹

A number of phase I/II clinical studies have now been initiated. Investigators in Boston (Harvard Medical School) assessed the safety and efficacy of TG101348 in a murine model of *JAK2*-V617F-induced PV.¹⁶⁰ Oral administration of this agent normalized splenic architecture, reduced or eliminated extramedullary haematopoiesis and also reduced the number of erythroid and myeloid progenitors. T-cell function was not adversely affected. There was some evidence that myelofibrosis could be reversed in the experimental system. Investigators in San Diego (University of California at San Diego) studied the mechanism of action of TG101348 in a different system involving xenogeneic transplantation into an immune compromised mouse.^{161,162} They showed that haematopoietic progenitor cells from patients with PV and human cord blood stem cells transduced with *JAK2*-V617F that differentiated along the erythroid lineage could be inhibited by TG101348, whereas differentiation of progenitors with *JAK2*wt were much less affected by the drug. Moreover, the investigators showed that TG101348 treatment decreased GATA-1 expression, which was associated with erythroid-skewing of *JAK2*-V617F-positive

progenitor differentiation. This agent has now been used in a phase I study to treat patients with myelofibrosis and could also prove useful for other Ph-negative myeloproliferative disorders.

Lestaurtinib was identified recently as a result of a multikinase screen.¹⁵⁷ It inhibited *JAK2*wt with an IC_{50} of 1 nM *in vitro*. It also inhibited the growth of HEL92.1.7 cells, which are dependent for growth *in vitro* on mutant *JAK2* activity. In a phase II clinical study, 22 patients with PMF and patients with myelofibrosis following PV or ET, who were *JAK2*-V617F positive, were treated with 80 mg twice daily by mouth. Median age was 61 years (range, 38–83 years) with median of two prior therapies (range, 0–5 years); 15 had abnormal cytogenetics, 18 had enlarged spleen (two had splenectomy) and eight were transfusion dependent. At median follow-up of 2 months, Clinical Improvement (International Working Group (IWG) criteria) was noted in 6 of 21 evaluable patients with reduction in spleen size, being the most common response (seen in five patients). Nausea/vomiting/diarrhoea were the most common side effects and dose reduction was required in four patients. Grade 3 toxicity occurred in two patients, thrombocytopenia in both patients and diarrhoea in one.¹⁶³

Another agent of potential clinical interest, XL019¹⁶⁴ is a potent and reversible inhibitor of *JAK2*-V617F. In cell lines, it downregulated STAT5 phosphorylation by both wt and activated forms of *JAK2*. It showed activity in a murine xenograft model. It has, therefore, been investigated in phase I/II study in patients with primary or secondary (post-ET/PV) myelofibrosis. Nine patients have been treated so far in escalating doses. Interestingly, patients with either *JAK2*-V617F or *MPL*-W515L mutation who had enlarged spleens, experienced significant spleen size reduction, unlike patients without a mutation. Several patients had decreases in elevated WBC or platelets, but no myelosuppression was observed. Unfortunately, at doses tested, most patients experienced mild-to-moderate neurotoxicity, central nervous system-related and/or peripheral. Lower doses and different schedules of this new therapy are being investigated.

Another agent that selectively inhibits *JAK2* is INCB01824.^{165,166} A phase I/II study of orally administered drug was started recently for patients with PMF and post-PV/ET myelofibrosis. Overall characteristics of the first 32 patients enrolled include median age of 65 years; 67% men; 47% PMF, 38% post-PV and 15% post-ET and 87% with *JAK2*-V617F mutation. The starting dose of 25 mg by twice daily mouth was demonstrated to be the maximum tolerated dose ($N=6$). Two patients had grade 4 thrombocytopenia in the 50 mg p.o. BID cohort ($N=5$), which defined the dose-limiting toxicity. No other significant drug-related toxicities were noted. An expanded cohort of 21 additional patients has also been enrolled at the maximum tolerated dose (MTD), all of whom completed at least 1 month of therapy. A rapid and significant reduction in splenomegaly was observed in the patients with palpable spleens at baseline with reductions of 53% at 1 month ($N=24$) and 76% at 3 months ($N=7$). In addition, the majority of patients had improvement in their Eastern Cooperative Oncology Group (ECOG) performance score to 0, and their constitutional symptoms resolved or were significantly reduced. Additional drug-related improvements included transfusion independence in the initial patient enrolled in the study, significant reduction in *JAK2*-V617F allele burden, marked reduction in proinflammatory and angiogenic cytokines, significant increases in haematopoietic growth factors and normalization of phosphorylated Stat3.

AZD1480 was developed by AstraZeneca as a specific ATP-competitive inhibitor of the *JAK2* kinase with an inhibition constant of 260 pM. In enzyme assays, the IC_{50} for *JAK2* is under 1 nM. The IC_{50} for *JAK3* is 14 nM, representing at least 14-fold

selectivity for *JAK2* over *JAK3*. In Ba/F3 cells engineered to cytokine independence by transfection of JAK family KDs fused to the TEL oligomerization domain, the GI_{50} for *JAK2* is 49 nM, as measured in an Alamar Blue viability assay. This is very similar to the IC_{50} for inhibition of STAT5 phosphorylation in these cells. The GI_{50} for the *JAK1*, *TYK2* and *JAK3* TEL fusion proteins is respectively 17-, 38- and 44-fold higher than that seen for TEL-*JAK2*.

As part of preclinical studies, the effect of AZD1480 was assessed on the *ex vivo* development of haematopoietic progenitor cells from PV patients. *In vivo* preclinical studies in a murine model of CMD have recently been reported.¹⁶⁷ Cultures were initiated from peripheral blood CD34+ cells isolated from phlebotomies, and normal control peripheral blood CD34+ cells were isolated from phlebotomies of hemochromatosis patients. The IC_{50} 's for BFU-E or CFU-e, both stimulated by stem cell factor, interleukin-3 and Epo and CFU-GM stimulated by stem cell factor and interleukin-3, were around 100–200 nM. No difference in sensitivity to AZD1480 was observed between progenitor cells derived from PV or control patients. However, because of the fact that progenitor cells from PV patients include malignant and normal cells in variable proportions, it was not possible to conclude from this result that malignant and normal cells display similar sensitivities to AZD1480. In the absence of Epo, only malignant progenitor cells from PV patients develop, giving rise to endogenous erythroid colonies, the hallmark of PV. The IC_{50} of these malignant erythroid progenitor cells was reduced fivefold relative to PV or normal BFU-E stimulated with 1 U/ml of Epo. Similar results have been reported with other *JAK2* inhibitors, including TG101209/101348^{159,168} and INCB018424.¹⁶⁵ These results could be explained by (1) a preferential inhibition of malignant versus normal progenitor cell growth by the inhibitors, or (2) the role of Epo in antagonizing *JAK2* inhibition. Genotyping of BFU-E colonies revealed that the normal and mutated BFU-E had similar sensitivity to AZD1480. Furthermore, drug sensitivity of BFU-E stimulated by the normal serum Epo concentration (14 mU/ml) was (1) five times lower than that of BFU-E, maximally stimulated by 1 U/ml of Epo, (2) similar to that of endogenous erythroid colonies, and (3) similar in PV and control patients.

Experience with chemically different *JAK2* inhibitors shows that (1) a variety of small molecules are highly potent inhibitors of *ex vivo* growth of CD34+ haematopoietic colonies, which do not discriminate in cell culture between normal and *JAK2*-V617F BFU-E, and (2) Epo antagonizes blockade of erythroid progenitor cell growth by *JAK2* inhibitors. These results have multiple consequences related to clinical trials. Firstly, although treatment of CML by imatinib is often cited as the prototype of successful treatment of MPD by a kinase inhibitor, the potential inability of *JAK2* inhibitors to selectively suppress the malignant clone at the level of committed progenitor cells in contrast to imatinib in CML may represent a major difference, with expected impacts in clinical management between the two therapies. Secondly, as PV is characterized by reduced Epo levels, *JAK2* inhibitors may be effective in selectively suppressing the malignant clone(s) until Epo levels normalize at disease alleviation. Indeed AZD1480¹⁶⁶ and TG101348¹⁶⁰ have been shown in a *JAK2*-V617F mouse model to be effective in suppressing erythrocytosis and reducing allele burden during polycythemia. However, it is not known whether the inhibitor will be selectively effective at eliminating the malignant cells once haemoglobin levels have normalized. Finally, in PMF, high Epo levels may blunt the effect of inhibitors on erythropoiesis and reduce potential side effects related to anaemia.

Conclusions

The *BCR-ABL1*-positive and the *JAK2*-positive CMDs (or MPNs) provide important paradigms for the earliest stages of tumorigenesis. Studies of their molecular pathogenesis reveal not only many similarities but also some unexpected differences. In both cases, the defining molecular abnormalities may or may not be the true initiating lesions. Genomic instability is a well-accepted feature of CML, but less clearly a feature of the *BCR-ABL1*-negative CMLs. Study of the mutations that occur in the *BCR-ABL1* gene and in other genes in the neoplastic clone should prove informative. An understanding of the mechanisms responsible for these commonalities and contrasts will benefit researchers studying both disorders and is likely to be of general relevance for cancer biology. The introduction of TKIs has been extremely effective in treating majority of patients with CML, and comparable results may be achievable in *JAK2*-positive CMDs.

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Disclosure

Paul Manley is a fulltime employee of Novartis Pharmaceuticals.

Workshop participants

This report was based on a workshop that took place in Puerto Rico on 12 and 13 December 2007, but it includes some data that became available after that date. The participants at the meeting in Puerto Rico included: Tiziano Barbui, Bergamo, Italy; Giovanni Barosi, Pavia, Italy; Nicholas Cross, Salisbury, UK; Alan Gewirtz, Philadelphia, USA; Sergio Giral, Houston, USA; John Goldman, London, UK; Anthony Green, Cambridge, UK; Oliver Hantschel, Vienna, Austria; Jonathan Harris, Philadelphia, USA; Ronald Hoffmann, New York, USA; Rudiger Hehlmann, Mannheim, Germany; Timothy Hughes, Adelaide, Australia; Catriona Jamieson, San Diego, USA; Pierre Laneuville, Montreal, Canada; Francis Lee (Bristol Myers Squibb, Wallingford, CT, USA); Ross Levine, Boston, USA; Paul Manley (Novartis), Junia Melo, London, UK; Tariq Mughal, Dallas, USA; Heike Pahl, Freiburg, Germany; Feyruz Rassool, Baltimore, USA; Giuseppe Saglio, Torino, Italy; Richard Silver, New York, USA; Radek Skoda, Basel, Switzerland; Ted Szatrowki (Bristol-Myers Squibb), William Vainchenker, Paris, France; Richard van Etten, Boston, USA; Alessandro Vannucchi, (Florence, Italy), Jean Luc Villeval, Paris, France; Richard Woodman (Novartis), The meeting was supported by unrestricted educational grants from Bristol-Myers Squibb, Novartis Pharmaceuticals and Wyeth Pharmaceuticals.

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